The University of Salford

School of Science, Engineering and Environment

PhD in Biological Sciences

Improving outcomes for obese children with Acute Lymphoblastic Leukaemia (ALL): a clinical collaboration to understand the influence of obesity on treatment success.

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ABSTRACT

Cancer Research UK has reported that obesity is the second highest risk factor for developing cancer after smoking. Studies suggest that almost three quarters of people are expected to be overweight by 2035. Children with ALL who are obese at diagnosis are less likely respond to standard cancer treatment, it is more difficult to achieve a complete remission in obese children, they are subject to higher minimal residual disease risk, poorer event free survival and increased risk of metabolic syndrome and cardiovascular disease later in life

This work has been designed to identify changes in adipokine profiles at diagnosis and after the intense phase of induction therapy to investigate if dysregulated adipokine profiles contribute to weight gain and MRD risk. To achieve this weight status and BMI was recorded and blood plasma was tested using human adipokine arrays at diagnosis and following induction therapy to establish if these changes correlated with weight gain or MRD risk.

We have shown that MRD risk status at the end of induction therapy is associated with lower weight rather than weight gain. We have identified a number of adipokines linked to MRD risk and weight gain in our patient cohort, these include Leptin, IL-8, pref-1, endocan and adiponectin in weight gain. Also, resistin, serpin A12, IGFBP4, cathepsin-S and Angiopoetin-2 in MRD risk. We have shown that leptin, IL-8, serpin A12 and angiopoietin-2 have a protective effect when incubated with the Molt4 ALL cell line and that resistin does not offer this protection.

Further experiments with leptin confirmed the activation of the PI3K/Akt pathway which is a shared pathway with the other adipokines of interest along with the JAK/STAT pathway. Leptin was unable to change the sensitivity of the chemotherapy drugs alone this may suggest that targeting single adipokines may not be the best course of future treatment stratification. Inhibitors of the PI3K/Akt pathway or the JAK/STAT pathway could be treatment options for adipokines highlighted in this study.

Future treatment options that show promise could include activators of AMPK like metformin, Bcl-2 inhibitors or small molecule inhibitors that target common pathways of chemoresistance which include autophagy, upregulation of drug efflux pumps or the UPR network. It is clear that more research is needed in these areas in the hope to improve outcomes for obese, overweight and MRD risk patients and to develop kinder drug treatments for paediatric ALL patients.

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2-DG- 2-Deoxy- d-glucose

AIDS- Acquired immune deficiency syndrome

ALL- Acute lymphoblastic leukaemia

AML-Acute myeloid leukaemia

AMPK- Adenosine monophosphate-activated protein kinase

BAT- Brown adipose tissue

cAMP- Cyclic adenosine monophosphate

CAR T- Chimeric antigen receptor therapy

CML- Chronic myelogenous leukaemia

CRP- C-reactive protein

EFS- Event-free survival

ER- Endoplasmic reticulum

GFR- Glomerular filtration rate

GRE- Glucocorticoid response elements

GWAS- Genome wide association study

HIV- Human immunodeficiency virus

HPV- Human Papilloma Virus

JMML- Juvenile myelomonocytic leukaemia

MAT- Marrow adipose tissue

MRD- Minimal residual disease

PBS- Phosphate buffered saline

PEG- Polyethylene glycol

STAT- Signal transducer and activator of transcription

T2D- Type 2 diabetes

TAO- Treatment acquired obesity

TAOW- Treatment acquired overweight

TLR- Toll like receptor

UPR- Unfolded protein response

WAT- White adipose tissue

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1.0 CHAPTER 1 INTRODUCTION

1.1 Introduction to Cancer

Cancer is a group of diseases that stem from unregulated cell growth; the cells often spread from a site of origin, or primary site and can invade or metastasise to other secondary sites within the body where they grow and proliferate (Pecorino, 2012). Depending on the origin of the cancer cell and the tissue it resides in, there are more than 200 different types of cancer (NHS UK, 2021). One of the traits of cancer cells, in comparison to normal cells, is their ability. to sustain chronic proliferation. Normal tissues produce growth promoting signals and carefully control the cell growth and division cycle, cancer cells disrupt this homeostasis and become masters of their own fate (Hanahan and Weinberg, 2011)

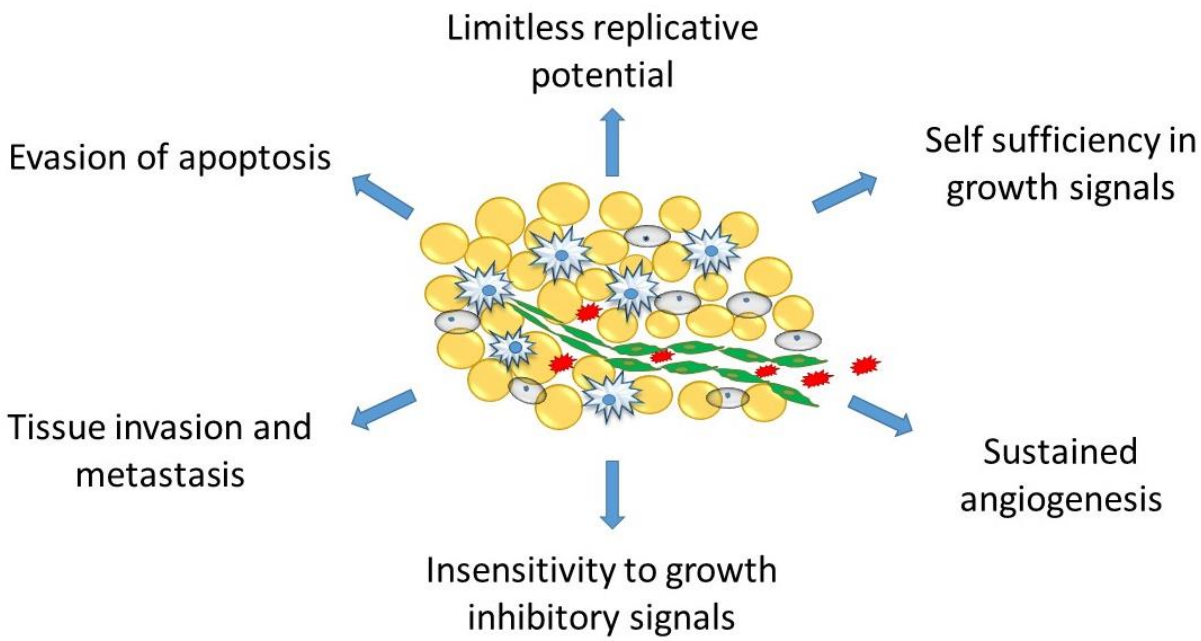

Figure 1.1: The Hallmarks of Cancer. (adapted from Hanahan and Weinberg, 2011) There are six hallmark features of cancer, each of which have undergone extensive research over recent decades, the mechanisms involved are; evasion of apoptosis, cells having a limitless replicative potential, self-sufficiency and an insensitivity to growth inhibitory signals, sustained angiogenesis, tissue invasion and metastasis. The different shapes and colours illustrate that there are many different cell types, in addition to tumour cells, found within the tumour microenvironment.

It has been proposed that cancer is essentially promoted by six alterations in cell physiology and behaviour which initiate malignant growth, see figure 1.1. These include a self-sufficiency in growth and development signals, lack of sensitivity to growth inhibitory signals, cells can evade apoptosis, an unending replicative potential, sustained angiogenesis and invasion of surrounding tissue and metastasis (Hanahan and Weinberg, 2000). Fundamentally, the initiating factors for this dysregulation are mutations within DNA, this can be an inherited genetic error, or it can arise from replication errors in DNA or RNA from cellular processes (Kai, 2016). It is rarely one error that contributes to the development of cancer, but rather several gene mutations and an accumulation of genetic errors over time (Bertram, 2000). An example of this would be the *BRCA1* gene associated with breast cancer. A person can inherit a mutation within this gene and have a high susceptibility to developing breast cancer (Mersch et al., 2015) but may not go onto develop cancer. It is more likely that a combination of factors such as other chance mutations which increase with age, lifestyle and environment, which would eventually contribute to the breast cancer forming (Kuchenbaecker et al., 2017).

1.1.2 Cancer Statistics

Cancer types, incidence and statistics vary between men and women (Kim et al., 2018). In the UK, statistics for men's cancer demonstrate the highest percentage of cancer types occur in the prostate; there were 48,588 new cases in 2017. Lung cancer was the second most common cancer in men at 24,881 cases, bowel at 23,516 head and neck 8,522, melanoma 8,174 respectively (Cancer research UK, 2020b). Women's statistics reported breast cancer as the most common at 54,722 cases, lung at 23,087, bowel at 18,565, uterus 9,494, and melanoma 8,001 (Cancer research UK, 2020a). Combined incidence of all new cancer cases in men and women totalled 367,167 in the UK between 2015-2017 (Cancer Research UK, 2020c, Cancer research UK, 2020a). Mortality data for 2016 reached 166,533 deaths in the UK between 2016 and 2018 (Cancer Research UK, 2020d, Cancer Research UK, 2018c). Statistics in America show that there are expected to be a total of 1,898,160 new cancer cases overall in 2021 and 608,570 estimated deaths (Siegel et al., 2021, Siegel et al., 2015) Worldwide, including developing countries, there was an estimated 19,300,000 new cancer cases (Sung et al., 2021) , globally, cancer is also a leading cause of mortality and in 2020 was responsible

for nearly 10 million deaths which equates to nearly 1 in 6 people dying from cancer (World Health Organisation, 2021).

1.1.3 Causes and risk factors

Cancer is a multifaceted disease and has many causes and risk factors. The leading contributing causes and risk factors, apart from inherited genetics, include smoking and exceeding the recommended weekly alcohol intake (Purohit et al., 2013), being overweight or obese (Katzke et al., 2015), exposure to ultraviolet light from the sun or sunbeds (Autier and Boniol, 2013), eating an unhealthy diet lacking in fruit and vegetables, too many processed meats and lack of physical activity (Kerr et al., 2017), infections such as HPV which can cause cervical cancer (Petry, 2014), hormone dysfunction (Brown and Hankinson, 2015), environmental air pollution (Santibáñez-Andrade et al., 2017), workplace risks such as asbestos (Gilham et al., 2016) and old age (Meucci et al., 2016). Cancer is on the increase globally, particularly in developing countries, and is often associated with adopting unhealthy sedentary lifestyles and consumption of calorie dense food (Jemal et al., 2010). However, most cancers have improved survival rates over the last 20 years largely due to earlier detection and screening programmes (Cedolini et al., 2014) although incidence is increasing overall.

1.2.1 Paediatric Cancers

In children, the types of cancer diagnosed are different to adult cancers. The highest percentage of cancer cases in children are related to blood cancers such as leukaemia's, also cancers related to the brain and lymph nodes (Syrimi et al., 2020). Unlike adult cancer, the risk factors for cancer in children are different as children do not have the same influences such as old age and lifestyle choices (American Cancer Society, 2021, Spector et al., 2015a). Children's cancer is largely attributed to inherited genetics and environmental factors, however due to the rarity of childhood cancer overall, the quality and quantity of evidence hinders research for pinpointing actual causes (Spector et al., 2015b). Despite this there are still a number of indications associated with developing childhood cancer which are discussed below. These observations are highlighted by recent transcriptome analyses to find novel genes associated with genotype to phenotype changes specific to paediatric malignancies

(Savary et al., 2020). Children are also more sensitive to some carcinogens due to developing bones and organs, for example they are at greater risk of developing cancer after exposure to ionizing radiation than adults (Kutanzi et al., 2016).

1.2.2 Paediatric Cancer incidence and statistics

The most common childhood cancers are cancers of the blood, such as leukaemia and lymphomas, cancers of the brain and central nervous system (Smith et al., 2010b). Figure 1.2 is adapted from (Children with Cancer UK, 2020) shows the most common types of cancer diagnosed in children each year by percentage in the UK, these are leukaemia's 30%, brain and spinal tumours 26%, lymphomas 11%, soft tissue sarcomas 6%, CNS tumours 5%, kidney tumours 5%, other carcinomas and melanomas 4%, germ cell and gonadal tumours 4%, retinoblastomas 3%, liver tumours 1% and others 1%. In total around 1,900 children are diagnosed each year (Cancer research UK, 2020e), where in the same time period there were 236 deaths reported, the most common age at diagnosis being 0-4 years old. Incidence is increasing overall though particularly in children, in the UK incidence rate for children have increased by 10% in the last decade (Cancer Research UK, 2020f). Global models in a recent report in the Lancet estimated there were 397,000 of childhood cancer cases worldwide in 2015 and that there will be an estimated 6.7 million childhood cancer cases by 2030 and that a massive 2.9 million cases will be missed in the same time period mainly in developing countries (Ward et al., 2019), this is mainly due to lack of infrastructure and health care in developing nations and also access to affordable medicines and treatment.

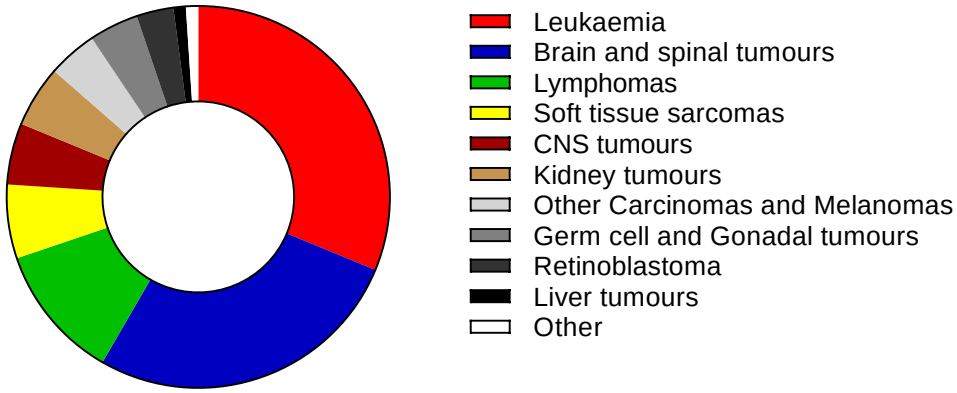

Figure 1.2: Types of childhood cancer diagnosed in the UK each year. The main types of cancer as a percentage from the 1,500 children diagnosed in the UK (adapted from Children with cancer.org).

1.2.2 Paediatric Cancer causes and risk factors

Factors found to be influencing the development of paediatric cancers have been explored in several studies; it is long been recognised that ionising radiation is a well-established cause of leukaemia's and other types of cancer in children including the prenatal exposure of parents to radiation sources (Folly et al., 1952). Population groups in Japan that survived atomic bombs showed a peak incidence of childhood leukaemia 6-8 years after their exposure which declined afterwards, whereas the risks of other cancers persisted for decades afterwards (Little, 2008). Children who were exposed to medical radiation and treated with X-rays for tinea capitis (ringworm) of the scalp, showed a higher rate of brain and skin cancers than the control group treated with topical medications (Shore et al., 2003). In the 1960s and 1970s, it was common place to X-ray pregnant mothers, studies have now shown this to be a causal factor in the development of childhood cancer (Boice and Miller, 1999) . Children that have previously had cancer radiation therapy have an increased risk of developing further malignancies following treatment (Neglia et al., 2001), a long term late effect which can affect childhood cancer survivors. Extensive research has shown that certain infections can cause cancer in children such as the Epstein Barr virus which can contribute to the development of lymphomas (Karajannis et al., 2003). Childhood cancer has also been linked to Hepatitis B and C (Kelly, 2006) and also the HIV AIDS virus (Chintu et al., 1995).

Other risk factors include; maternal smoking and parental, particularly paternal, passive smoking (de Smith et al., 2017, Shu et al., 1996), environmental chemicals and pesticides (Chen et al., 2015), maternal reproductive history i.e. mothers age at birth (Wang et al., 2017), mothers weight and diabetes risk (Contreras et al., 2016) and high birth weight which is an established risk for childhood leukaemia (O'neill et al., 2015).

Despite the wealth of evidence presented above suggesting causes and risk factors for developing childhood cancer, it remains difficult to predict which children will go onto develop cancer (Moorman, 2016). There are major benefits using meta-analysis and pooling of raw data from epidemiological studies by groups such as the Childhood Leukaemia International Consortium (CLIC) which help calculate the risk of less studied factors and associations and their cumulative effects (Metayer et al., 2013). These risk factors include; non-ionizing radiation (Calvente et al., 2010), traffic pollution (Heck et al., 2013) and exposure to light at night from screen usage which can disrupt melatonin production and circadian rhythms (Stevens, 2012). Although there have been no conclusive studies linking childhood cancer to mobile phone usage, scientists act on the side of precaution due to the susceptibility of children exposed to increasing levels of electromagnetic frequencies at a younger age and recommend additional research in the face of scientific uncertainty (Kheifets et al., 2005). Large scale epidemiological studies are collecting data so that we can begin to answer questions such as, "Do we have enough evidence about risk factors to move on to prevention?"

1.2.3 Paediatric Cancer prevention?

Recent evidence has suggested that acute lymphoblastic leukaemia (ALL) could be preventable and has evolved from a multifactorial cause (Greaves, 2018). The Greaves 'two hit model' suggests that ALL occurs in a sequence of events which includes inherited genetics, lack of early microbial exposure and an initiating event such as a common childhood infection. Greaves' study concludes that there is a mismatch between evolutionary adaptions of the immune system and our clean contemporary lifestyles, suggesting that childhood ALL may be a preventable cancer. Known protective factors against developing childhood cancer include attendance at day care (Ma et al., 2002), children that have been breastfed (Bener et al., 2008), pet ownership and farm animal contact especially in early life (Ajrouche et al., 2015).

The results of these studies support the hypothesis that events that promote the maturation of the immune system early in life have a protective role against childhood cancer.

1.3 Leukaemia's and lymphomas in children

There are four types of leukaemia which affect children, the two main types are Acute Lymphoblastic Leukaemia (ALL) and Acute Myelogenous Leukaemia (AML) (American Cancer Society, 2019). Approximately 80% of children with leukaemia are diagnosed with ALL and around 20% of children are diagnosed with AML. Both cancers develop quickly over days and weeks (Bloodwise UK, 2018). The two rarer forms of leukaemia in children are Chronic Myelogenous Leukaemia (CML), which is extremely rare in children and develops over a period of months years, and Juvenile Myelomonocytic Leukaemia (JMML), which accounts for 1-2% of cases each year (Dana-Farber Cancer Institute, 2018).

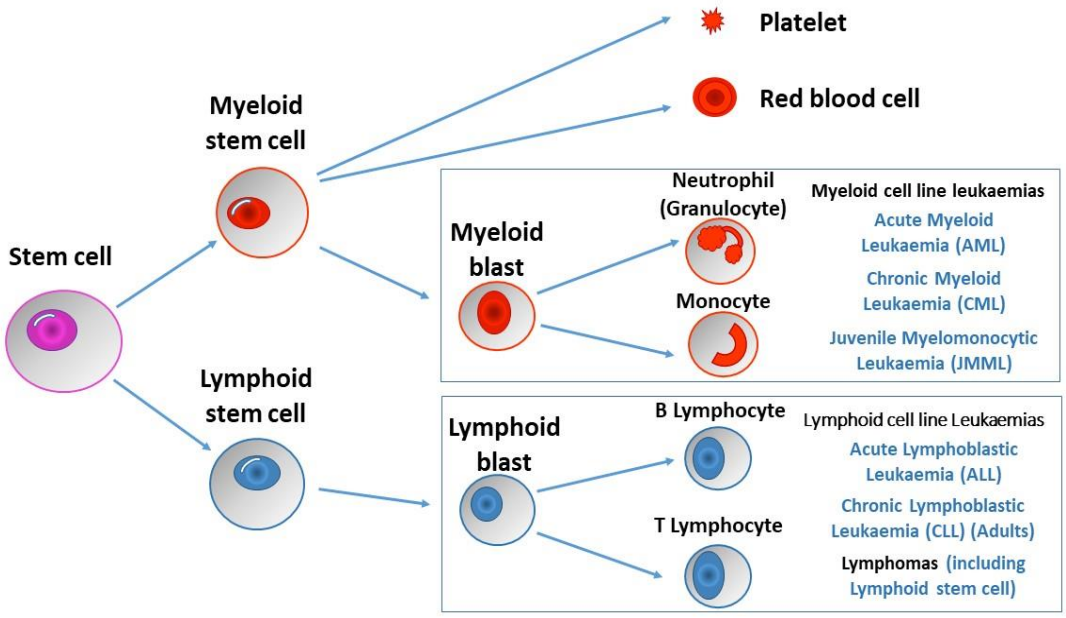

Figure 1.3: Childhood leukaemia and lymphoma types and the cells they originate from. CLL, Lymphomas and ALL B and T cell disease derive from lymphoid blasts from the lymphoid stem cell line, CML, JMML and AML derive from myeloid blasts which are from the myeloid stem cell line, these are also precursors to platelet and red blood cells. (adapted from Cancer Research UK 2018).

Leukaemia's develop from different progenitor cells, the leukaemia cells can develop from myeloid and lymphoid stem cells, blasts, or immature precursor cells to their final cell type,

for example ALL can develop from precursor B and T lymphocytes (Arber et al., 2016), figure 1.3. Lymphoma is a type of cancer of the lymphatic system. The lymphatic system is a network of vessels, tissues and organs which runs through the body carrying lymph fluid which contains white blood cells which attack invading pathogens (Children with Cancer UK, 2018c). Lymphoma occurs when a particular lymph node produces abnormal lymphoid cells, these are the same cells that are involved in the development of leukaemia but develop in the lymph system and not the bone marrow (Aifantis et al., 2008). There are two types of lymphoma; Hodgkin and non-Hodgkin lymphoma; Hodgkin lymphoma mainly occurs in the lymph nodes of the head and neck and is formed in specific Reed-Sternberg cells (Marcotte et al., 2014). Non-Hodgkin lymphoma can occur in any lymph node within the body and is made up of a number of subtypes for example; Large cell Lymphoma, Lymphoblastic Lymphoma and Burkitt's Lymphoma (Sandlund et al., 1996). The development of both types of lymphoma have been linked to viruses such as the Epstein Barr virus (Gandhi, 2006), also in several areas of the world, including Africa, Burkitt's Lymphoma is endemic (Burkitt and O'Conor, 1961). Lymphoma and leukaemia are also treated with similar protocols and drugs (Amylon et al., 1999), the UKALL2011 trial protocol, discussed later in the introduction, also includes guidelines for treating lymphoma in children (Goulden et al., 2017)

1.3.1 Acute Lymphoblastic Leukaemia (ALL)

Acute lymphoblastic leukaemia (ALL) is a cancer of the blood and accounts for almost 80% of childhood leukaemia's, there are around 400 new cases per year in the United Kingdom in children aged between 0-14 years old (Cancer Research UK, 2018a). Globally, there were a total of 53,000 recorded cases in adults and children in 2016, this number is expected to increase to 56,000 cases by 2020 (Bethlehem Solomon, 2017). More than half of the children diagnosed are under five years old, with peak rates between 2-5 years old. It is the only form of leukaemia that is more common in children than in adults, but has a higher mortality rates in adults (Sallan, 2006). Survival rates have improved dramatically since the 1960's where at the time childhood leukaemia was incurable. Now, due to improvements in care and investment in research, the five-year survival rate is approaching 90% in children (Pui et al., 2018).

1.3.1.1 ALL development

The development of ALL involves the abnormal proliferation of immature cells from the lymphoid lineage. Abnormal cells can development from lymphoid stem cells, blast and precursor B and T lymphocytes (American Cancer Society, 2018). The bone marrow produces a number of cells derived from stem cells which are released into the blood. A stem cell has the ability to differentiate into the three main blood cell types; healthy non-cancerous cells go onto develop into white blood cells which are used by the immune system to fight infection and release antibodies, red blood cells which carry oxygen around the body and platelets which are crucial to the clotting cascade and aid blood clotting in injury and recruitment of white blood cells in wound healing (King et al., 2018, Children with Cancer UK, 2018a), this is summarised in figure 1.3. In ALL, this process is disrupted, and the bone marrow makes too many lymphocytes, these lymphocytes are immature and do not develop correctly and never differentiate into mature B and T lymphocytes (Bürgler and Nadal, 2017). They begin to overcrowd the bone marrow making less room for other cells such as red blood cells and platelets. When the bone marrow becomes overwhelmed with the dysfunctional cells they spill out into the blood and disrupt the cell balance (Steele and Narendran, 2012). Patients become prone to infection because they do not have enough mature white blood cells, are anaemic due to low red blood cell numbers and are also prone to bleeding disorders because of reduced platelets (NHS UK, 2018). ALL develops from precursor B-cells and precursor T-cells (Arber et al., 2016), around 85% of cases derive from B-cells and 15% from T-cells (Chiaretti et al., 2014) each subtype has its own set of risk factors and genetic mutations which can contribute to disease progression and outcome. Table 1.1 shows a selection of the types of B and T-ALL and prognostic outcome. Although prognostic outcome can be less favourable in particular genetic subtypes, this has improved in recent years due to the introduction of newer protocols and treatments (Board, 2019). Even with these improvements T-cell disease is still notoriously difficult to treat (Raetz and Teachey, 2016).

Table 1.1: Genetic subtypes of ALL and their prognostic outcome information. Genetic abnormalities and cell origin can impact the prognostic outcome in ALL adapted from (Terwilliger and Abdul-Hay, 2017). *patients with this genetic abnormality were not included in the clinical study described later in this thesis.

	Genetic abnormality	Prognostic outcome
B Cell	Hyperdiploidy	Good
B Cell	Hypodiploidy or near Haploidy <39 chromosomes	Poor
B Cell	BCR-ABL Philadelphia Chromosome *	Poor
B Cell	MLL rearranged Translocation between chromosomes 4 and 10	Poor
B Cell	Translocation between chromosomes 12 and 21 ETV6-RUNX1	Good
T Cell	Various Mutations in NOTCH, PI3K/Akt/mTOR, JAK/STAT & MAPK pathways	Poor

1.3.1.2 ALL causes and risk factors

The precise pathogenic events leading to the development of ALL in specific individuals are largely unknown (Macmillan UK, 2018a). Studies in the paediatric population have identified a subset of genetic syndromes that predispose children to ALL, including Down's syndrome (Chessells et al., 2001), Fanconi anaemia (Swift, 1971), Bloom syndrome (Willis and Lindahl, 1987), ataxia telangiectasia (Boultwood, 2001) and Nijmegen breakdown syndrome (Pastorczak et al., 2011). Greaves two hit model puts suggests that genetic initiating events are identifiable in most cases of childhood ALL, but these alone are not sufficient for ALL to manifest and secondary "hits" are required (Greaves, 2018). It is suggested that ALL occurs in a sequence of events which begins with inherited genetics. Genetic drivers or first hits of ALL include the mutation and fusion of gene ETV6-RUNX1 (Zuna et al., 2011), loss of function of Ikaros in the development of BCR-ABL1 ALL (Mullighan et al., 2008), and hypodiploidy (loss of chromosomes) which creates a covert pre-leukemic clone (Paulsson et al., 2015). Interestingly it has been shown that 1% of the population have the ETV6-RUNX1 genetic mutation, but not

all of these individuals go on to develop ALL (Studd et al., 2017). The second hit involves lack of early microbial exposure, which fails to correctly prime the early developing immune system, and then an initiating event such as a later common childhood infection (Swaminathan et al., 2015). This introduces the critical secondary mutations which go onto trigger the development of ALL. Until more research is undertaken in this area for prevention-based medicine and lifestyle changes, children who develop ALL will consequently undergo a combination of cytotoxic chemotherapeutics with a wide range of lasting long-term side effects.

1.3.1.3 ALL treatment

Treatment for ALL can be divided into three phases, induction, consolidation and maintenance therapy.

Induction therapy - the aim of induction therapy is to get the patient into early remission. It is an intensive phase of treatment and involves using a combination of chemotherapy drugs which include vincristine or daunorubicin, L-asparaginase and a steroid, dexamethasone or prednisolone, figure 1.4. The goal of this stage is to clear as many leukemic blasts as possible from the blood and bone marrow. This stage lasts 4 weeks and childhood patients are admitted to hospital for this period.

Consolidation therapy - It is possible that some leukemic cells will remain after induction therapy, this stage aims to clear any remaining cells in the blood and bone marrow. Chemotherapy drugs are administered again, although at this point drugs may be changed to avoid tumour cells becoming resistant to them. This stage can last anything from 8 weeks to months and often the patient will be allowed to go home and treated as an outpatient.

Maintenance therapy - The final stage of therapy aims to prevent relapse and involves low dose chemotherapy and steroids. This stage can take up to two years and aims to keep the patient in full remission long term (Goulden et al., 2017).

Figure 1.4: Standard ALL treatment protocol and chemotherapy drugs used. Treatment is split into three phases, a high dose intensive induction phase with includes steroids, then a second consolidation phase which looks to consolidate results of induction therapy. After this a longer maintenance phase which monitors disease and reduces chances of relapse. (adapted from UKALL2011 study)

1.3.1.4 UKALL2011 Study

Patients within the UK, and the patient cohort recruited in our study, also follow the treatment guidelines for the United Kingdom National Randomised Trial for Children and Young Adults with Acute Lymphoblastic Leukaemia and Lymphoma 2011 (UKALL2011), the study recruited patients from April 2012 to December 2018, with data analysis ongoing to present (ISRCTN clinical registry, 2019). The overall aims of the study were to determine if

minimal residual disease (MRD) risk needed further refinement based on risk stratification and treatment type to improve overall survival and to reduce the burden of therapy in paediatric patients and young adults during treatment for ALL and Lymphoblastic Lymphoma. The objectives of the study were to reduce toxicity by swapping the lower dose of dexamethasone given for the full 28 days of induction and introduce a shorter 14-day course of higher dose dexamethasone. Secondly, to omit vincristine and dexamethasone pulses during intrathecal treatment to the central nervous system in maintenance therapy and reduce burden by using high doses of methotrexate. Finally, for low risk MRD patients, the study hopes to limit the toxicity of therapy by introducing a single delayed intensification, this will reduce overall burden and unnecessary treatment to lower risk patients (Goulden et al., 2017).

1.3.1.5 UKALL2011 Induction phase regimens

At diagnosis, patients are risk stratified by the National cancer institute (NCI) guidelines. Patients are risk assessed from initial blood tests; standard risk patients are below age 10 with a white cell count less than 50 x 10^9/l, and are assigned to regimen A which is a three-drug induction including vincristine, peg-asparaginase and dexamethasone. High risk patients are classed aged over 10 years old with a white cell count over 50x 10^9/l, and receive induction regimen B, which is four drug treatment including vincristine, peg-asparaginase, dexamethasone and daunorubicin. Poor cytogenetic risk patients receive regimen C induction therapy which is the same therapy as regimen B. This induction therapy starts at day 15 and is for high risk cytogenic patients under 10 years old with standard risk white cell count, or Down syndrome patients with a slow early response. Minimal residual disease (MRD) is measured at the end of induction on day 29 by taking a bone marrow sample and testing using Real Time Quantitative PCR by using probes to detect translocated genes or other associated genetic abnormalities. Patients are classified as MRD low risk on day 29 with a blast cell count <0.005% and high risk on day 29 with a blast cell count > 0.005%. (Information from UKALL2011 trial protocol).

1.3.1.6 Cytogenic risk

Cytogenic risk is a classification used to group patients depending on their risk of severe disease due to underlying genetic abnormalities which drive B and T-cell disease and is a strong predictor of prognosis and relapse (Moorman et al., 2010). Cytogenic risk groups in this patient cohort are classified as good, intermediate, high and the strongest predictor of poor outcome, T-cell disease, taken from UKALL2011 trial protocol, table 1.1 shows overview of common genetic abnormities and risk. In the UKALL2011 trail high risk cytogenetics are considered to be intrachromosomal amplification of chromosome 21 (iAMP21), t(17;19), (q22;p13)/TCF3, (E2A)-HLF, MLL rearrangement and near haploidy (<30 chromosomes) and low hypodiploidy (30-39 chromosomes).

1.3.1.7 Minimal residual disease (MRD)

During treatment patients are tested for their MRD risk, which is an independent prognostic factor for childhood ALL and lymphoma and can be used to predict the overall response to treatment and a potential complete remission (Luskin et al., 2018). The concept of MRD was first demonstrated in the early 1980's when it was discovered that patients with leukaemia could have measurable levels of leukemic blasts circulating in their system at various stages of treatment and these could be measured to predict treatment outcome and guide it (Bradstock et al., 1981). This procedure has become highly valuable in routine diagnostics, with up to date technologies such as Next Generation Flow being used for sensitivity and detection of residual leukemic blasts and to standardise results across clinics (Flores-Montero et al., 2017). This trial has used real time quantitative PCR for analysis of *Ig* and *TCR* gene rearrangements which are used to identify residual ALL cells. This measurement of early response to treatment has the greatest prognostic outcome than any other test to date, and has been the basis of many trials which aim to reduce the number of toxic drugs given unnecessarily to patients with good responses to induction therapy (Rytting, 2014). This has led to trials to reduce the steroid treatment and more toxic therapies (Biondi et al., 2000, Jacquy et al., 1997). The UKALL2011 trial, from which the patients are recruited to for this study, uses MRD risk to calculate the effectiveness of treatment and adjusts ongoing

treatment intensity accordingly after induction phase. Section 1.3.2 details the drugs used in combination treatments within the study and their mechanisms of action.

1.3.2 Chemotherapy treatments

1.3.2.1 Vincristine

Vincristine is a cytotoxic chemotherapy treatment from the family of drugs called Vinca Alkaloids which are plant alkaloids originally derived from the Madagascar periwinkle *Catharanthus roseus* (Noble, 1990). Vinca alkaloids have been one of the most effective treatments of leukaemia's and solid tumours since they were discovered in the 1950's (Moudi et al., 2013). Vincristine is one of the main chemotherapeutics used in combination for ALL and is administered in all three stages of treatment , figure 1.4. It is also used in treatment for acute myeloid leukaemia, Hodgkin and Non-Hodgkin lymphoma, lung and breast cancer solid tumours and neuroblastoma (NICE National Institute for Health and Care Excellence, 2018). Vincristine's mechanism of action involves binding irreversibly to microtubules and spindle proteins which form in the S phase of the cell cycle this in turn impedes the formation of the mitotic spindle (Mohammadgholi et al., 2013), when the mitotic spindle is unable to form the cells arrest in the G_2/M phase of the cell cycle and are unable to divide which induces apoptosis (Groninger et al., 2000). The most severe side effect of vincristine is accidental intrathecal administration which causes severe toxicity and often death (Reddy et al., 2011). Less serious, but still significant, side effects are neurotoxicity, neuropathy and seizures which often improve after treatment (Gomber et al., 2010).

1.3.2.2 Daunorubicin

Daunorubicin is an anthracycline drug and was first discovered in the 1960's when it was isolated from a soil based antibiotic microbe called *Streptomyces peucetius* (Cassinelli, 2016). Daunorubicin is an anti-tumour antibiotic and used in combination therapy of ALL and AML (Macmillan UK, 2018b). Its primary role is an enzyme inhibitor of Topoisomerase II involved in DNA synthesis (Hortobagyi, 1997). Topoisomerase II relaxes supercoils in DNA to enable transcription, by blocking its action daunorubicin prevents the resealing of DNA and inhibits

cell replication (Pommier et al., 2010). Daunorubicin lacks the specificity of other chemotherapeutics and can produce significant off-target toxicity (Polgár et al., 2018).

Figures suggest that 50 to 60% of childhood cancer patients will be treated with anthracyclines during their cancer treatment (Smith et al., 2010a). Because the side effect profile of daunorubicin is prominent in the cardiovascular system, there is a large proportion of childhood cancer survivors receiving this treatment with the risk of developing cardiac disease later in life (McGowan et al., 2017). Patients may benefit from the use of many of the small molecule inhibitors that have recently come onto the cancer drug market rather than risk the toxic profile of anthracyclines (McGowan et al., 2017). Further to this, results from the ELSPAR and FRALLE 2000 trials, where 1,128 patients were recruited, demonstrate daunorubicin can safely be omitted from precursor B-cell ALL standard risk patients, and in higher risk patients the addition of new drugs should be considered to reduce long term side effects (Baruchel et al., 2012). Daunorubicin can be administered in all three stages of treatment and to higher risk patients in induction category B and C as previously discussed, see figure 1.4.

1.3.2.3 Dexamethasone/Prednisone

Dexamethasone and prednisone are glucocorticoid steroid medications used in treatment of inflammatory and immune conditions, as well as cancer in conjunction with chemotherapy treatment (Nieman and Kovacs, 2011). Glucocorticoids have been used in the treatment of cancer for over 50 years due to their proapoptotic actions in haematologic cancers (Lin and Wang, 2016). They bind to intracellular glucocorticoid receptors in lymphocytes which then translocate to the target GRE site in the nucleus and upregulate genes promoting apoptosis therefore arresting cell growth (Schoneveld et al., 2004). Although evidence suggests that dexamethasone and prednisone exert their effect due to their proapoptotic actions, the mechanism of action is far more complex involving a number of cellular pathways and changes in gene expression (Pufall, 2015). The pathways include repressing the transcription factors activating protein 1 (AP1) and nuclear factor-Kβ or NF-kβ (Tissing et al., 2003) which are involved in cell cycle arrest and apoptosis (Harmon et al., 1979). A major clinical issue with glucocorticoids is drug resistance, where treated cells release calcium ions which induces cytochrome C release to trigger apoptosis, this in turn upregulates the expression of calcium

binding proteins and the antiapoptotic proteins Bcl-2 and Mcl-1. This then inhibits free cytosolic and mitochondrial calcium ion signalling and increases glucocorticoid resistance making the cells resistant to the original apoptotic signals (Hagelstein et al., 2009, Wei et al., 2006). Side effects of steroid treatment in ALL include increased appetite, excessive sleeping or difficulty sleeping, weight gain, fluid retention, indigestion and heart burn, increased risk of infection and mood disorders (Cancer Research UK, 2019b). More serious complications can include steroid-induced psychosis, bone toxicities and fractures, osteonecrosis and severe bone pain (Moghadam-Kia and Werth, 2010).

In patients receiving dexamethasone treatment for ALL, the quality of life (QoL) was found to be reduced in treatment overall but was aggravated when dexamethasone was administered as part of the protocol (de Vries et al., 2008). In addition to this steroids, and in particular dexamethasone, can be a cause severe bacterial and fungal infections and was significantly associated with death in induction therapy in the ALL2000 BFM /AIEOP study at higher doses 10mg/m^2 (Schrappe et al., 2008). With these findings it is difficult determine if dexamethasone is superior to prednisolone in treatments; both work in a similar way and in certain patients, like standard risk BCP ALL, prednisolone is superior. However, in T-cell ALL, dexamethasone is proven still superior in spite of the side effects and the higher induction related death rate (Möricke et al., 2016).

The following conclusions were taken from the ALL2000 BFM study (Carlson, 2016); The five cumulative relapse rates were 10.8% for patients receiving dexamethasone and 15.6% for patients receiving prednisone, extramedullary relapse had the largest impact. This was counterbalanced as patients receiving dexamethasone had a significantly higher rate of induction related death at 83.9% compared to 80.9% receiving prednisone. The overall 5-year survival rate there was no difference with 90.3% from dexamethasone and 90.5% from prednisone. Writing about the study (Hunger, 2016) speculated that dexamethasone improved event-free survival but not overall survival because more patients treated initially with prednisone were salvaged following relapse, this was because dexamethasone treatment had the superior effect on preventing extramedullary relapses which are easier to treat than marrow relapses. Children with relapsed ALL respond well to treatment and are more curable even with marrow relapse, these were prevented by dexamethasone. To further add to the options, when we consider patients over 10 years of age receiving the

anthracycline containing 4 drug induction the ALL2000 BFM trial have chosen to use prednisolone to reduce overall toxicity and combined induction therapy due to addition of anthracyclines in this age group moving forward (taken from UKALL2011 protocol). The rational for dexamethasone use in the UKALL2011 trial is to use dexamethasone in all stages of therapy using a shorter course of higher dose dexamethasone (14 days) in induction therapy and randomised pulsed dexamethasone in later stages to reduce toxicity while maintaining efficacy (taken from UKALL2011 Protocol).

Table 1.2: Toxicity of dexamethasone based induction in UKALL2003. (Information taken from the UKALL2011 trial protocol)

	A Induction	B/BC/AC Inductions	TOTAL
Number of patients treated	1126	1128	2287
Induction treatment related mortality	7	27	34
Induction steroid related adverse events	9	26	35
Induction steroid contributory adverse events	44	67	111

Table 1.2 shows the findings of the dexamethasone-based toxicity in the induction phases of the UKALL2003 trial which has helped guide the more up to date UKALL2011 protocol. Recruitment for the shorter 14 day induction phase of dexamethasone was stopped early due to concerns that there was a non-significant increase in induction treatment related deaths in NCI standard risk ALL patients, short phase dexamethasone 9/503 (1.8%), standard phase dexamethasone 4/503 (0.8%) (p=16) even though the study found that the shorter induction schedule of dexamethasone 10 mg/m^2/day x 2 weeks is no less toxic than 6 mg/m^2/day x 4 weeks in children and young adults with ALL (Goulden et al., 2017). The UKALL2011 study is trialling split dose dexamethasone for patients aged 10 or over who are at greater risk of developing Osteonecrosis, the outcome of these findings is ongoing. It is clear that even with a large amount of evidence from various studies tailoring steroid therapy in patients with ALL remains a challenge.

1.3.2.4 Pegylated L-Asparaginase

Pegylated L-asparaginase is a chemotherapy drug used in the treatment of ALL. It has been used in the management and protocols of childhood ALL and non-Hodgkin lymphoma since 1994 and has a strong antileukemic effect (Mondelaers et al., 2017). Pegylated L-asparaginase is a form of asparaginase covalently linked to a molecule called polyethylene glycol (PEG). When a substance is pegylated it is modified by adding PEG (Silverman et al., 2010). This changes the chemical properties of the drug molecule and can change the way it behaves in the body such as its hydrophobicity or reduced immune response in the patient due to less antibodies being produced in the patient in its L-asparaginase form, it increases the time in circulation with reduced side effects increasing its efficacy (Goodsell, 2005). L-asparaginase is an enzyme which breaks down an amino acid called asparagine (Müller and Boos, 1998), ALL cells are dependent on asparagine for protein synthesis and with less asparagine available in the blood from treatment this inhibits the growth of cancer cells (Pinheiro et al., 2002). The pegylated form of the drug is safer than the previous more toxic L-asparaginase (Piatkowska-Jakubas et al., 2008). Side effects from pegylated L-asparaginase include allergic reactions and hepatic dysfunction but these are lessened in the pegylated form (Keating et al., 1993). Pegylated L-asparaginase is administered in all three stages of treatment, figure 1.4.

1.3.2.5 Methotrexate

Methotrexate is a chemotherapy and an immune system suppressant drug and is used to treat cancer a number of autoimmune diseases such as rheumatoid arthritis and crohns disease (Friedman and Cronstein, 2018). The drug is used to treat types of cancer including leukaemia, lymphoma, lung cancer and breast cancer (Bryan, 2018). Methotrexate's mechanism of action involves inhibition of purine metabolism by interfering with folic acid synthesis which in turn disrupts nucleic acid synthesis, this then inhibits DNA and RNA synthesis and results in cell death (Tian and Cronstein, 2007). Methotrexate can be administered orally and by intrathecal injection, this is to treat cancer that has entered the cerebrospinal fluid (Olmos-Jiménez et al., 2017). Methotrexate is given as an intrathecal injection in the consolidation phase and orally and by intrathecal injection in the maintenance phase, see figure 1.4.

1.3.2.6 Cytarabine

Cytarabine is a chemotherapy agent used in the treatment of acute leukaemia's, CML and non-Hodgkin lymphoma and tumours associated with these blood cancers (National Cancer Institute, 2019b). Cytarabine is a cytosine analogue and is incorporated into DNA where it interferes with DNA polymerase and DNA chain elongation (Kufe et al., 1980), this in turn blocks synthesis and subsequently cell division causing cell death (Li et al., 2017). Cytarabine is given intravenously or subcutaneously during maintenance and consolidation phases, see figure 1.4.

1.3.2.7 Mercaptopurine

Mercaptopurine is a chemotherapy drug used in the treatment of ALL and non-Hodgkin lymphoma (Bökkerink et al., 1993) and the autoimmune diseases crohns disease and ulcerative colitis (Wahed et al., 2009). Mercaptopurine competes with purine derivatives and in turn is converted to inosine monophosphate (TIMP) (Stet et al., 1993). TIMP then goes onto to inhibit an enzyme involved in the *de novo* synthesis of purine synthesis, this in turn disrupts DNA and RNA function and causes cell death (Fernández-Ramos et al., 2017). Mercaptopurine is orally administered during maintenance and consolidation phases of treatment, see figure 1.4.

1.3.2.8 New therapeutic options in ALL

The drugs discussed previously are the main chemotherapeutics used in ALL paediatric treatment therapy, many of these drugs used in the ALL regimens were developed over 30 years ago and while they have led to increased survival rates patients can be left with lifelong conditions, known as late effects, and still be at risk of relapse (Kızılocak and Okcu, 2019). In recent years there has been a call for a more personalised approach to ALL and targeted treatment, particularly of higher risk T-ALL patients (included in this cohort) and patients with Philadelphia chromosome positive B-ALL (not included in the patient cohort). The following review gives up to date information on targeted therapies and newer drugs and trials (Phelan and Advani, 2018). Advances include; drugs used in immunotherapeutic approaches, such as rituximab, epratuzumab and blinatumomab which target the cell surface antigens CD20, CD22

and CD19 respectively, epratuzumab has been used safely in paediatric ALL trials (Raetz et al., 2015). There has also been a major breakthrough in personalised medication in ALL using Chimeric antigen receptor (CAR) T cells derived from the patient's own T-cells (Rogosic and Ghorashian, 2020). The cells are genetically modified and returned to the patient to target a chosen antigen on the surface of their lymphoblasts and directly kill the circulating cells or tumour cells. Although effective, this treatment is costly and not feasible to introduce as treatment for every ALL patient and particularly not feasible as treatment in the developing world.

Other new drug treatments in clinical trials in ALL target receptors or pathways within the cell; mTOR inhibitors, PI3K/Akt inhibitors are options to block the PI3K/Akt/mTOR signalling pathway which has shown to be activated in B-cell ALL (Neri et al., 2014) and also T-cell ALL (Evangelisti et al., 2020). Janus kinase-signal transducer and activator of transcription (JAK-STAT) inhibitors have also been evaluated in ALL subtypes that have amplification of the JAK-STAT signalling pathway, ruxolitinib and dasatinib are currently in clinical trials (Tasian et al., 2018). Drugs that target the B cell lymphoma-2 (Bcl-2) proteins which regulate apoptotic cell death have been developed over the past few years, Navitoclax and Venetoclax are Bcl-2 inhibitors which shift the balance of the cell to a proapoptotic phenotype and induce cell death. Venetoclax is currently in clinical trials for paediatric relapsed ALL (Place et al., 2018). Other research has shed light on targeting the unfolded protein response (UPR) which combats chemoresistance as a treatment option in acute leukaemia's (Masouleh et al., 2015), in B-cell ALL the use of the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide along with Methotrexate or 2-deoxy-D-glucose (2-DG) activated prolonged ER stress which in turn induced a proapoptotic phenotype from the UPR network . A similar response was observed in T-ALL using the AMPK activator metformin where it was used alongside 2-DG, here the UPR activated apoptosis causing increased T-cell death (Leclerc et al., 2013). Recent research is proving insight into the efficacy of autophagy inhibition in ETV6-RUNX1 B-cell ALL, treatment with the autophagy inhibitor hydroxychloroquine significantly reduced cell proliferation and survival (Polak et al., 2019). Advances in high throughput screening techniques have led to the identification of many potential targets of small molecule inhibitors in the treatment of acute leukaemia's and ALL, these can be used alone or in combination with existing therapies, the following studies look at targeting chemoresistance

in leukemic stem cells and in ALL cells using newly identified small molecule inhibitors (Dieck et al., 2019, Tremblay et al., 2020)

1.3.3 ALL treatment side effects and survival statistics

ALL treatment is effective in children, but chemotherapy is toxic and has the potential to damage developing organs within the body and leave children with late effects, including subclinical metabolic disorders early in life even after successful cancer treatment (Schwartz, 1999). During treatment patients often present with the following side effects; general weakness, fatigue, high temperature, infections, bruising or bleeding easily, swollen lymph nodes, pain in the joints and bones, breathlessness due to anaemia, swollen stomach due an enlarged liver or spleen, hair loss and pale skin (Ness et al., 2011). Furthermore, dexamethasone increases appetite which causes weight gain, which, although positive for stimulating appetite in patients, nutritional interventions are recommended to promote healthy choices during treatment (Warris et al., 2017). The Dexadays study of ALL patients demonstrated that dexamethasone contributes to the development of metabolic syndrome and markers of early insulin resistance just 4 days after induction treatment (Warris et al., 2016). Long term survivors of ALL are also at greater risk of obesity (Razzouk et al., 2007) and obesity related diseases such as hyperlipidaemia, high blood pressure, diabetes mellitus and cardiovascular disease (Barnea et al., 2015). A recent study has implied that there is 'no safe dose' of anthracycline drugs in terms of cardiotoxicity for paediatric cancer patients as treatment related damage is irreversible with cumulative anthracycline dosage throughout stages of treatment (Bansal et al., 2018). In the US the National Institute of Cancer support the Childhood Cancer Survivor Study (CCSS) is a multi-institute research initiative which tracks a large cohort of patients across the country, their research also includes many of the less studied problems faced in life for childhood cancer survivors such ongoing pain, mental health status, being underweight or obese and coping with reduced activity levels or limitations (Ness et al., 2017). Their study concluded that overall self-reported health status has not improved even with treatment designed to reduce toxicity, research also included what has been shown to help survivors like exercise (Scott et al., 2018). As discussed previously, survival rates have improved dramatically since the 1960's when leukaemia was considered incurable, now with current chemotherapy regimens over 90% of patients achieve full remission from

treatment, survival rate is highest in children diagnosed under the age of 4 years old (Children with Cancer UK, 2018b). This study will look at the links between weight gain and treatment success, becoming obese or overweight and how it relates to ALL will be discussed in the next sections.

1.4 Obesity

Obesity is recognised by the World health organisation (WHO) as a global epidemic and is now termed "globesity" and almost three quarters of people worldwide are expected to be overweight by 2035 (World Health Organisation, 2018). In the UK alone, five million people are expected to be morbidly obese by 2035 (Keaver et al., 2018). It is predicted that 700,000 new cases of obesity related cancer will be reported by 2035 (Cancer research UK, 2018b). Major drivers for the rise in obesity levels include excessive calorie intake (Hall et al., 2012), changes in eating habits such as fast food diets, sugary drinks and increased levels of dietary fat (Prentice and Jebb, 2003). Lack of exercise and sedentary lifestyles also contribute to the growing number of overweight or obese individuals (Tremblay et al., 2010). In a small number of cases, genetics can predispose a person to obesity; genome wide association studies (GWAS) have identified a number of candidate genes which, when interacting with environmental and lifestyle choices, can contribute to increased body fat (Choquet and Meyre, 2011). Candidate genes that are associated with obesity in adults and children include *POMC, LEPR* (Ayers et al., 2018). Other genes linked to obesity development in children include *SIM1*, the following study found early onset obesity was associated with copy number variations (CNV) in the gene *SIM1* (Windholz et al., 2017), another study found frame shift deletions in the *MC4R* gene in early onset obesity (Loid et al., 2020). Body mass index (BMI) is a measurement used to predict how healthy a person is by measuring their height and weight, the units of BMI are expressed as kg/m^2 which is mass divided by the square of height (Nuttall, 2015). BMI measurements fall in the following categories which are standards used and developed by The World Health Organization, see table 1.3.

Table 1.3: WHO definitions of obesity and associated BMI range. (Information taken from The World Health Organisation).

BMI, kg/m^2 range	WHO classification
<18.5	Underweight
18.5-24.9	Normal
25.0-29.9	Overweight/pre-obesity
>30.0	Obesity
>40.0	Morbid obesity

The BMI calculator can also be used to work out if a child is at a healthy weight, children are also put into a percentile range by calculating a child's BMI percentile, their age and sex then comparing the statistics to other children in that percentile category. In children and young adolescents, a BMI percentile that falls below the 5th percentile is underweight and above the 95th percentile obese, when a child falls between 85th and 95th percentile they are in the overweight category, being in the 95th percentile is also specific indicator of body fatness in children (Freedman and Sherry, 2009). There has been disagreement as to whether BMI is the best indicator of body fatness (Liu et al., 2013), in people with a high proportion of muscle tissue BMI measurement can be misleading due to their higher weight but lower body fat percentage (Mazic et al., 2009). Whilst BMI is not a direct measurement of adiposity it is generally accepted that in a large percentage of the general population who are in the obese and underweight category BMI does correlate with body fatness (De Schutter et al., 2011) less so in the overweight category. Obesity is associated with an increase in adipose tissue and circulating adipokine levels and can have detrimental effects in the body and a negative effect on health (Balistreri et al., 2010).

1.4.1 Obesity and Adipose tissue

Adipose or fat tissue was originally characterised as having little to no basic physiological function, mainly a storage vessel for energy and fatty acids for times when excess nutrients are needed and insulation to protect from cold temperatures. One of the main functions of adipose tissue is to store excess nutrients such as free fatty acids and triacylglycerols during

times of fasting (Carobbio et al., 2017). Currently, it is recognised that adipose tissue is a very complex organ in its own right with roles that regulate the immune system, the endocrine system and to maintain energy balance and metabolic systems (Booth et al., 2015). The heterogenous tissue, comprised of adipocytes, pre-adipocytes, macrophages, mesenchymal stem cells and immune cells such as B and T-cells (Lenz et al., 2020) , secretes physiologically active factors, known as adipokines, which can influence a raft of processes within the body (Taylor, 2021). Adipose tissue can be separated into four different types of tissue; white adipose tissue (WAT) which is localised subcutaneously and surrounds internal organs and has a specialised role in storing energy and is involved in weight regulation (Heyde et al., 2021). Brown adipose tissue (BAT) situated in the paracervical and supraclavicular regions, its main role is regulating body temperate in response to energy intake from food and cold weather (Duong et al., 2017). Beige adipose tissue which consists of BAT and can appear in WAT cell areas and increase whole body energy expenditure (Wu et al., 2013). Finally, Marrow adipose tissue (MAT) which represents over 5% of the total bone marrow mass (Hindorf et al., 2010). MAT was historically thought to be an inactive filling of the bone marrow cavity but has now shown to be increased in diseases such as obesity and diabetes (Veldhuis-Vlug and Rosen, 2018). More importantly, like WAT, MAT is metabolically active and produces its own secretory molecules such as adipokines which act systemically in the body and locally within the bone (Cawthorn et al., 2014). MAT has been shown to contribute to increased circulating levels of adiponectin (an adipokine) during caloric restriction (Cawthorn et al., 2014). Obesity is a proinflammatory condition where increased numbers of hypertrophied adipocytes contribute to low grade systemic inflammation and higher circulating levels of pro inflammatory cytokines or adipokines (Makki et al., 2013). This low grade systemic inflammation is considered the hallmark in the pathogenesis of insulin resistance and type 2 diabetes in humans (Ouchi et al., 2011). Obesity can modify adipose tissue and change the way it functions, these changes include expansion and recruitment of inflammatory immune cells and induction of fibrosis and angiogenesis (Herold and Kalucka, 2020), together this is linked with increase aggressiveness of cancer and creates the perfect setting for cancer cells to thrive (Zhang et al., 2016). Further to this, there is a potential role of adipose tissue in general attracting ALL cells to potential niche environments such as the bone marrow to support cancer progression and tumour growth and providing a source of fuel for ALL (Tucci et al., 2021). How circulating levels of adipokines and low-grade inflammation promotes

cancer progression may be important to consider its role in disease outcome. The next sections will introduce some key adipokines and those that have been implicated in cancer and in ALL.

1.4.2 Adipokines

Adipokines are messenger molecules similar to hormones that are secreted from adipocytes in adipose tissue and exert their effects on other cells in the body (Sarmento-Cabral et al., 2017b). Adipokines contribute to a number of biological functions within the body; when the body is healthy, adipokines help regulate appetite, energy expenditure, metabolism, blood pressure and immune function (Sarmento-Cabral et al., 2017a). In conditions such as obesity, dysregulated adipokine secretions are the proposed link between higher levels of body fat and cancer, cardiovascular and inflammatory disease (Codoner-Franch and Alonso-Iglesias, 2015). Adipokines can act in an autocrine, paracrine, and endocrine manner and an individual adipokine can function in all three ways (Francisco et al., 2018). Adiponectin, a well-studied classical adipokine can act in an autocrine manner to prevent the release of insulin resistance inducing factors which have an important anti-diabetic function (Dietze-Schroeder et al., 2005). Leptin can work in a paracrine manner in the cancer microenvironment (Hosney et al., 2017) and Resistin can act in an endocrine manner to modulate insulin resistance throughout the body (Zaidi and Shirwany, 2015). Adipokines can also act as novel biomarkers in obesity-related disease and can be monitored to predict outcomes in disease (Rajandram et al., 2019). A number of adipokines have been linked to cancer development including adiponectin (Yu et al., 2019a), leptin (Gu et al., 2019b) and resistin (Avtanski et al., 2019). The following sections explain a selection of the adipokines of interest to this study and their relationship to cancer and ALL.

1.4.2.1 Adiponectin

Adiponectin is considered to be the “Guardian angel adipokine” and an anti-inflammatory factor due to its protective role within the body (Taliaferro-Smith et al., 2013). Adiponectin is low in many conditions such as cardiovascular disease and obesity and these reduced levels have a key role in the development of obesity-related conditions. This includes cancers associated with obesity (Nigro et al., 2018, Yu et al., 2019b), cardiovascular disease (Kyrou et

al., 2017), insulin resistance (Yadav et al., 2013) and type 2 diabetes (Lindberg et al., 2015). Adiponectin has been shown to be the most promising adipokine in developing novel intervention strategies in cancer and obesity-related disorders this has led to the development of the adiponectin agonists ADP355 and AdipoRon which have shown similar effects to endogenous adiponectin on the downstream AMPK and PPAR-α signalling pathways (Okada-Iwabu et al., 2013, Otvos et al., 2015). Studies have also successfully used the PPARy agonists rosiglitazone and pioglitazone to elevate levels of adiponectin in animals and humans to improve outcomes in disease (Combs et al., 2002). More recent research is investigating the role of the mTOR pathway in ALL by developing anti-mTOR compounds which would counteract the effects of low adiponectin in patients with ALL (Simioni et al., 2019). Figure 1.5 provides an overview of the pathways involved. Low adiponectin known as hypoadiponectinaemia is associated with ALL (Moschovi et al., 2010a) in this study low adiponectin and high resistin and leptin were recorded at diagnosis and in successful therapy were slowly restored to normal levels. The following studies have also reported low levels of adiponectin in ALL (El-Baz et al., 2013b, Ma et al., 2016, Srivastava et al., 2015b). Further research has focused on activating AMPK in ALL cells which is reduced in hypoadiponectinaemia, and has studied the effects of 5-amino-4-imidazolecarboxamide riboside (AICAr) which can induce apoptosis in ALL cells (Sengupta et al., 2007). More recently AICAr has been studied further to understand it's cytotoxic mechanisms revealing that AICAr impairs pyrimidine biosynthesis and supresses ribosomal RNA leading to cell cycle arrest and apoptosis in ALL cells (Du et al., 2019).

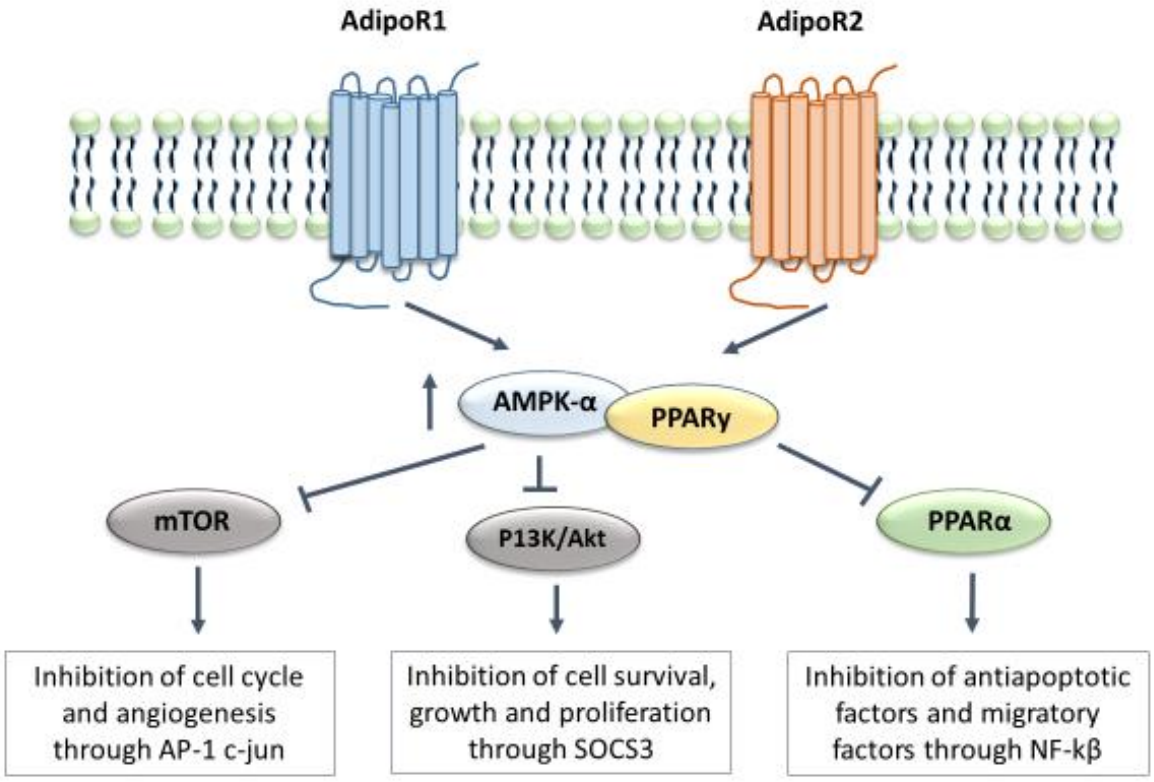

Figure 1.5: Adiponectin receptor signalling pathways. The activation of adiponectin receptors activates AMPK which blocks the proliferative effects of PI3K/Akt/mTOR pathways and the PPARα pathway which blocks anti apoptotic pathways through NF-kβ signalling, low levels of adiponectin reverse this action and activate the downstream proliferative pathways, adapted from (Azamar-Llamas et al., 2017)

1.4.2.2 Leptin

The adipokine leptin was the first adipokine to be discovered in 1994 and is primarily associated with feeding behaviour and appetite control (Castracane and Henson, 2006). Leptin is strongly associated with obesity and weight gain; blood levels of leptin correlate positively to the amount of adipose mass in a patient (Ekmen et al., 2016). This also leads to a condition called leptin resistance which maintains a high detectable level of leptin in the blood of obese patients (Knight et al., 2010). Leptin in itself is considered to be a proinflammatory adipokine and it can also initiate the production of other proinflammatory adipokines such as IL-6 and TNFα and MCP-1 (Wang et al., 2019). The biologically active form of the leptin receptor, Ob-R, activates intracellular pathways including STAT3, MAPK, P13K/Akt/mTOR and ERK1/2 (Achkar et al., 2019) , see figure 1.6. When activated in cancer, and cancers associated with obesity, high leptin levels can change chemotherapy sensitivity

(Ma et al., 2019). In this study high leptin levels upregulated the micro RNA miR-342-3p which potentiated gemcitabine resistance. A further study in endometrial cancer identified that leptin activated the STAT3 ERK1/2 pathways in cancer and induced proliferation of Ishiwaka cells, the group used kinase inhibitors to abolish the activation of ERK1/2 and STAT3 pathways this blocked the effects of leptin (Liu et al., 2011).

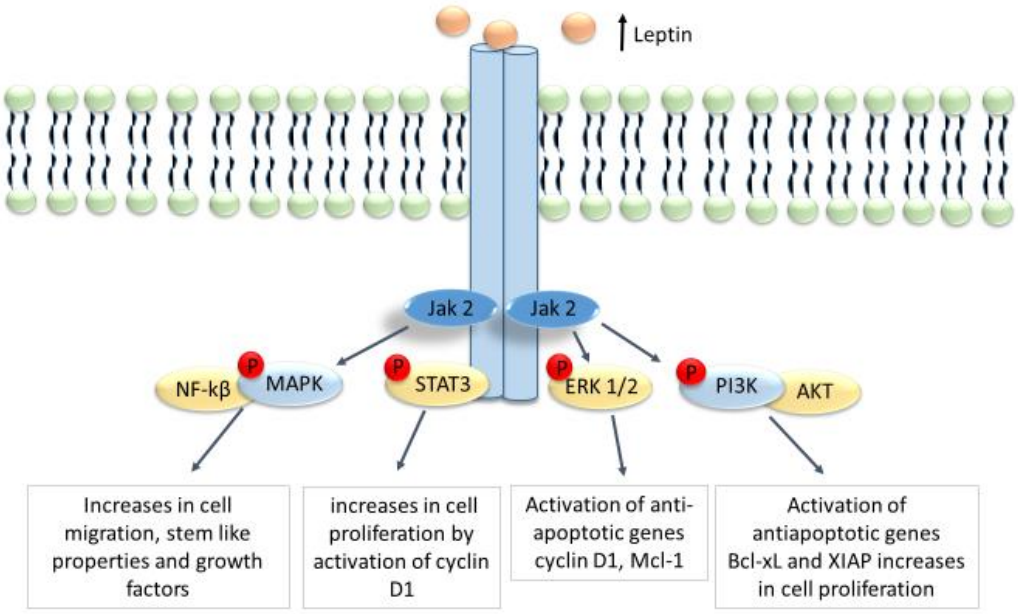

Figure 1.6: Leptin receptor signalling pathways. Increased levels of leptin activate the downstream proliferative pathways of NF-kβ and MAPK, STAT3, ERK1/2 and PI3K/Akt/mTOR adapted from (Jiang et al., 2014)

Although leptin is an adipokine of interest in cancer, the current understanding of leptin in ALL is mixed. Leptin levels are reported to be dysregulated in ALL; significantly higher leptin levels between overweight ALL patients compared to normal weight range patients have been reported (Skoczen et al., 2011), in fact leptin levels have been shown to be raised in ALL, and are higher than levels recorded in AML patients (Shahramian et al., 2016). However, no associations of the individual patients leptin receptor genotype contributed to leptin dysfunction but they did find a possible association between raised levels of leptin after 7 days treatment of high dose steroids (Tavil et al., 2012). In a further study, obesity was reported as a complication of ALL treatment and was associated with elevated levels of

circulating leptin, suggesting that weight and BMI should be considered during the course of treatment (Zareifar et al., 2015). New therapeutic approaches to controlling leptin physiology in patients, found that fasting blocks the development of ALL and this effect was due to upregulation of leptin receptors throughout the body, thus decreasing circulating levels of leptin (Lu et al., 2017). A further study in mice reported that switching to a low-fat diet with a reduced calorie intake improved outcomes in their pre-clinical model and improved outcome on vincristine based treatment, although this study did not include leptin it suggests the importance of calorie restriction during treatment of ALL (Tucci et al., 2018).

1.4.2.3 Resistin

Resistin is an adipokine released from several types of cell including adipocytes (McTernan et al., 2002). Murine resistin is primarily expressed from adipocytes (Hartman et al., 2002) whereas human resistin is predominantly expressed by macrophages and peripheral blood mononuclear cells (PBMC) and is associated with macrophages in the tumour microenvironment (Gong et al., 2018b). Resistin has been shown to be involved in the pathological response in cardiovascular disease, T2D, inflammation (Cobbold, 2019), it can also promote cancer progression by enhancing cancer cell proliferation in a number of cancers (Housa et al., 2008, Malvi et al., 2018, Zaidi and Shirwany, 2015). Research has recently discovered more about the biological function of resistin and its functional receptors in humans. The receptors discovered are adenylyl cyclase-associated protein-1 (CAP-1) (Lee et al., 2014) and toll-like receptor 4 (TLR4) , both receptors have been implicated in cancer progression (Zhang et al., 2019). Other receptors include ROR1 which serves as a receptor in murine 3T3-cells and their development (Sánchez-Solana et al., 2012) and decorin, a receptor on WAT, where it may regulate white adipose tissue expansion (Daquinag et al., 2011). The pathways involved after activation of the receptors CAP-1 and TLR4 include activation of intracellular cAMP, increased Protein kinase A (PKA) and NF-kβ (Lee et al., 2014) and activation of STAT3 by the TLR4 receptor which increases cell survival, see figure 1.7.

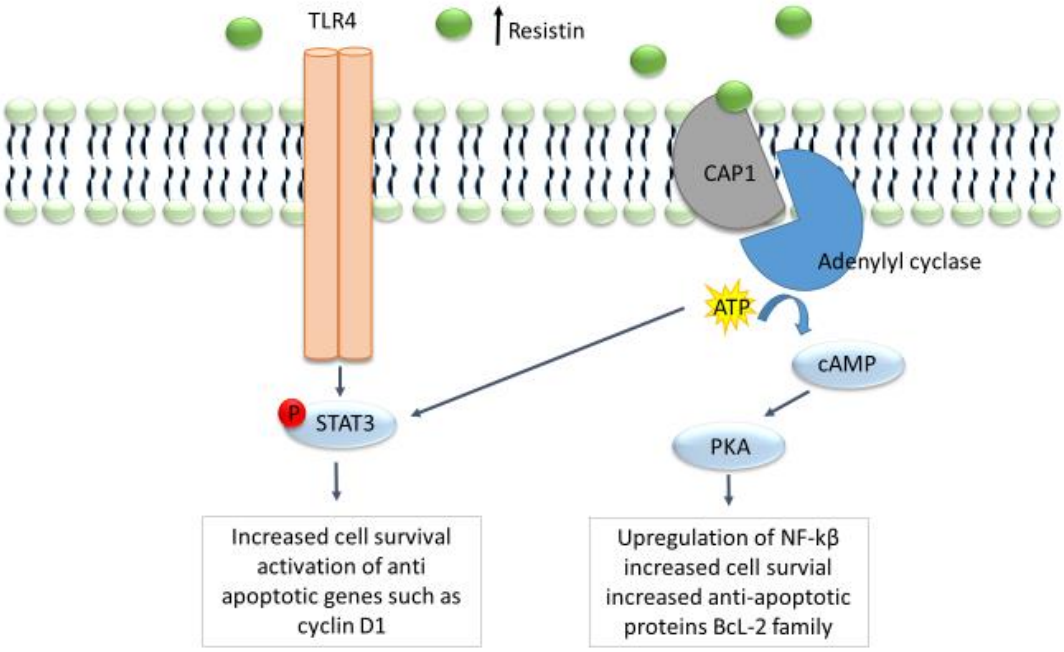

Figure 1.7: Resistin receptor signalling pathways. Resistin has been shown to act through the TLR4 receptor where it activates STAT3, also the CAP1 receptor which activates the antiapoptotic NF-kβ prosurvival pathway, information adapted from (Lee et al., 2014) (Zhang et al., 2019)

In a study to asses biomarkers in children with ALL, resistin levels were similar in obese and non-obese patients (Srivastava et al., 2015b). In a further study low adiponectin and high resistin at diagnosis was implicated in ALL pathogenesis, the group suggested the results may serve as a potential diagnostic marker to detect disease relapse (El-Baz et al., 2013b). Finally in a study investigating adipokines in plasma concentrations throughout therapy, resistin levels were high at diagnosis along with high leptin and low adiponectin and were progressively restored by the end of therapy with a decrease in plasma resistin levels (Moschovi et al., 2010a).

1.4.2.4 Serpin A12 (Vaspin)

Serpin A12 or vaspin is an adipocyte-derived serine protease inhibitor which is associated with impaired insulin resistance and obesity in adults (Youn et al., 2008). In children, a study found significantly increased levels of vaspin in obese children compared to their lean counterparts (Yin et al., 2019). Circulating vaspin levels have been shown to bind with the cell surface

receptor GRP78 which relocates from the ER membrane during events that cause ER stress (Nakatsuka et al., 2012). This response is a consequence of the unfolded protein response which is activated by an accumulation of unfolded proteins and oxidative stress in a number of cancers and increases cancer cell stemness and chemoresistance (Dauer et al., 2019). When the GRP78 receptor binds to vaspin this activates the PI3K/Akt signalling pathway, see figure 1.8, this leads to increased cell survival. GRP78 relocation to the cell surface as part of the unfolded response has been shown to activate PI3K/Akt signalling in other cancers (Fu et al., 2014, Zhang et al., 2013). In a study targeting the GRP78 receptor in leukaemia and lymphoma cell lines, the ALL cell lines Molt4 and CEM-CCRF both responded to the drug BMTP-78, a GRP78 binding peptide by reduced cell viability even at low molar concentrations of the drug (Staquicini et al., 2018). A further study confirmed high expression of GRP78 on the surface of human ALL B-cells where it was associated with vincristine resistance and disease relapse, this was accomplished by using HSPA5 inhibitor epigallocatechin gallate, which inhibited the antiapoptotic actions of GRP78/HSPA5 (Uckun et al., 2011).

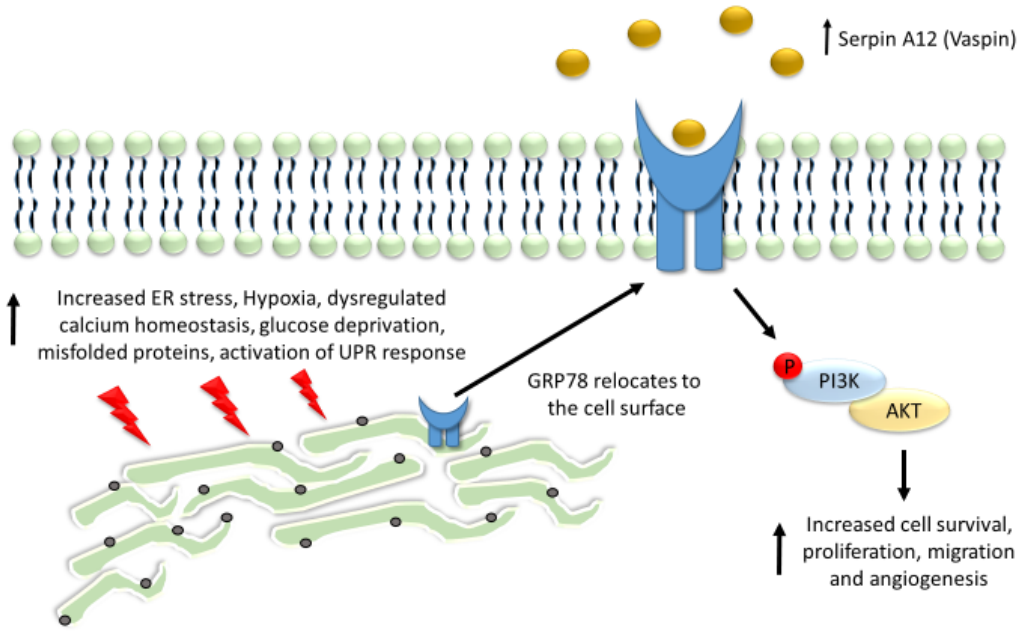

Figure 1.8: GPR78 receptor signalling pathways. Part of the UPR response linked to ER stress and potential relocation of the GRP78 receptor to the cell membrane which activates the PI3K/Akt/mTOR pathway.

1.4.2.5 Interleukin-8

IL-8 is a pro-inflammatory cytokine that acts through the receptors CXCR1/2 to activate a number of downstream pathways including PI3K/Akt and JAK/STAT3 in cancer (Liu et al., 2016). Studies investigating immature AML stem cells have shown that IL-8 is an adverse factor in the development of the disease (Schinke et al., 2015), less is known about the specific effects in ALL cells. A study looking at the interactions between ALL cells and bone marrow microenvironment found that the bone marrow microenvironment had increased levels of IL-8, the increased levels had a positive impact on bone marrow mesenchymal (BMMS) cells which increased their adhesiveness to All cells which in turn favoured ALL cell survival, it was noted that IL-8 had no direct impact on ALL cells alone without the presence of BMMS cells (de Vasconcellos et al., 2011)

1.4.2.6 Endocan/ESM-1

Endocan is a proteoglycan which is expressed in obese states at higher levels and also in metabolic cardiovascular diseases (Klisić et al., 2021). Endocan has been linked to poor outcomes in many cancers, in breast cancer overexpression promoted tumorigeneses by inducing cell proliferation, invasion and metastasis through NF-kβ and STAT3 pathways in mouse studies (Jin et al., 2020). Endocan levels in acute leukaemia's are highly expressed and return to normal levels after chemotherapy and increase again at relapse so has been suggested as a potential biomarker (Xu et al., 2014a). In ALL, higher circulating endocan levels in paediatric patients have been directly implicated in sepsis with statistically significantly higher levels recorded in patients with febrile neutropenia (Kiral et al., 2016). Research published this year studied endothelial dysfunction and mean common carotid artery thickness in a group of paediatric ALL survivors which correlated with high endocan levels. Patient triglycerides, cholesterol, blood glucose were all significantly higher in survivors compared to the control group, the consequences of these findings have put the patients at risk of endothelial dysfunction and development of premature atherosclerosis (Sherief et al., 2021)

1.4.2.7 IGFBP4

Insulin-like growth factor binding proteins (IGFBPs) can interfere with and modulate the biological actions of other insulin like growth factors (Allard and Duan, 2018). IGFBP4 has been shown at higher concentrations to inhibit the mouse tumour cell proliferation effects of insulin growth factor 1 (IGF1) (Ryan et al., 2004). In a study investigating the relevance of IGFBP4 in acute lymphoblastic leukaemia, levels were significantly reduced at diagnosis compared to healthy subjects and further reduced at day 33 after induction therapy compared to diagnosis, however this study found no correlation with ALL risk group (Wex et al., 2005).

1.4.2.8 Pref-1/DLK1

Pref-1/DLK1 protein is described as the gatekeeper of adipogenesis and its expression is high in preadipocytes and its expression is downregulated in adipocyte differentiation which in turn inhibits differentiation (Smas and Sul, 1993), so high expression levels inhibit differentiation of adipocytes. Research has confirmed that the DLK1 acts as an antagonist of the NOTCH1 receptor (Baladrón et al., 2005), but can potentiate growth of mesenchymal cells through activation of ERK1/2 so it could be dependant of cell type (Nueda et al., 2007). Other studies have confirmed that there is a mixed role depending on the cell involved, the following study confirmed that DLK1 activated NOTCH signalling in lung cancer which led to increased proliferation (Li et al., 2014). Studies in ALL show how exogenous DLK1 protein activates upregulation of the NOTCH1 receptor which in turn stimulates the proliferation of the ALL cell line CEM-CCRF, and activation of this receptor upregulated the downstream targets *c-myc* and *NF-κB* (Wei et al., 2013). Studies have suggested the use of NOTCH1 and PI3K/Akt/mTOR inhibitors as a dual treatment strategy in T-cell disease due to the interactions between the two pathways (Khoshamooz et al., 2020)

1.4.2.9 Cathepsin-S

Cathepsin S is a proteolytic enzyme involved in cancer cell proliferation, migration and invasion, it has been targeted in a number of diseases including cardiovascular and arthritis (Wilkinson et al., 2015). There is limited information on its role in ALL, in other cancers it has

been implicated due to its immune suppressive activity where its knockdown or inhibition promotes anti-cancer immunity (Fuchs et al., 2020). In a study in colorectal cancer, using the small molecule inhibitor Fsn0503h which targeted antibody mediated cellular toxicity via cathepsin S, the inhibitor was capable of blocking angiogenesis, endothelial tube formation and tumour cell invasion (Kwok et al., 2011).

1.4.2.10 Angiopoietin-2

Angiopoietin-2 is a growth factor involved in angiogenesis which exerts its effects by interacting with the angiopoietin/Tie2 signalling axis (Felcht et al., 2012). Angiopoietin-1 acts as the main agonist and target for the Tie2 receptor where angiopoietin-2 acts as an antagonist for this receptor (Maisonpierre et al., 1997). In more recent studies angiopoietin-2 alone at higher expression levels can act as an agonist for the Tie2 receptor but when expressed together angiopoietin-2 mainly acts as an antagonist for the Tie2 receptor (Kim et al., 2000). A study in childhood ALL found that angiopoietin-1 was low at diagnosis and angiopoietin-2 was high, in response to treatment this finding was reversed with higher levels of angiopoietin-1 and reduced levels of angiopoietin-2 at day 33 of treatment (Karakurt et al., 2016). Activation of the Tie2 receptor is an activator of the downstream PI3K/Akt pathway in oncogenesis (Park et al., 2010).

1.4.3 Consequences of obesity

There are many medical consequences of obesity, being overweight or obese can have a negative effect on many systems in the body. Obesity is associated with reduced lifespan compared to an individual within the normal weight range (Nagai et al., 2012). Health conditions and co-morbidities related to being overweight include cancer, metabolic syndrome, type 2 diabetes, stroke, high blood pressure, heart disease which includes coronary heart disease from atherosclerosis, congestive heart failure and myocardial infarction (Pantalone et al., 2017). Other outcomes from being obese include asthma, reproductive disease, osteoarthritis, chronic back pain, pulmonary embolism, gall bladder disease and mental health issues (Pi-Sunyer, 2009). Many of the cardiovascular disease diagnoses stem from complications of type 2 diabetes, this causes high blood sugar and poor insulin handling in the body, this also contributes to kidney disease, neuropathy and

retinopathy (Papatheodorou et al., 2018). The pattern of distribution of weight in obese individuals can contribute further to complications, visceral fat or fat that accumulates around the waist has a strong association with cardiovascular disease compared to subcutaneous fat which is distributed around the hips and lower body (Elffers et al., 2017). This can be measured by the waist to hip ratio where a higher waist to hip ratio is strongly linked to cardiovascular disease and complications of obesity (de Koning et al., 2007). This visceral fat that is stored around the waist and organs in the abdominal cavity is also referred to as abdominal obesity and is highly active and the main contributor to metabolic syndrome (Paley and Johnson, 2018). Obesity is also a risk factor for developing cancer and when an obese patient is diagnosed with cancer it can predict a poorer outcome (Petrelli et al., 2021), this will be discussed in the next section.

1.5 Obesity and Cancer

Recent research links obesity to an increased risk of cancer and also an increased risk of cancer related death, including studies in younger populations born after 1985 (Sung et al., 2019). A number of epidemiological studies have shown that obesity is linked directly to a number of cancers and links to poor prognosis once diagnosed, one such study involved a cohort of 1.2 million UK women where results indicated that up to 50% of cancer cases in post-menopausal women were attributable to being overweight or obese (Reeves et al., 2007). One of the first studies to confirm the link between obese cancer patients and mortality was in 2003, the study confirmed that mortality rates for obese men and women with a BMI over 40 were 52% and 62% higher respectively (Calle et al., 2003). Many studies since then have shown correlations between cancer and obesity (Arnold et al., 2016). Cancer research UK have reported that more than 1 in 20 cancers are caused by excess body weight and it is the second biggest preventable cause of cancer in the UK (Cancer Research UK, 2019a).

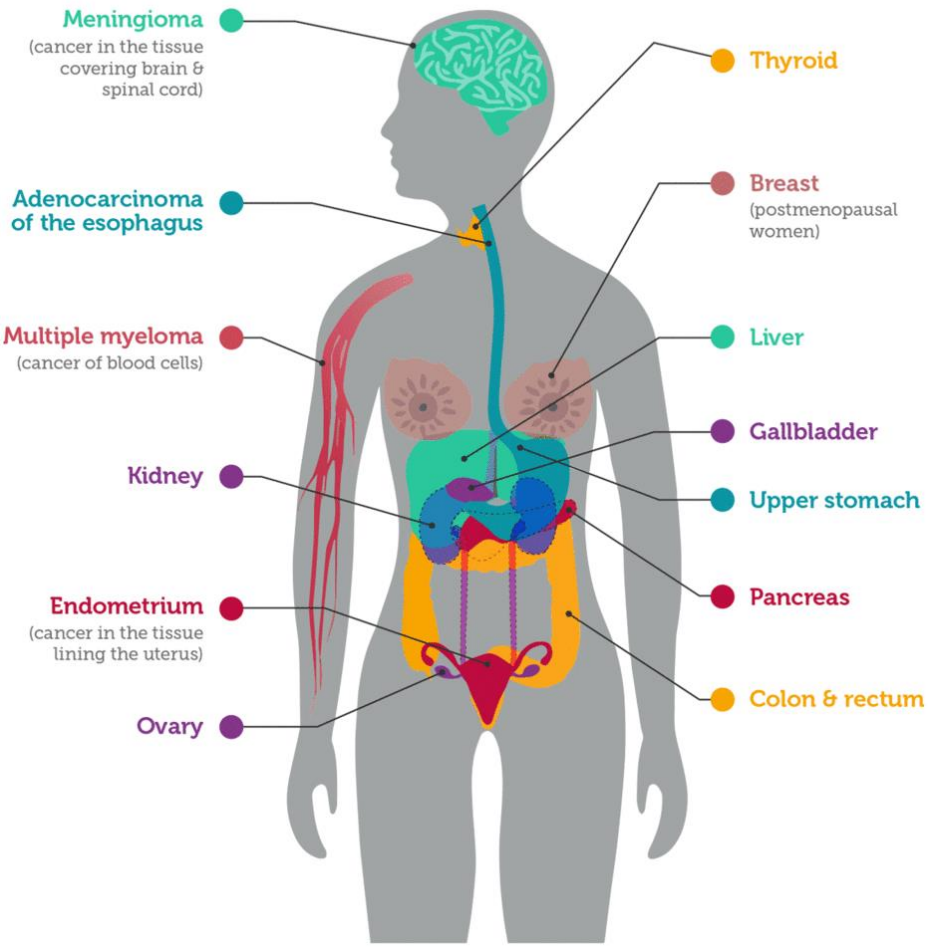

Figure 1.9: The 13 types of cancer associated with obesity. Obesity contributes to poorer outcome in the following cancers, Meningioma, thyroid cancer, oesophageal cancer, breast cancer, multiple myeloma, kidney cancer, endometrium and ovarian cancer also cancer of the liver, gallbladder, upper stomach, the pancreas, colon and rectum. (National Cancer Institute, 2019a)

Obesity is known risk factor for 13 types of cancer which include meningioma (Takahashi et al., 2019), adenocarcinoma of the oesophagus (Murphy et al., 2017), thyroid cancer (Siqueira et al., 2019), breast cancer (Ecker et al., 2019), liver cancer (Loo et al., 2017), gallbladder cancer (Li et al., 2016), multiple myeloma (Went et al., 2017), kidney cancer (Laaksonen et al., 2019), upper stomach cancer (Kim et al., 2019), pancreatic cancer (Gruber et al., 2019), colon and rectal cancer (Matsui et al., 2019), ovarian cancer (Ghasemi et al., 2019) and cancer of the endometrium (Kitson et al., 2019), see Figure 1.9. Cancers associated with obesity are often in close anatomical proximity to adipose tissue stores in the body which creates a hospitable environment for cancer cells and developing tumours (Laurent et al., 2019). This is particularly prevalent in breast cancer due to adipose tissue naturally residing in the breast,

the crosstalk between the tissues in this case can interfere with breast cancer drugs (Bougaret et al., 2018). Studies in obese multiple myeloma patients implicate the adipocyte-rich bone marrow as a source of protection for cancer cells (Trotter et al., 2016). The adipose tissue niche and its relation to ALL and its interactions will be explained further in section 1.5.2.1 “adipose tissue as an ALL cell niche”. Recently research has linked obesity to paediatric ALL outcomes, this will be discussed in section 1.5.1.

1.5.1 Obesity and paediatric ALL outcome

Research into ALL and obesity confirms that obesity impacts ALL outcomes and can increase the risk of dying prematurely and decrease the chance of an event free survival (EFS), the study by (Orgel et al., 2014b) obtained these results from a cohort of 198 patients. The group hypothesized that obesity before or during induction therapy was associated with MRD risk. The findings confirmed that children who were overweight or obese were at risk of a lower chance of EFS regardless of MRD risk, and newly diagnosed obese children were at an increased MRD risk and poorer EFS. Previous to these findings, the group showed that the duration of weight extremes during the intensive phases early in treatment independently predicted EFS (Orgel et al., 2014a). In the cohort of 2,008 children the results indicated that the influence of weight extremes, i.e., being obese, overweight or underweight, were not set at diagnosis and were also influenced by treatment related toxicity and if weight could be addressed in induction and the intensive consolidation treatment phases it could improve survival rates and EFS. Another study (Butturini et al., 2007) found that preteenagers and adolescents were at higher risk of relapse when obese. A further study confirmed that obesity and being overweight was an independent risk factor for relapse and could be partly explained by the antiapoptotic properties linked to adipocytes; leptin increased levels of Bcl-2 and Pim-2 which acted to protect leukaemia cells from treatment (Gelelete et al., 2011). Contradictory to this, obesity and BMI at diagnosis did not predict poorer response or relapse after therapy in a 2017 study, but obese patients were at risk of reduced overall survival due to treatment-related mortality (Eissa et al., 2017b). A recent study involving 55 patients diagnosed in a single institution between 2006 and 2012 evaluated the risk in patients with a BMI percentile greater than >95 during pre-maintenance therapy confirmed that obesity was a risk factor for developing hypertension, high blood sugar levels and febrile neutropenia

admissions, these factors lead to reduced doses of chemotherapy which can compromise efficacy of treatments and even lead to death (Meenan et al., 2019). Children with ALL are also at an increased risk of becoming obese due to treatments, (Chow et al., 2007, Zhang et al., 2014, Zhang et al., 2015a) and as such should be taken into account during treatment and long term care afterwards. Further to this, a study found that BMI only increased in standard risk ALL patients in the first month of therapy or the induction phase and this was greater in patients receiving dexamethasone and by the end of therapy the obese patients had returned to normal weight range (Lindemulder et al., 2015), suggesting intervention to diet or physical activity is best timed during therapy especially due to the amount of long term survivors living to increasing ages. It is widely accepted that patients are at risk when obesity occurs before, during and after treatment and this has the potential to impact their health long-term (De Pergola and Silvestris, 2013). ALL has an excellent cure rate but as discussed in this introduction obesity can impact the outcome and success of treatment, toxic combinations of chemotherapy can further the risk of death and EFS and also the long-term health of ALL survivors. Taking everything discussed about side effects of combined chemotherapy regimens and outcomes it is important that we are considering individual patient status when designing clinical trials and treatments, this includes cell count at diagnosis, age (over 10 years), three or four drug induction therapy, dexamethasone or prednisolone as a choice of steroid, poor early responders, relapsed ALL patients and if their leukaemia is of B-cell or T-cell in origin. It would also be important to include children who present as obese or become obese with treatment as another subgroup of patients that could benefit from a tailored chemotherapy protocol, possibly including newer drugs and/or tailoring of more toxic current drugs. Should obese patients be treated with the same protocols or should we intervene and adjust protocols to suit this increasing cohort of patients? This remains an important question under the threat of 'globesity'. The underlying mechanisms associated with obesity and ALL will be discussed in the next sections.

1.5.2 Why might obesity results in poorer outcomes for ALL patients?

Obesity can contribute to poorer outcomes in obese patients, the next sections will cover the key points and mechanisms behind this in more detail. I have introduced that obesity results in higher levels of body fat which contributes to low grade inflammation, also that dysregulated levels of adipokines can contribute to disease progression. Some of the mechanisms behind this includes adipocytes secreting chemoattractants which entice ALL cells into adipose tissue and create a protective environment (Pramanik et al., 2013a). Adipocytes then communicate with ALL cells and supply them with fuel such as fatty acids and amino acids (Tucci et al., 2021). Adipocytes can secrete molecules which prevent apoptosis in ALL cells (Sheng and Mittelman, 2014) and can also absorb chemotherapeutic drugs and deactivate them promoting the survival of ALL cells (Sheng et al., 2017). Adipokines have also been shown to change sensitivity of chemotherapeutics boosting survival of cancer cells (Qiu et al., 2018b), see figure 1.10.

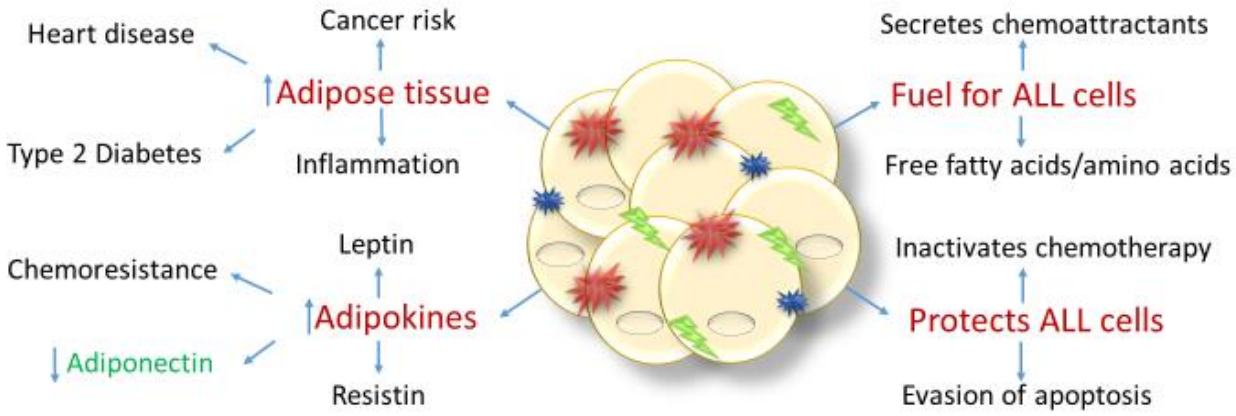

Figure 1.10: Obesity increases Adipose tissue which dysregulates adipokine secretion. This dysregulation provides fuel for ALL cells, it can also protect ALL cells by inactivating chemotherapy and driving evasion of apoptosis and chemoresistance. The figure summarises adipose tissue as a heterogenous tissue with associated cells, these include immune cells B-cell and T-cells, macrophages, mesenchymal cells, pre adipocytes and stem cells.

1.5.2.1 Adipose tissue as a cancer and ALL cell niche

Adipose tissue is able to attract ALL cells via secretion of chemo-attractants and create a protective environment which allows ALL cells to grow and proliferate (Pramanik et al., 2013b). Adipose tissue in the form of WAT is a niche site for haematological malignancies, this was confirmed previously in a mouse model where injected leukaemia cells congregated into the fats pads of the mice and were then shown to be resistant to vincristine in experiments (Behan et al., 2009). This is in addition to the bone marrow niche, where the disease initially resides and proliferates (Askmyr et al., 2011). WAT is also a metastatic site for cancers such as breast cancer due to the close proximity in the breast which is one of the sites of WAT (Dirat et al., 2011). Many other cancers reside in areas adjacent to adipose tissue such as the ovaries, prostate and uterus, also the pancreas, colon and liver as previously discussed. In the case of prostate cancer, peri prostatic adipose tissue, which resides close to the prostate and is often infiltrated by prostate cancer cells, promotes an aggressive tumour biology within this microenvironment (Ribeiro et al., 2012). In a study investigating the interactions between adipose tissue and multiple myeloma (MM) cells, adipocytes were extracted from obese and super-obese, defined as a BMI between 35-40 kg/m^2, patients and co-cultured with MM cell lines, the MM cells proliferated faster in the superobese to obese media; the superobese media enhanced tube formation in endothelial cells and expression of matrix mellanoproteinases (MMPs) which is indicative of angiogenesis and epithelial to mesenchymal transition respectively. Conclusively, it was suggested that the MM micro environment should be further evaluated as a potential therapeutic target (Bullwinkle et al., 2016). A further study suggests that the biologically active adipose stem cells (ASCs) isolated from abdominal fat, which express leptin through oestrogen stimulation, increased breast cancer growth and concluded that abdominal fat and abdominal obesity induced significant changes in ASCs (Strong et al., 2013), summarised in figure 1.11. Studies regarding the ALL cell niche and preferred WAT sites for ALL cells were limited to the studies discussed in this section but recent research is shedding more light on the supportive role of MAT and the bone marrow microenvironment in the progression of ALL and its preferred site for development and chemoprotection (Dander et al., 2021, Ma et al., 2020)

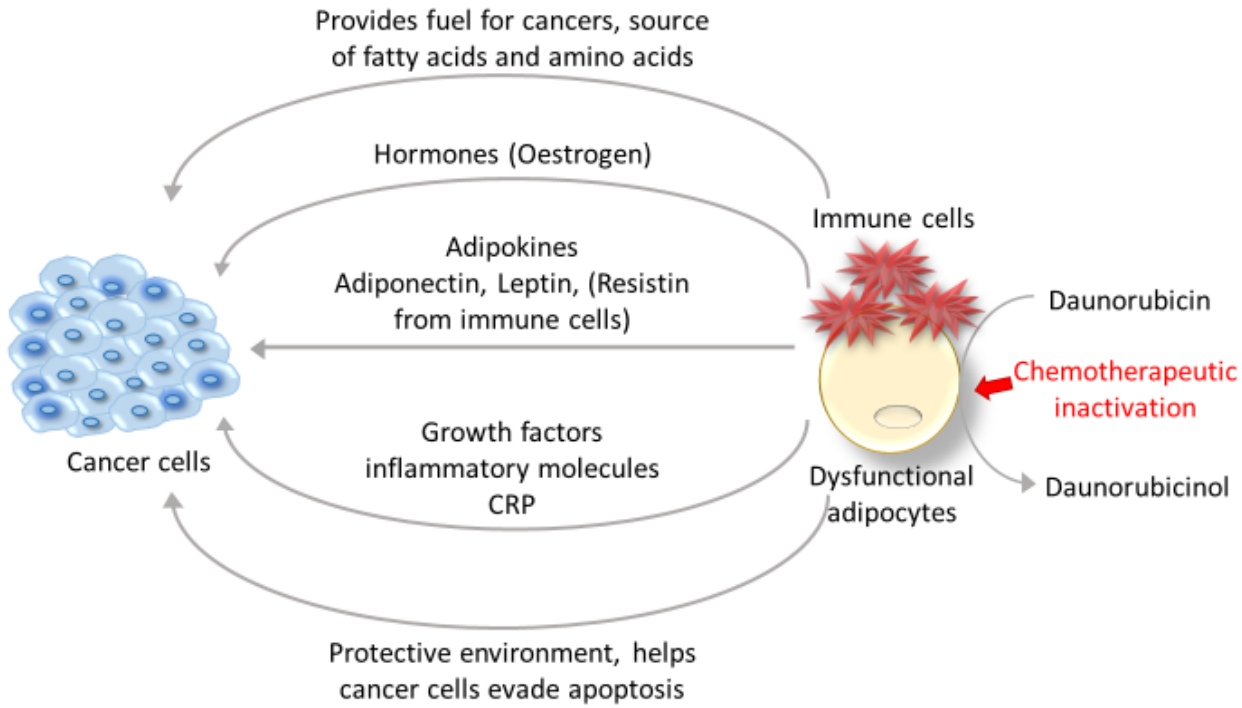

Figure 1.11: Dysfunctional adipocytes, immune cells and their interaction with cancer cells. Dysfunctional adipocytes attract immune cells and release several substances which are protective of cancer cells, these includes secretion of adipokines, growth factors, hormones which can help cancer cells evade apoptosis and inactivate chemotherapy (adapted from Sheng et al 2017).

1.5.2.2 Adipose tissue as a source of Fuel for cancer and ALL cells

It is considered that adipocytes may support the consistent supply of sufficient energy and nutrients for cancer cells to proliferate (Huang et al., 2018a). To aid this exchange of nutrients, adipocytes secrete adipokines which attract tumour cells, where they provide lipids which can exacerbate tumour growth and promote metastasis (Nieman et al., 2011). Cancer cell metastasis requires very high energy demands and lipids are an excellent source of energy for cells to adapt to changing environments and carry out tasks such as reprogramming protein expression, aiding cellular processes and releasing further signalling proteins (Sant'Anna-Silva et al., 2018), see figure 1.11. In some cases, disrupting the pathways involved in energy metabolism can have a detrimental effect on cancer cell fate, as it has been shown that supressing ATP citrate lyase (ACLY), a key enzyme that initiates *de novo* lipid synthesis in cancer cells such as osteosarcoma, prostate, cervical and lung cancers, inhibits tumour growth

and metastasis. Inhibition of ACLY has been achieved using MicroRNAs, as miR-22 is a negative regulator of the ACLY gene (Xin et al., 2016).

Cancer cells also exploit lipid sources exogenous to them; metastasis-initiating cells have been shown to rely on lipids to promote metastasis, furthermore, blocking the fatty acid receptor, CD36, on oral carcinoma cells using neutralising antibodies resulted incomplete inhibition of metastasis in immunodeficient mice (Pascual et al., 2017). In a study using *in vivo* and *in vitro* models of AML, it was shown that AML blasts activate lipolysis in adipocytes and by using FABP4 shRNA knockdown it prevented AML cell proliferation. Further to this, knockdown of carnitine palmitoyl transferase IA (an enzyme involved in the oxidation of long chain fatty acids) in an AML patient derived xenograft, improved survival rates in mice (Gharpure et al., 2018). It would be interesting to know if the results of this finding could be transferred to our understanding of the FABP4 fatty acid binding protein in relation to ALL.

ALL cells have been shown to stimulate adipocyte lipolysis to obtain a supply of adipocyte derived free fatty acids to supplement *de novo* lipogenesis and for molecular building blocks (Tucci et al., 2014). Part of the treatment protocol for ALL includes L-asparaginase this depletes levels of asparagine and glutamine available to the leukaemia cell, and has been shown to break down asparagine and glutamine into aspartic acid and glutamic acid, thus disrupting ALL cell homeostasis (Kitoh et al., 1990). Adipocytes are also a source of amino acids; adipocytes produce asparagine and glutamine which increase levels available to ALL cells and protects leukaemia cells from L-asparaginase treatment (Ehsanipour et al., 2013). Since leukaemia cells can infiltrate adipose tissue it was concluded that high local levels of asparagine and glutamine was protective of ALL cells. Recent research has suggested that adipose tissue stem cells (ASCs) can negatively influence haematological malignancy, in a study by Lee *et al.* (2018) NOD/SCID mice were co-injected in the peritoneum with an ALL cell line expressing firefly luciferase ALL/fLuc cells and ASCs or normal fibroblasts as a control. Mice injected with ALL/fLuc cells and ASCs showed significantly higher bioluminescence intensity in the intraperitoneal area and larger tumour masses than control mice, the findings of this study concluded that the results should be considered when developing cell therapies using ASCs as these cells were attracting the ALL cell line (Lee et al., 2018). Research published this year confirmed that adipocytes provide a source of free fatty acids which supported the

growth and proliferation of patient B-ALL cells, the study concluded that this source of fatty acids could contribute to chemoresistance in ALL (Tucci et al., 2021).

1.5.2.3 Adipose tissue can disrupt chemotherapy pharmacokinetics

Drug pharmacokinetics (PK) can also be influenced by obesity. Excess adipose tissue can accumulate drugs in adipocytes, and this in turn increases the volume of distribution available to the drug (Cheymol, 2000). It has previously been shown that a protein called alpha 1-acid glycoprotein is increased in obesity and binds to the basic positively charged drugs in the plasma, meaning less of the drug is available in the body, that is, it has a low bioavailability (Blouin et al., 1987). Further to drugs binding to plasma proteins, renal and hepatic clearance of drugs is impaired in obesity, this is due to increased activity of the enzyme cytochrome P450 CYP2E1 which accelerates drug detoxification in the liver and increases the glomerular filtration and in the kidneys (Brill et al., 2012, Emery et al., 2003). Together, these findings suggest altered PK of water soluble drugs would speed up excretion from the kidneys. With these scenarios taken into account, obesity could be associated with impaired efficacy of a number of different medications.

At present there are no known studies where the effects of obesity and PK in children with leukaemia, although a study by Hijiya *et al.* (2006) reported no difference in calculated means of systemic clearance and intracellular levels of the thioguanine nucleotide levels and methotrexate polyglutamates, which are both treatments used in ALL, within the four different BMI groups; underweight, normal, overweight and obese (Hijiya et al., 2006). The commonly used treatment vincristine is a lipophilic agent and maybe sequestered in adipose tissue which could lead to altered tissue distribution and build up in the fat tissue of obese individuals. PK modelling of injection of control and obese mice with titrated vincristine showed that obesity impaired overall exposure of the ALL cells to vincristine, in this study a single dose of vincristine proportional to body weight was injected and blood and tissue levels were measured over a 24 hour period. By 3 hours there were significantly higher levels of vincristine measured in the white adipose tissue of obese mice in comparison to the control mice (Behan et al., 2010). Vincristine is metabolised by the hepatic enzyme cytochrome P450 CYP3A subfamily, with approximately 80% of the drug metabolised in the liver and excreted in the faeces and a further 10-20% metabolised by the kidneys and excreted via urine

(Drugbank, 2018). Although in obese animal models this enzyme has shown both increased and decreased activity so further work would be needed to confirm this (Maximos et al., 2017, Tomankova et al., 2017).

In more recent studies, it has been observed that adipocytes sequester and metabolise the ALL chemotherapeutic daunorubicin. This expands on the work by Sheng and colleagues where it was shown that adipocytes protect ALL cells from daunorubicin via an oxidative stress response (Sheng et al., 2016). The group went onto determine that adipocytes clear daunorubicin from the tumour microenvironment by upregulating the enzymes aldo-keto reductases and carbonyl reductases which effectively metabolise daunorubicin into its inactive metabolite daunorubicol, see figure 1.11. The study concluded that aldo-keto reductases and carbonyl reductases can be expressed in higher levels in obese individuals and have the ability to metabolise several drugs with implications across many diseases and not just cancer. They suggested that adipocyte absorption of chemotherapeutic drugs is contributing to poorer survival outcomes in cancer (Sheng et al., 2017).

1.5.2.4 Dose capping in obese individuals

Further to adipose tissue and increased levels of body weight disrupting chemotherapy, pharmacokinetics clinicians face the dilemma of dose capping in children with obesity which can reduce the therapeutic effects of drugs (Kendrick et al., 2010). Chemotherapy treatments have a range of side effects and even more care is taken with children to avoid toxicity and long term problems with organs and the nervous system (Gade et al., 2015). There are different methods used to dose individuals correctly to body weight when outside of normal parameters, such as ideal body (within normal BMI percentile range >5 <85) even if they are overweight (De Baerdemaeker et al., 2004), creatinine clearance and GFR rate (Stevens et al., 2009), or drugs are dosed proportionally to body surface area (Sawyer and Ratain, 2001), or a combination of these methods.

There are differences in opinion between clinicians on how to maximise the efficacy of drugs in overweight children, with some agreeing that it is suitable to dose to ideal body weight and lean mass in the obese population or body surface area (Callaghan and Walker, 2015, Mulla and Johnson, 2010). Vincristine, commonly used in ALL therapy, comes under separate strict dose capping guidelines to body surface area and is generally capped at 1.4 to 1.5mg/m^2 with

a maximum dose of 2mg/m^2 a week regardless of the child's size (electronic Medicines Compendium (eMC), 2018). Due to these dose capping restrictions, obese ALL paediatric patients' risk being under dosed without an effective dose of in the case of vincristine.

1.5.2.5 Apoptosis

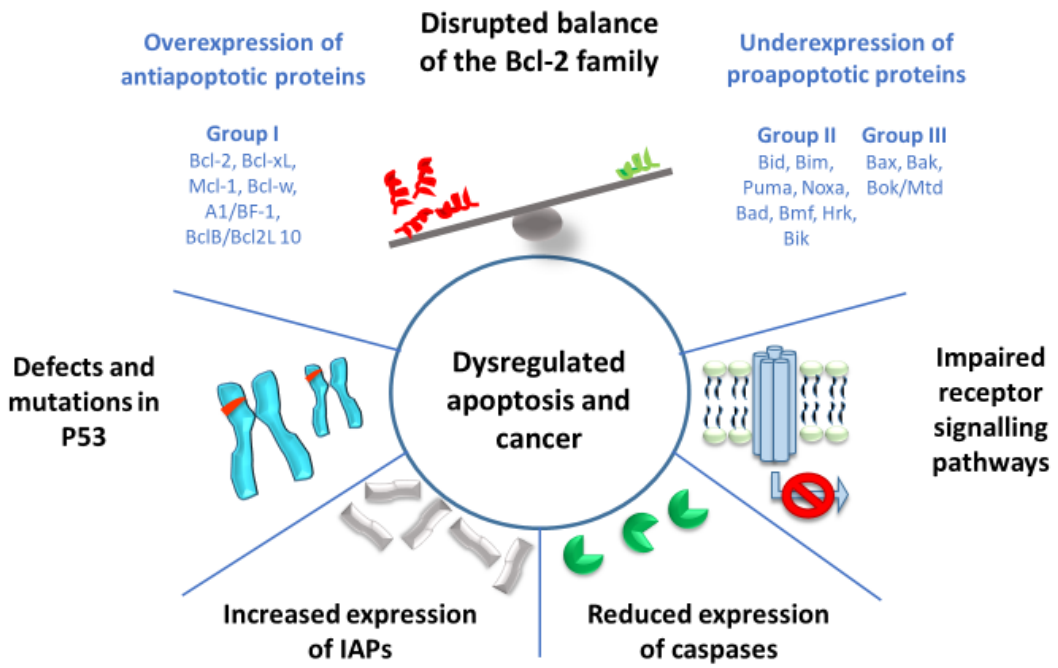

Figure 1.12: Dysregulated apoptosis and cancer. Drivers of dysregulated apoptosis include impaired death receptor signalling, reduced expression of caspases, increased expression of inhibitors of apoptosis, defects and mutations in the p53 gene and disrupted balance of the Bcl-2 family of proteins where there is overexpression of antiapoptotic proteins and under expression of pro-apoptotic proteins, adapted from (Wong, 2011)

Apoptosis is a form of programmed cell death which is characterised by several features including cell shrinkage, membrane blebbing, condensation of chromatin and fragmentation of DNA, the cell is eventually engulfed by neighbouring phagosomes (Fiers et al., 1999). Apoptosis has a role in many biological processes such as embryogenesis, the normal aging processes (Vaux and Korsmeyer, 1999) and diseases such as Alzheimer's disease (Obulesu and Lakshmi, 2014) and cancer (Vaux et al., 1988). In cancer, there is a loss of balance between growth and division of the cell and cell death due to dysregulation of cellular mechanisms; one hallmark of cancer is that cells which should have died evade apoptosis and go onto proliferate (Hassan et al., 2014). An example of how this occurs would be downregulation of

the tumour suppressor *TP53*; reduced expression of this gene results in decreased apoptosis and enhanced tumour development and growth (Bauer and Helfand, 2006). The loss of function or mutation of *TP53* has been linked to many types of cancer (Gasco et al., 2002, Rodrigues et al., 1990, Tyner et al., 2012) and the targeting of apoptotic mechanisms has been the basis of extensive research in the treatment of cancer and overcoming of chemotherapy drug resistance (Rathore et al., 2017). Further to defects and mutations in the *TP53* gene, one of the major areas of interest in cancer biology is the Bcl-2 family of proteins. When this family is out of balance it can tip the cell towards an antiapoptotic phenotype involving overexpression of the group I proteins under expression of group II and III (Campbell and Tait, 2018). The Bcl-2 family of proteins is well studied for their regulation of programmed cell where they can inhibit or promote apoptosis by interfering with the mitochondrial membrane (Reed et al., 1998). They were first discovered in 1984 in a variant of B-cell lymphoma, its discovery identified the Bcl-2 protein as an inhibitor of apoptosis (Tsujimoto et al., 1984). The Bcl-2 family can be divided into pro and antiapoptotic groups, shown in figure 1.12, the anti-apoptotic proteins include Bcl-2, Bcl-xL and Mcl-1, (group I), the group of pro apoptotic proteins include Bid, Bim, (group II) , Bax and Bak, (group III) (Carrington et al., 2017). Other regulators of apoptosis also include inhibitors of apoptosis (IAPs), overexpression of which can lead to increased tumour cell survival, chemoresistance and poor prognosis (Silke and Meier, 2013). Reduced expression of caspases has been confirmed in pancreatic cancer (Jakubowska et al., 2016) and finally impaired death receptor pathways are implicated in cancer progression, changes with death receptor signalling was involved in multidrug resistance in the breast cancer cell line MCF7, in this study (Antoon et al., 2012) the death receptor signalling pathway was significantly altered with increased NF-kβ expression and blocked extrinsic apoptosis. Recent research in ALL implicated impaired death receptor signalling which led to reduced effectiveness of (CAR) T therapy, using genome wide association study (GWAS) screening to look for loss of function genes it was found that there was reduced levels of death receptor associated genes which caused (CAR) T cell treatment failure (Singh et al., 2020), see figure 1.12 for an overview of dysregulated apoptotic mechanisms and cancer.

1.5.2.6 Adipose tissue inhibits apoptosis

Increasing evidence supports the role of adipose tissue in the inhibition of apoptosis in cancer cells and major driver in chemoresistance (Lehuédé et al., 2019). In a study relating to Acute Monocytic leukaemia (AMoL) a metabolic role was discovered for bone marrow adipocytes by supplying fuel and aiding evasion of apoptosis in leukemic blasts, the study concluded that bone marrow adipocytes prevented spontaneous apoptosis in AMoL cells by increasing fatty acid β-oxidation (FAO) also by upregulation of the *PPARG*, *FABP4*, *CD36* and *Bcl-2* genes. Co-culture of both cell types increased expression of the adiponectin gene and its downstream targets AMPK and p38 MAPK, and upregulated antiapoptotic chaperone heat shock proteins. Blocking FAO with etomoxir increased apoptosis in the AMoL cells and the study suggested that blocking FAO in bone marrow adipocytes maybe a novel therapeutic strategy for AMoL therapy (Tabe et al., 2017). A set of experiments which co-cultured OP-9 derived adipocytes with chronic lymphocytic leukaemia cells (CLL) caused the CLL cells to become resistant to dexamethasone avoiding apoptosis, the CLL cells were highly resistant to dexamethasone in comparison to the serum free conditions alone (Tung et al., 2013). In a study on breast cancer published by Iyengar *et al.* (2003) the breast cancer cell line MCF-7 was treated with adipocyte-conditioned media containing a range of adipokines, this resulted in upregulation of A20 and NFkB which are both anti-apoptotic signals (Iyengar et al., 2003). In research involving ALL cells Behan *et al.* (2009) have shown that adipocytes protect ALL cells from chemotherapeutic drugs such as vincristine, daunorubicin, nilotinib and dexamethasone, in the study it was found that adipocyte protection of ALL cells was associated with the upregulation of the pro-survival signals Bcl-2 and Pim-2, which are part of the intrinsic apoptosis pathway, and an increased phosphorylation of the proapoptotic protein Bad which indicates its deactivation (Behan et al., 2009). Oxidative stress is strongly linked to apoptosis and is a potent apoptotic inducer (Kannan and Jain, 2000), in a further study by Sheng *et al.* (2016), the group showed that in co-culture experiments adipocytes protected ALL cells from oxidative stress promoting survival. The human adipocyte cell line Chub-S7 protected human ALL cells in a transwell culture system without direct cell to cell contact against daunorubicin suggesting a secreted factor was key in this response Further to this daunorubicin treatment led to increased apoptosis in human BV173 cells, this was reversed in the presence of 3T3-L1 mouse adipocytes (Sheng et al., 2016). These studies have suggested a direct effect of

adipocytes on cancer cells demonstrating that adipocytes may be boosting proliferation and helping cancer cells evade apoptotic mechanisms through various mechanisms, and so could be a causal factor for the development of chemoresistance. This thesis seeks to investigate this further.

1.5.3 Mechanisms of chemoresistance

1.5.3.1 Unfolded protein response

The unfolded protein response (UPR) is a cellular system activated in response to ER stress (Corazzari et al., 2017), the stressors that activate the system include glucose and energy deprivation, accumulation of misfolded proteins and adaptive responses to drugs such as chemotherapy and it is a major driver of chemoresistance in cancer therapy (Avril et al., 2017). The pathway is described as a "double edged sword" as under acute condition the UPR network acts in a pro survival manner, but under more continuous chronic stress it shifts the balance to a pro-apoptotic phenotype in the cancer cell (Gorman et al., 2012). During unstressed conditions there are three main sensors (PERK, ATF6α and IRE-1α) which are bound and inactive to the GRP78 receptor (Mei et al., 2013). When stress occurs in the ER they are activated and involved in the mechanisms of chemoresistance in their own pathways (Oslowski and Urano, 2011). For the purpose of this work we will focus on the role of the GRP78 protein and its relocation to the cell membrane and then its subsequent activation by exogenous ligands (Ge and Kao, 2019) one being SerpinA12 which is discussed previously in section 1.4.2.4, this can then activate the downstream PI3K/Akt pathway and increase cell survival and proliferation. In a study using a mouse model knockout of GRP78 and PTEN (an antagonist of the PI3K/Akt pathway) it restored normal development of leukemic blasts, also in the study overexpression of GRP78 led to cytosine arabinoside resistance against apoptosis and knockdown of GRP78 sensitised AML cells to the drug (Wey et al., 2012). In a study of murine B-ALL, BCR-ABL1 cells were generated and taken from a mouse model with a genetic deletion of the GRP78 gene (described as HSPA5 in the study) deletion of the *HSPA5* gene reduced the leukemic burden significantly compared to control mice (Masouleh et al., 2014)

1.5.3.2 Autophagy

Autophagy is a conserved mechanism in cells which promotes survival in times of stress (Das et al., 2012) , when autophagy processes are increased inside cancer cells this allows the cells to promote tumour growth and escape metabolic stressors such as nutrient deprivation and chemotherapy drugs (Buchser et al., 2012). The role of autophagy in cancer does remain controversial (Bhutia et al., 2013) as there is dual role for autophagy and timing in cancer development is important to trigger either cancer cell death or survival (Bhutia et al., 2013). Early in cancer development autophagy acts to promote cell death but once cancer has established autophagy acts to protect the cancer cell (Li et al., 2020). Studies in ALL have demonstrated that autophagy inhibition could be a useful targeted therapy for ETV6-RUNX-1 B-cell ALL, the study achieved this by using the drug hydroxychloroquine (Polak et al., 2019). Other research has confirmed that autophagy inhibition sensitizes ALL cells to L-Asparaginase. In this study L-asparaginase induced autophagy which protected the ALL cells, and when cells were then exposed to autophagy inhibitors this led to increased cell death (Takahashi et al., 2015).

1.5.3.3 Upregulation of drug transporter efflux pumps

A major driver of chemoresistance in cancer is the upregulation of transporter pumps which cause the efflux of cancer drugs which are removed from cancer cells promoting their survival (Liu et al., 2018). There are three main types of pump that are involved in multidrug resistance which are members of the ABC binding cassette transporter family (Feldman et al., 2015). In cancer roles for the multidrug resistance protein (MRP1), the breast cancer resistance protein (BCRP) and the P-glycoprotein (P-gp) are well established (Choi, 2005). Due to the major contribution to multidrug resistance there has been interest in recent studies for the development and use of new compounds that inhibit the ABC transporter family of transmembrane proteins, (Zheng et al., 2021). In childhood ALL a study aimed to investigate the expression profile of multidrug resistance protein due to its implications in treatment failure and relapse. The findings of the study confirmed a significant upregulation of mRNA expression of *MDR1, MPR1, ABCA2* and *ABC3* which correlated with increased MRD risk a year into treatment; the study called for genetic evaluation of the pumps to help mitigate risk in these high risk patients (Rahgozar et al., 2014).

1.5.3.4 Adipokines and mechanisms of chemoresistance

The relationship between serum adiponectin levels in patients, the risk of subsequent metabolic disease and cancers is linked as discussed in section 1.4.2.1. In a recent study it was revealed that there is a link between low adiponectin levels and colon cancer, in that high adiponectin levels inhibited proliferation in colorectal HCT116 cells *in vitro* (Huang et al., 2018b). Following incubation with recombinant adiponectin, the percentage of cells in G1/G0 cell cycle phase increased along with the expression of p21, cleaved-caspase-3 and the number of apoptotic cells, expression of cyclin D1 and phosphorylated NF-kBp65 were downregulated. This demonstrated that adiponectin shifted the balance of apoptosis to inhibit the growth of colorectal cells and promoted apoptosis.

Adiponectin increases the efficacy of chemotherapy drugs in breast cancer via induction of autophagosomes, increased ratio of LC3BI to LC3BII and decreased expression of the ubiquitin binding protein, SQSTM1/p62, in this study these are proteins involved in the autophagy pathways and blocking these pathways reversed the action of adiponectin induced autophagy. These events were regulated by the initiating master kinase STK11/LKB1. Knockout of this protein in the study also reversed the protective effects of adiponectin and decreased levels of apoptosis in MCF7 and MDA-MB-231 breast cancer cells (Chung et al., 2017). The following study established a critical role for the AMPK/FoxO3A axis has been identified in adiponectin-induced apoptosis in cancer cells. Adiponectin treatment increased p27 and decreased levels of cyclin D1 in hepatoma (HepG2) and breast cancer cells (MCF-7) which was associated with increased cancer cell death. Ablating the *FoxO3A* gene reversed this effect. Gene silencing of *AMPK* prevented nuclear relocation of FoxO3A indicating a role for AMPK signalling upstream of FoxO3A, the group concluded that the AMPK/FoxO3A induces the anti-proliferative effect in the two cells lines studied (Shrestha et al., 2016).

Hypoadiponectinemia is implicated in multiple myeloma and promotes tumour progression, In a set of experiments prolonged exposure of 96 hours to adiponectin activates protein kinase A which increases AMPK, promoting cell cycle arrest and apoptosis. Supplementing with palmitic acid the human and mouse MM cell lines were protected from apoptosis, suggesting that adiponectin boosts survival in multiple myeloma cells by potentially supressing lipogenesis (Medina et al., 2014). Adiponectin promotes cell survival and inhibits

apoptosis in other cancers including endometrial cancer where the antiproliferative effects of adiponectin were blocked by compound C, an AMPK inhibitor. This study concluded that the adipoR1 receptor had a greater role then adipoR2 receptor in apoptosis of the endometrial cell line Ishikawa 3-H-12 (Zhang et al., 2015b). Adiponectin also inhibits the growth of gastric cancer (Ishikawa et al., 2007) and cervical cancer (Xie et al., 2011), in this cervical cancer study adiponectin-induced apoptosis in HeLa cells was investigated. Using RT-PCR to measure the expression of apoptosis related genes it was shown that after 48 hours exposure to adiponectin, levels of the apoptotic gene *BAX* was upregulated the anti-apoptotic gene *Bcl-2* was reduced.

Leptin is involved in the pathogenesis of many cancer types including breast cancer, pancreatic cancer, prostate and colon cancer (Candelaria et al., 2017). A study using cervical cancer HeLa cells demonstrated that leptin correlated positively with c-myc and Bcl-2, both anti-apoptotic proteins. In the study recombinant leptin significantly activated gene transcription for the anti-apoptotic proteins Bcl-2 and c-myc, and furthermore silencing leptin with siRNA inhibited expression of Bcl-2 and c-myc and reduced proliferation of the cell line (Yuan et al., 2013). Leptin can also stimulate growth of ovarian cancer through mechanisms inhibiting apoptosis, leptin upregulated the expression of cyclin D1 and the antiapoptotic proteins Mcl-1. This effect was mediated by activation of JAK2 non-receptor tyrosine kinase and downstream P13K/Akt and MEK/ERK1/2, inhibition of these cellular pathways abolished the effect of Leptin (Chen et al., 2013b). Leptin has been shown to contribute taxol chemoresistance in ovarian cancer by reducing the number of cells in G2/M phase of the cell cycle which promoted cancer cell growth (Gu et al., 2019a)

Resistin contributes to the growth of cancers and is involved in the proliferation and metastasis of cancers (Hsieh et al., 2014). Higher circulating levels of resistin in cancer patients is associated with decreased sensitivity to chemotherapy drugs in a number of different cancers (Qiu et al., 2018a). The particular cellular mechanisms underlying this resistance are varied, but often involve induction of cell proliferation due to evasion of apoptosis (Deshmukh et al., 2017). Resistin significantly attenuated doxorubicin induced apoptosis in breast cancer cells which increased breast cancer cell survival, in the study addition of recombinant resistin upregulated autophagy pathways in the cell and promoted cell survival, this activated the

signalling pathways AMPK/mTOR/ULK1 and JNK, suggesting resistin antagonism could be a novel treatment for doxorubicin resistance in breast cancer (Liu et al., 2017).

Further to this, resistin inhibited chemotherapy-induced apoptosis in myeloma cell lines, in this study treatment of the cell lines with the chemotherapy drug melphalan caused significant decreases in the expression of Bcl-2 and Bcl-xL antiapoptotic proteins, whereas addition of resistin enhanced their expression. In addition resistin increased the expression of ABC transporters which removed intracellular chemotherapy accumulation leading to drug resistance in the patient derived cells and MM cell lines (Pang et al., 2017). The levels of serum resistin in pancreatic patients have been found to positively correlate with pancreatic cancer tumour grades. Silencing CAP1 and/or TLR4 resistin receptors using siRNA elicited a significant reduction in cell proliferation, which was highest when silencing both receptors simultaneously. Furthermore, resistin decreased pancreatic cancer cell sensitivity to gemcitabine via its receptors and downstream pathways by activation of STAT3 (Zhang et al., 2019). This pathway is known to activate the anti-apoptotic Bcl-2 family of proteins (Kang et al., 2015). Another study looked at obesity and breast cancer in post-menopausal women because of the higher concentrations of resistin detected, this is positively associated with the risk of breast cancer. The study investigated the effect of resistin on EMT and demonstrated that its inflammatory actions are mediated by the resistin receptor CAP1. The results demonstrated that resistin influences metastatic potential by inducing EMT and cell stemness partly by the CAP1 receptor (Peiser et al., 2003).

As introduced in this section there are many reasons and mechanisms as to why obesity may contribute to poorer outcomes in ALL . Adipose tissue, in particular obese adipose tissue can provide a protective niche for ALL cells to survive which can help cells evade apoptosis, they can also provide fatty acids and amino acids as a source of fuel. Other mechanisms include the disruption of chemotherapy pharmacokinetics, adding to this there is the issue of dose capping of chemotherapy drugs in obese children. More recent research is beginning to shed light on how adipokines through various mechanisms can interfere with the efficacy of chemotherapy protocols in a range of cancers. These mechanisms include activation of the unfolded protein response, changes in autophagy pathways in the cell, blocking autophagy processes can increase cancer cell death, upregulation of drug transporter pumps which aid removal of chemotherapy drugs from the cell. Another major factor is the role of adipokines

in the evasion of apoptosis for example leptin is known to activate gene transcription of anti-apoptotic proteins of the Bcl-2 family adding to the complexity of the mechanisms of chemoresistance.

1.6 Case for study

Improvements in the treatment of ALL since the 1960's has been one of the success stories in childhood cancer treatment. The cure rate for paediatric patients has increased to almost 90%. There are still many challenges to overcome treating paediatric ALL patients, they include difficult to treat patient subgroups, chemotherapy side effects during treatment and long-term late effects from treatment such as cardiovascular disease later in life even in children who have been successfully cured (Kızılocak and Okcu, 2019).

The obesity epidemic is a ticking timebomb with nearly three quarters of the UK population expected to be obese by 2035 having a huge impact on health and obesity related diseases including cancer, these statistics include children. Obesity has been linked to developing cancer in adults and while obesity is not linked to the initiation of ALL, studies in recent years indicate that it does impact the chance of overall survival and event free survival in paediatric patients. Secretions from fat tissue include adipokines and they have been shown to be dysregulated in obesity and cancer. In ALL, adipokines such as adiponectin, leptin and resistin have been studied but less is known about others, and more importantly the underlying changes which occur at a cellular level when adipokines are changed in obese patients with ALL.

This study aims to identify a pattern of expression of adipokines in overweight and obese individuals with ALL before and after induction therapy, and to also look into the underlying cellular changes that occur when adipokine expression profiles are changed to see if those changes could confer a pro-survival phenotype in ALL cells. With this information we will begin to build knowledge so that we can combat MRD risk in obese, overweight and weight gain patients with ALL, and their associated risk factors with new or existing treatments.

1.6.1 Aims and Objectives

The aim of this study is to investigate the impact of obesity and becoming overweight on adipokine expression profiles and treatment outcomes during in the induction phase of treatment in childhood acute lymphoblastic leukaemia patients. This may unlock key information which will benefit obese and overweight ALL patients in the future, perhaps by providing evidence for a tailored treatment regimen, or by identifying new treatment targets or biomarkers in obese and overweight patients.

The overall objectives of the study were:

1. **To determine how induction phase treatment influences adipokine expression and if changes correlate with MRD risk and BMI.**

   Patient plasma samples were obtained at day 0 and day 29 of induction treatment and adipokine expression levels quantified using an adipokine antibody array. BMI data was collected at day 0, and BMI and MRD data were collected at day 29.

2. **To investigate potential patterns in adipokine expression profiles between obese, overweight and typical weight patients.**

   We set out to identify potential patterns in adipokine expression within the patient cohort, i.e. patients who present as non-obese and do not gain weight during the induction phase of treatment, patients who present as non-obese and gain weight as part of the induction treatment (treatment-acquired obesity) or become overweight. These are examples of particular subgroups of patients but there are many ways we could analyse the patient data. Patients that present as obese or overweight at diagnosis could shed light on potential biomarkers for increased risk during treatment, this information has been included in the appendix and referred back to for significant adipokines within chapter 5, table 7.2. We also set out to identify if any particular adipokines could be suitable biomarkers of patients who go on to have high MRD risk at the end of induction, this information has also been recorded in the appendix, table 7.3.

3. To investigate the phenotypic impact of altered adipokine levels on ALL cell lines.

Adipokines identified in objective 2 will be cultured with ALL cell lines in order to investigate if they could have an impact on cell growth and viability. Adipokines in the MRD risk group of patients and the weight gain group of patients were further researched to identify if they could be driving disease by activating pathways known to be protective of ALL cancer cells. This was achieved by designing a trypan blue exclusion assay to measure cell growth and viability in reduced FBS conditions, part of this assay was to collect the cells that had been exposed to a particular adipokine and test them using western blotting to confirm activation of pathways. Further to this cells were incubated with selected adipokines and increasing concentrations of vincristine or daunorubicin to determine if the adipokines could change the sensitivity of the chemotherapy drugs.

2.0 CHAPTER 2 METHODS

2.1 Culturing and differentiation of 3T3-L1 mouse fibroblast into adipocyte-like cells

3T3-L1 are adherent embryonic fibroblasts derived from mice, the L1 is a continuous sub strain of 3T3 (Swiss albino) developed through clonal isolation, the cells undergo a pre-adipose to adipose like conversion when differentiated using the method described below (Ruiz-Ojeda et al., 2016). 3T3-L1 cells were purchased from American Tissue Culture Collection (ATCC). The cells were supplemented with base medium Dulbecco's Modified Eagles Medium DMEM, catalogue number 30-2002 (ATCC, USA), to make up complete pre-adipocyte expansion medium it was supplemented with Bovine Calf Serum FCS (ATCC, USA) 10% 100µL/mL, Penicillin and Streptomycin (Biosera) 1% 10µL/mL, see table 2.1

2.1.1 Subculture and trypsinisation of cells

Subculture techniques and trypsinisation of cells were only performed on cells in a preadipocyte fibroblast state up to differentiation, once plated in 24 well plates for differentiation cells were not subcultured. Cells were always kept under 70-80% confluence. Cells were cultured in complete pre-adipocyte expansion medium, see table 2.1, media was changed every 2-3 days. Cells were kept under regular observation every 1-2 days and were split when 70-80% confluent. Cells were split by removing media and washing with 5mL of sterile PBS (Sigma Aldrich). To detach cells from the flask 2.5mL of trypsin (Thermo Fisher) was added and the flask was incubated for 2-3 minutes. To neutralise the Trypsin 10mL of pre-adipocyte expansion media was added to the flask, cells were collected and spun at 125 x g for 5 minutes. After harvesting the cell pellet was resuspended in 10mL of pre-adipocyte expansion media. Cells were counted, see procedure below and new flasks were reseeded at 3 x 10^3 cells/mL in a T75 flask.

2.1.2 Cell counting

Cells were counted using a trypan blue exclusion assay. From the resuspended cell pellet obtained at each passage 20µL of cell suspension was mixed with 20µL of trypan blue solution

(Biosera), from this 20µL was loaded onto the haemocytometer to count the cells. In the presence of trypan blue dead cells will uptake the dye and will appear blue in the centre, this can distinguish dead cells as live cells will be clear. Viable were counted on the four corners of the haemocytometer at x10 magnification using a light microscope (Nikon, Digital sight). To determine the cells per mL count, the average of the 4 live cell counts were taken and multiplied by 2 (dilution factor for trypan blue) and displayed as cell per mL. Step 1 and 2 equations were followed to seed a new flask.

Step 1:

$$\begin{array}{|c|c|}\hline A & B \\ \hline C & D \\ \hline\end{array} \qquad \frac{A+B+C+D}{4} \; X \; 2 = \; cell\,concentration \times 10^4$$

Step 2:

$$Volume\ of\ cell\ solution\ required\ from\ flask = \frac{cell\ concentration\ required \times Volume\ of\ cell\ suspension\ required}{Concentration\ of\ cell\ solution\ in\ flask}$$

2.1.3 Differentiation procedure

Cells from passage number 7 were seeded at a density of 2 to 3 x 10^3 cells/mL in a T75 flask and incubated in a humidified atmosphere at 37°C with 5% CO_2. It was important that cells are not allowed to become confluent as this will start the process of differentiation when in a contact inhibited state (Zhu et al., 2017). Medium was refreshed every 2-3 days. To start the differentiation procedure cells were harvested at 70-80% confluence and seeded in pre-adipocyte expansion medium in a 24 well plate at 2 x 10^4 cells/well in 1mL of medium per well. Cells were left to grow for a further 48 hours and refreshed again with pre-adipocyte expansion medium. Cells were incubated as a confluent culture for a further 72 hours. Growth medium was removed, and an identical volume of differentiation medium was added to each well (1mL). Published research recommends the following protocol for successful differentiation (Zhao et al., 2019). Differentiation medium made up of Dulbecco's Modified Eagles Medium, catalogue number 30-2002 (ATCC, USA), supplemented with Fetal Bovine Serum FBS (Gibco) 10%, dexamethasone (Sigma Aldrich) 1.0µM methylisobutylxanthine IBMX (Sigma Aldrich) 0.5mM, these drugs were dissolved using dimethyl sulfoxide (DMSO) (Thermo

Fisher) before adding to the differentiation medium. Insulin (Sigma Aldrich) 1.0µg/mL as purchased see table 2.1. Differentiation is initiated by changes in gene expression, microarray analysis of 3T3-L1 cells during differentiation show a number of upregulated genes such as GLUT4 glucose transporter 4 and insulin like growth factor genes compared to preadipocytes (Guo and Liao, 2000). After 48 hours the differentiation medium was removed and replaced with adipocyte maintenance medium, Dulbecco's Modified Eagles Medium (DMEM), catalogue number 30-2002 (ATCC, USA), supplemented with Fetal Bovine Serum FBS (Sigma Aldrich) 10%, insulin (Sigma Aldrich) 1.0µg/ml, see table 2.1. Differentiated cells were kept in the 24 well plate in continuous culture, maintenance medium was replaced every 48-72 hours.

Table 2.1: 3T3-L1 media formulations used for pre-adipocytes, differentiation and adipocyte maintenance.

Pre-adipocyte expansion medium	**Differentiation medium**	**Adipocyte maintenance medium**
90% DMEM	90% DMEM	90% DMEM
10% Bovine Calf Serum (FCS)	10% Fetal Bovine Serum (FBS)	10% Fetal Bovine Serum (FBS)
1% Penicillin/Streptomycin	1.0µM Dexamethasone	1.0 µg/mL Insulin
	0.5mM Methylisobutylxanthine (IBMX)	1% Penicillin/Streptomycin
	1.0 µg/mL Insulin	
	1% Penicillin/Streptomycin	

Cells were checked using light microscopy (Nikon, Eclipse TS100) after 0, 7, 14, 21 days and considered fully differentiated when lipid droplets were observed. Images were taken using Nikon, Digital sight at X40 magnification.

2.2 Oil red-O staining of 3T3-L1 cells

To confirm 3T3-L1 cell differentiation into adipocytes and production of lipids, Oil red-O stain (Sigma Aldrich) was used to highlight the presence of lipids droplets in differentiated cultured cells. Oil red-O is a lipophilic diazo dye which stains lipid material in samples red (Proescher, 1927). It has been used in tissue and cell culture settings to discern adipocytes from other cell types for over 40 years (Green and Kehinde, 1975), more recently as a measure of adipocyte differentiation (Ramirez-Zacarias et al., 1992). A working stock of 60 % Oil Red-O was obtained by mixing Oil red-O solution with double distilled DD water. Cells were then fixed in a 10% paraformaldehyde (Sigma Aldrich) solution made up with PBS (Sigma Aldrich): the media was removed completely from the wells and the cells were rinsed with PBS, wells were aspirated completely and 1ml of 10% paraformaldehyde fixative solution was added to each well, the plate was incubated at room temperature for 30 minutes. Cells were washed twice with PBS and aspirated completely before adding the Oil Red-O working solution and incubated at room temperature for 1 hour on a rocking platform. The staining solution was removed, and cells washed 3 times with PBS, cells were then ready to image using Nikon, Digital sight at X40 magnification and photographed using Nikon, Eclipse TS100.

2.3 Mouse Adipokine Arrays

To determine levels of secreted adipokines from differentiated 3T3-L1 adipocytes the cell supernatants from cells plated in a 24 well plate were collected 24 hours after seeding before reaching a contact inhibited state to compare with levels after differentiation and at weeks 1, 2, 3, and 4 to establish the optimum time point for collection for future experiments. Adipokines were measured using Mouse adipokine arrays (ARY013 R & D Systems Bio-Techne Ltd). The ARY013 mouse array measures 38 different adipokines, appendix table 7.4, spotted in duplicate on the array, appendix figure 7.45. Following the manufacturers protocol each array membrane was incubated on a rocking platform in provided 4-well dish in 2ml of buffer 6 blocking buffer. During incubation 1ml of sample was prepared individually and adjusted to a final volume of 1.5ml with buffer 6 and 15µl of mouse detection biotinylated antibodies then incubated for an hour. Blocking buffer 6 was aspirated from the array compartments and each sample with the biotinylated detection antibodies was added, this allowed mixing of the adipokines and the detection antibodies with the capture antibodies pre-spotted on

the membrane, the arrays were incubated overnight at 4^{o}C on a rocking platform, see figure 2.1 for a visual representation of the procedure. Next arrays were washed 3 times on a rocking platform at room temperature for 10 minutes in individual containers followed by a 30-minute incubation step with 1:2000 diluted Streptavidin HRP and the wash step repeated. Arrays were incubated with 1ml of prepared Chemi Reagent Mix for 1 minute then covered with a plastic sheet avoiding air bubbles. Arrays were imaged using a G-Box (Syngene software) for suggested exposure time of 10 minutes which detects chemiluminescence in each spot of the array. Images were saved as .bmp files and analysed using Quickspots tool (Western Vision Software for R & D Systems). An average of the pixel intensity of each duplicate spot was taken representing each adipokine, the average of the Negative control spots was used to calculate the background signal, this was then subtracted from all average pixel intensities and saved in an excel file. The relative changes in adipokine levels could then be compared.

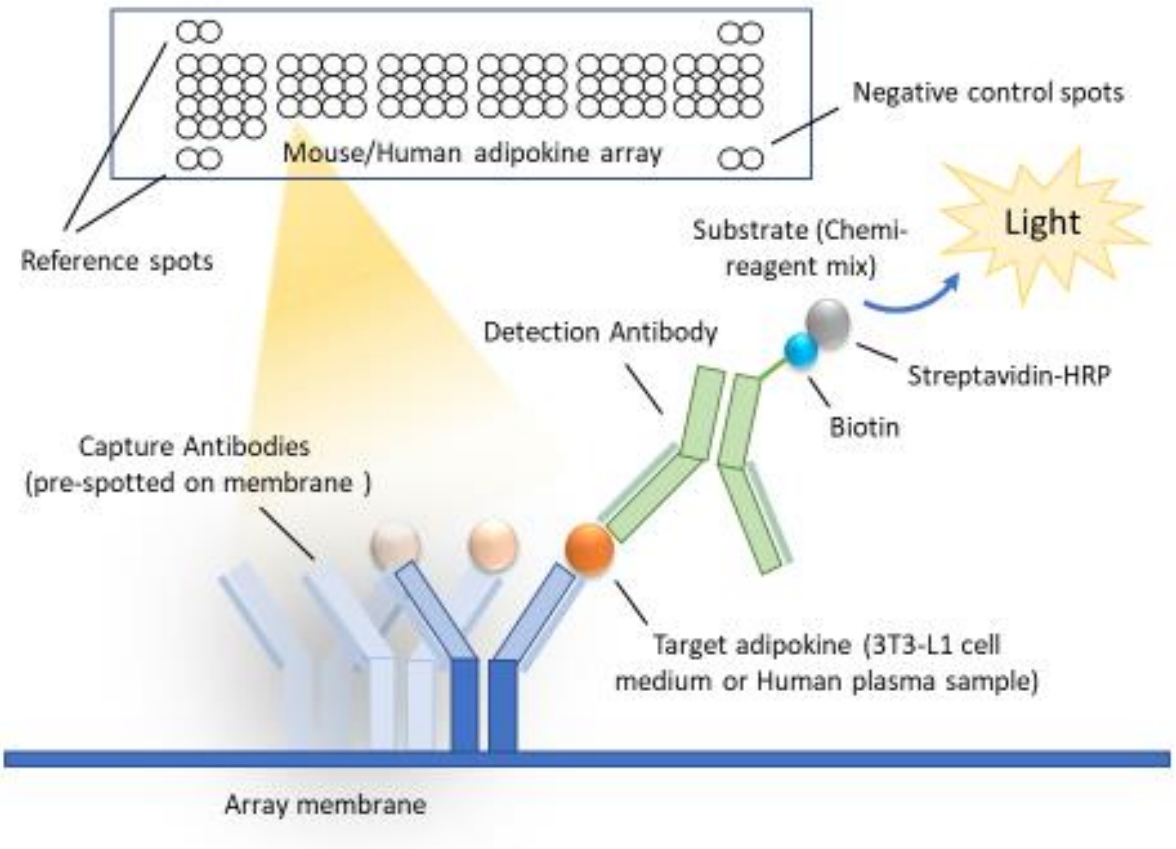

Figure 2.1: Representation of Human and mouse antibody arrays. Capture antibodies are pre-spotted in duplicate on nitrocellulose membranes. Arrays are incubated with sample and biotinylated detection antibodies, after further incubation with streptavidin-HRP chemiluminescence reagents are added to the membrane this produces a light signal on each spot which corresponds with the amount of protein bound. The negative control has no bound antibodies and acts as the background signal and the positive controls are used to align the array on the Quickspots analyses tool and to demonstrate the array has been incubated in Streptavidin-HRP and displays the maximum chemiluminescence signal. Adapted from R & D systems proteome profiler antibody array image.

2.4 Patient Sample and Data Collection

Patients were recruited in collaboration with Royal Manchester Children's Hospital. The data in the study has been anonymised to conform to data protection (Data Protection Act 1998) and parents/carers have given informed consent. NHS Health Research Authority ethical approval has been awarded: IRAS code 16/NS/0134. The study has also been approved by The University of Salford Research Ethical approval panel application number STR1718-01. We have recruited 38 patients in a 3.0 year period of this current study. In this pilot study we did not recruit patients to obtain healthy control blood due to the invasiveness of obtaining blood from young children. Blood samples were obtained by qualified medical professionals as part

of the standard induction therapy protocol to minimise unnecessary invasive procedures. 5mL blood samples were taken at day 1 of the induction therapy and day 29 when the induction therapy was complete, patients were also weighed at day 0 and day 29. The patient study design was made to be as minimally invasive as possible to reduce the impact on the children recruited to the study and also to collect data regarding adipokine profiles early on in treatment in this intensive part of therapy. A schematic of the study design can be seen in figure 2.2.

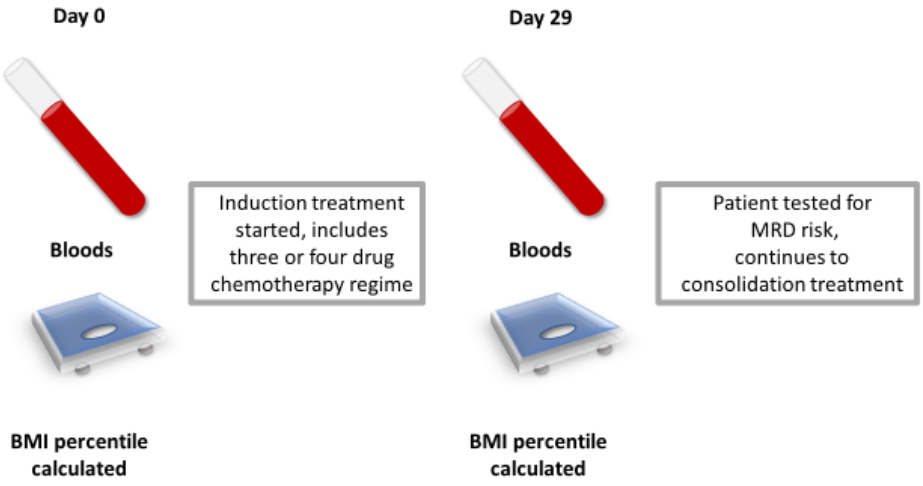

Figure 2.2: Schematic to show patient blood and weight collection for study design. Patients have bloods taken on day 0 and day 29, the beginning and end of induction therapy and are weighed at the same time, patients then are given an MRD risk status and move onto consolidation treatment.

From the whole blood samples plasma was extracted by research nurses and stored at -80°C for use in the study. Further to this as part of treatment weight and height were taken and BMI recorded at day 1 and day 29 which is then used to calculate the child's BMI percentile which is a measure of their weight in relation to age, sex and height compared with other children in the same categories (Daniels, 2009). Additionally, due to potential outcomes discussed in the introduction regarding toxicity and potential side effects of regimen A or regimen B where Daunorubicin is administered this has also been recorded. Patients were also given a category for MRD risk which was low risk or MRD risk. The MRD risk status was given after bone marrow sampling on day 29, patients with a blast cell percentage count <0.005% were considered low risk and a blast cell percentage count >0.005% were considered MRD risk Patients then are given an MRD risk status and move onto consolidation treatment.

These parameters are taken from the UKALL2011 trial protocol and summarised in table 2.2 with patient information and demographics.

Table 2.2: Summary of patient information and demographic. Table shows, age at diagnosis, gender-male or female, BMI at diagnosis, BMI at day 29, cytogenetic risk and minimal residual disease risk and % weight change, n=38.

Clinical variable	Number of patients n=38
Age	6.5 ± 3.8 years
Sex	24 males 14 females
Number in Induction category A/B	Induction A: 15 Induction B:23
BMI at Diagnosis (Kg/m^2)	16.6 ± 2.0
BMI at day 29 (Kg/m^2)	17.2 ± 3.3
Cytogenic risk	good: 18 intermediate:17 high risk: 1 T-cell disease: 1
MRD risk	22 low risk 15 MRD risk 1 no markers
% Weight change	Range -11.93 to +37.24, mean 6.03 ± 11.30

2.5 Human Adipokine Arrays

To determine levels of secreted circulating adipokines from patient samples plasma was obtained at day 0 of induction therapy and at day 29 when induction therapy was complete. The array approach was used to establish the range of all adipokines secreted at day 0 and day 29 to identify any novel patterns in expression. Adipokines levels were measured using Human Adipokine Arrays (ARY024 R & D Systems Bio-Techne Ltd). The ARY024 Human Array

measures 58 different adipokines, appendix table 7.5, spotted in duplicate on the array, array coordinates displayed in appendix figure 7.46. Following the manufacturers protocols each array membrane was incubated on a rocking platform in provided 4-well dish in 2ml of buffer 6 blocking buffer. During incubation 500µL of sample was prepared individually and adjusted to a final volume of 1.5mL with 500µL of buffer 6 and 500µL of buffer 4 also 30µl of human detection biotinylated antibodies then incubated for an hour. Blocking buffer 6 was aspirated from the arrays and each sample with the biotinylated detection antibodies was added and incubated overnight at 4^{o}C on a rocking platform, see figure 2.1 for a visual representation of the procedure. Next arrays were washed 3 times for 10 minutes in individual containers followed by a 30-minute incubation step with 1:2000 diluted Streptavidin HRP and the wash step repeated. Arrays were blotted and 1ml of prepared Chemi Reagent Mix was added for 1 minute then covered with a plastic sheet avoiding air bubbles. Arrays were imaged using a G-Box (Syngene software) for suggested exposure time of 10 minutes which detects chemiluminescence in each spot of the array. Images were saved as bmp files and analysed using Quickspots tool (Western Vision Software for R & D Systems). An average of the pixel intensity of each duplicate spot was taken representing each adipokine, the average of the Negative control spots was used to calculate the background signal, this was then subtracted from all average pixel intensities and saved in an excel file. The relative changes in adipokine levels can then be compared. For patient data analyses adipokine pixel intensity was normalised to the average of the three reference spots and changes in pixel intensity was calculated as Log2 fold change.

2.6 Cell culture of Human ALL cell lines Molt4 and CEM-CCRF

CEM-CCRF cells are a human ALL cell line originally obtained from a 4-year-old female, they are a T-Lymphoblast and grow in suspension in culture and are commonly used for studying ALL. In this study, the majority of the patients have ALL of B cell origin, however the only cell line of paediatric B cell origin available from tissue collections is CCRF-SB and this is infected with herpes virus and therefore not used in this study (Mendoza-Santiago et al., 2019). The CEM-CCRF cells were purchased from American Tissue Culture Collection. The cells were supplemented with base medium RPMI-1640, (Biosera), to make up a complete medium it was supplemented with Fetal Bovine Serum FBS (Gibco) 10% 100µL/mL, Penicillin and streptomycin (Biosera) 1% 10µL/mL, Sodium Pyruvate (Gibco) 1% 10µL/mL, Glutamine

(Lonza) 1% 10µL/mL. Cells were seeded at a density of 2 to 3 x 10^5 cells/mL in a T75 flask with 10ml of complete medium and incubated in a humidified atmosphere at 37°C with 5% CO2. Cell medium was refreshed every 2-3 days and fresh medium was added at 20-30% by volume each time. Cells were counted using a trypan blue viability assay (Sigma-Aldrich. USA) and kept at a live cell density between 2 to 3 x 10^5 and 1 to 2 x 10^6 viable cells/mL. Molt4 cells were purchased from ATCC and cultured in the same conditions as the CEM-CCRF cells. The Molt4 are also a T lymphoblast derived from a 19-year-old male, both cell lines are used extensively in ALL research.

2.7 Trypan blue viability assay and sample collection

To determine the viability of Molt4 cells in the presence of various adipokines trypan blue viability assay tests were completed every day over the course of a five day experiment to check cell viability. The following adipokines were tested; leptin, IL-8, resistin, serpinA12 and angiopoietin-2. Trypan blue is an azo dye which is impermeable to healthy cell membranes therefore it will only enter a cell which is unhealthy or dead with a compromised membrane (Strober, 2001). When cells are visually examined under a light microscope unviable cells will appear a blue colour in the cytoplasm due to trypan blue binding to intracellular proteins and viable cells will appear clear (Stoddart, 2011). The use of trypan blue live dead assays are inexpensive and the most commonly used method to count cells and calculate cell viability in a population of cells (Piccinini et al., 2017). Cells were collected on day 3 and used for western blot analysis to check for activation of the PI3K/Akt pathway. Molt4 cells were subcultured and 20µL of cell suspension was mixed with 20µL of trypan blue solution (Biosera), from this 20µL was loaded onto the haemocytometer to count the cells. Viable and dead cells were counted on the four corners of the haemocytometer at x10 magnification using a light microscope, Nikon, Digital sight. To determine the cells per mL count, the average of the 4 live cell counts, and the 4 dead cell counts were taken and multiplied by 2 (dilution factor for trypan blue) and displayed as cell per mL. The following equation was used to work out the percentage cell viability.

$$\text{Percentage Viability} = \frac{\text{Number of viable cells} \times 100}{\text{Total number of cells (viable count + non viable count)}}$$

To establish the effect of individual adipokines on the viability of Molt4 cells the following experiment was set up. Molt4 cells were seeded into a six well plate (Starstedt) at 200,000 cells/mL in 0.3% FBS with 2mL in each well this was classed as day 1. After 24 hours on day 2 cells were treated with an adipokine at doses of 500ng/mL, 50ng/mL, 5ng/mL and no adipokines in the control well. Adipokine solutions were prepared by mixing the lyophilized pellet with DD water and stored in aliquots at -20^{o}C. Cells were gently pipetted up and down to ensure all cells were in solution and 20µL was taken to perform a trypan blue viability assay. On day 3 1mL of cells were collected by gently pipetting up and down in the wells to ensure all cells were in solution from each well and proteins extracted for western blot, a further 20µL was taken to complete a trypan blue viability assay. Trypan blue viability assays were completed on day 4 and 5 as above, see figure 2.3 for plate layout.

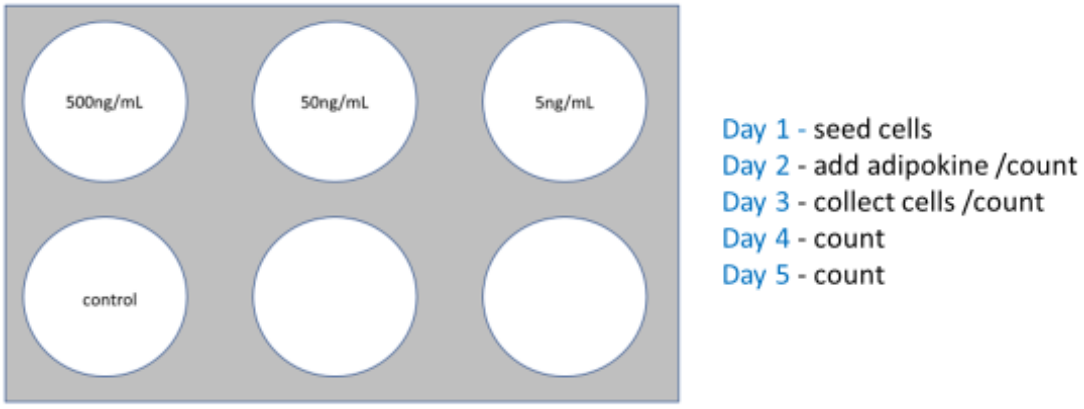

Figure 2.3: Trypan blue viability plate set up. Molt4 cells were seeded into a six well plate at 200,000 cells/mL in 0.3% FBS with 2mL in each well (day 1) after 24 hours cells were treated with an adipokine at doses of 500ng/mL, 50ng/mL, 5ng/mL and no adipokines in the control well (day2). On day 3 1mL of cells were collected from each well and proteins extracted for western blot. Trypan blue exclusion assays were completed on day 4 and 5.

2.8 Protein extraction and quantification

For western blotting, 1 mL of cell solution was removed from each well containing the treated and control cells and prepared for SDS page. Cells were centrifuged for 5 minutes at 1200rpm, after removing the media the pellet was washed with PBS and centrifuged again at 1200rpm. The supernatant was carefully removed, and samples were lysed in 100 µL triple lysis buffer (50mM Tris-HCI pH 7.5, 150mM, NaCI, 0.1% SDS, 1% NP40) containing phosphatase and protease inhibitor cocktail (Santa Cruz Biotechnology) this cocktail was used to stop proteases and phosphatases degrading proteins during the extraction process (Bass et al., 2017), the

samples were then placed on ice for 30 minutes. Samples were then centrifuged at 13000rpm at 4^{o}C to remove insoluble material. The protein concentration of the supernatant was then quantified using a Bradford protein assay (Bio-rad), this assay determines concentrations of proteins by the colour change of Coomassie G-250 from brownish orange to blue (Sedmak and Grossberg, 1977). Coomassie G-250 is brownish orange in colour when unbound to protein with an absorbance maximum of 470nm, when bound to protein this colour shifts to blue and can be measured on a calibrated photospectrometer (Jenway 6305) at a 595nm wavelength, this measurement is proportional to the amount of protein in the sample. The concentration reading allows the mass of the protein to be calculated by using a BSA curve (linear relationship between optical density and protein concentration/µg) which is calculated from using standards covering the linear range of the Bradford assay.

2.9 Western blotting

Samples containing 30µg of protein were boiled for 10 minutes with laemmli SDS sample buffer (Alfa Aesar). Laemmli SDS sample buffer is a loading buffer containing glycerol and bromophenol blue. Glycerol is dense and allows the sample to sink into the wells when loading, whilst bromophenol blue, aids tracking progress during electrophoresis (Mahmood and Yang, 2012). Heating and using laemmli SDS buffer also denatures the secondary/tertiary structures within the sample but preserves the negative charge of amino acids (Bass et al., 2017), the protein can then move through the electric field. Samples were then fractionated by SDS-PAGE using Trans-blot kit (Bio-rad) and 1x Tris/Glycine/SDS running buffer (Bio-rad). Following electrophoresis, the samples were transferred to an immune-Blot PVDF membrane (Bio-rad) using 1x Tris/Glycine transfer buffer (Bio-rad). When transferred Ponceau red stain (Sigma-Aldrich) was used to detect protein transfer onto the membrane, this was then rinsed with DD water and blocked with 5% non-fat powdered milk in TBST, (10nM Tris, pH 8.0, 150nM NaCl, 0.01% Tween 20) (Bio-rad) for one hour which prevents nonspecific antibodies binding to the membrane (Mahmood and Yang, 2012), it was then exposed to primary and secondary antibodies. Membranes were incubated overnight with primary antibodies against AKT (1:1000), phospho-AKT (1:1000) and GAPDH (1:10000) (Cell signalling). Membranes were washed for 3 x 10 minutes with PBS Tween 0.1% and incubated with anti-rabbit (GE healthcare) and anti-mouse (Sigma-Aldrich) secondary antibodies at 1:5000 dilution for one hour, then a final 3 x 10 minute wash step with PBS Tween 0.1%. Secondary antibodies used

were horseradish peroxidase conjugated and the reactivity of the proteins detected using chemiluminescence (Thermo Fisher Supersignal West femto). Images were captured using a G-Box (Syngene software). Images were then processed using ImageJ software to quantify the density of protein bands which reflect the relative amounts of protein compared to the loading control.

2.10 MTS assay

An MTS assay was used to check the viability of cells in suspension, it is a sensitive colorimetric assay based on the presence of metabolically active cells (Mosmann, 1983). NAD(P)H-dependant dehydrogenase enzymes in viable cells cause a reduction of the tetrazolium compound in the MTS solution which in turn produces a coloured formazan product which is then measured at an absorbance of 490nm (Cory et al., 1991), metabolically active cells produce a dark brown colour in each well compared to a lighter colour with less active or dead cells. This one step assay was performed to determine the viability Molt4 cells in the presence of drug treatments.

Prior to starting the assay, the cells were counted with Trypan Blue to determine live cell numbers. Cells were seeded in a clear 96 well plate at a density of 2 x 10^5 viable cells/mL in 0.3% FBS with 100µL in each well and incubated for 24 hours. Drug doses were prepared on a separate plate in a serial dilution, for vincristine this selected concentrations were 20nM, 10nM, 5nM, 2.5nM, 1.25nM, 0.625nM, 0.312nM, 0.156nM and 0.078nM with a negative control of untreated Molt4 cells and a positive control of colchicine (Sigma Aldrich) at a concentration of 500µM. 100µL of vincristine dilutions were added back to the Molt4 cell plate and incubated for a further 72 hours. This was also completed with the drug daunorubicin (Alfa Aesar) at concentrations ranging from 200nM, 100nM, 50nM, 25nM, 12.5nM, 6.25nM, 3.12nM, 1.56nM and 0.78nM. Overall DMSO concentration used for drug dilution across the plate was less than 1%. To complete the cell proliferation assay cells were incubated with 20µL/well of MTS solution (The CellTiter 96 Aqueous One Solution, Promega) for 3 hours in 37°C. Absorbance was read using an Omega Fluostar plate reader (Omega Fluostar, Omega software) at 490nm and 690nm. To process the data background absorbance was subtracted, the average absorbance of the treated wells was divided by the average absorbance of the negative control. The cell viability is expressed as a percentage change from

the negative control. The average absorbance of the treated wells was divided by the average absorbance of the no drug control for each condition on the plate not the negative control. Figure 2.4 shows an example of the plate layout, pale blue vincristine alone, dark blue vincristine and selected adipokine, pale green daunorubicin alone, dark green daunorubicin and selected adipokine, yellow are no drug controls for each condition, red are positive colchicine controls and grey are negative controls.

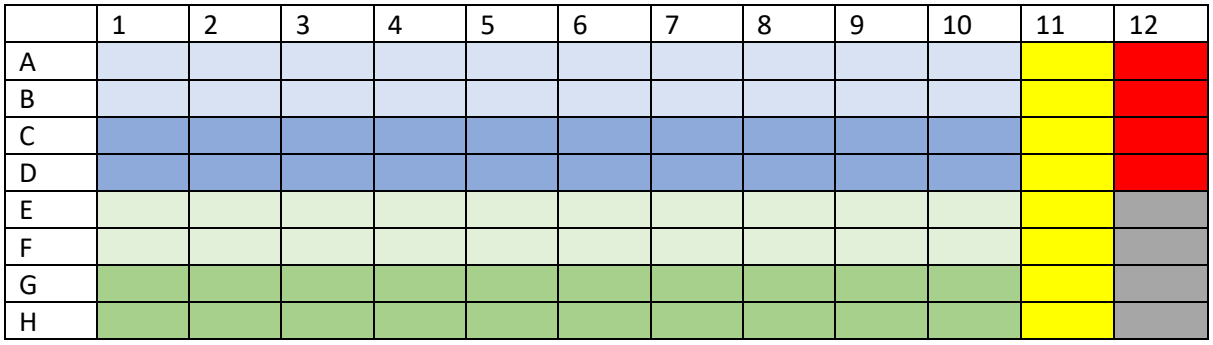

Figure 2.4: Example plate layout for chemosensitivity assays. Pale blue vincristine alone concentrations 20nM to 0.078nM A + B 1-10, dark blue vincristine and selected adipokine C + D 1-10, pale green daunorubicin alone concentrations 200nM to 0.78nM E + F 1-10, dark green daunorubicin and selected adipokine G + H 1-10, yellow are no drug controls for each condition, red are positive colchicine controls and grey are negative controls.

2.11 MTT assay

An MTT assay was used to determine the cell viability of adherent 3T3-L1 cells in response to the ALL chemotherapy drugs vincristine and daunorubicin. An MTT assay is a colorimetric assay and works on the same principle as an MTS assay as described previously, this is more suited to adherent cell lines and involves a two-step approach. The MTT reagent is reduced in this assay to an insoluble purple formazan product in the presence of metabolically active cells (Riss et al., 2016), the purple colour as marker for cell viability is then solubilised with DMSO and the absorbance measured at 570nm. 3T3-L1 cells were seeded in a 24 well plate (Starstedt) at 20,000 cells per well and differentiation was induced as previously described in section 2.1.3. When the cells were 3 weeks old the media was replaced with 1ml of media containing vincristine or daunorubicin, concentrations of each drug ranged from 100µM, 50µM, 25µM, 12.5µM, 6.25µM, 3.12µM, 1.562µM, 0.78µM, 0.39µM and 0.19µM. Cells were

then incubated for 96 hours at 37°C and 5% $C0_2$. To complete the cell proliferation 200µL of MTT solution (5mg/mL) was added to each well of the 24 well plate and then incubated for 3 hours at 37°C. The MTT solution was removed carefully leaving the purple formazan crystals which are an indicator of cell viability and then solubilized by the addition of 200µL of DMSO. The solutions were transferred to a 96 well plate (Starstedt), absorbance was read using an Omega Fluostar plate reader at 570nm and 690nm. To process the data background absorbance was subtracted, the average absorbance of the treated wells was divided by the average absorbance of the negative control on each plate.

2.12 Cell lines used in this study

2.12.1 3T3-L1

The 3T3-L1 cell line is a pre-adipocyte fibroblast-like cell line that was originally developed from mouse swiss 3T3 cells, the L1 strain is a continuous sub-strain which was developed through clonal isolation (Green and Meuth, 1974). The cells are a well-established model widely used in the field of adipose research in particular white adipose tissue research and obesity related conditions (Chang et al., 2016) (Guo et al., 2019). The cells can be continually cultured in a non-contact pre-adipocyte state where they have the potential to be differentiated into mature adipocytes and accumulate lipids droplets (Green and Meuth, 1974). The differentiation process involves allowing cells to become confluent then treating them with a cocktail of insulin, dexamethasone and a phosphodiesterase inhibitor 1-methyl-3-isobutyl xanthine (IBMX), cells can then be maintained as mature adipocytes to be used in experiments (Zebisch et al., 2012). During the differentiation process a number of genes are upregulated which drives the switch to a mature adipocyte phenotype by controlling cell growth and lipid metabolism (Gerhold et al., 2002).

2.12.2 CEM-CCRF

CEM-CCRF is a continuous T cell acute lymphoblastic leukaemia line originally isolated from the buffy coat fraction of a 4-year-old female after relapse from a multidrug chemotherapy regime in 1965 (Foley et al., 1965). Since CEM-CCRF was originally isolated, it has been used experimentally to produce variants, these include CEM-C1/2 which is resistant to the

topoisomerase inhibitor Camptothecin (Kapoor et al., 1995). Also the closely related clone CEM-C7 which is highly sensitive to glucocorticoids (Medh et al., 2003).

2.12.3 Molt4

The Molt4 cell line was originally isolated from the peripheral blood of 19-year-old male patient who was suffering from T-cell ALL during a relapse of the disease, the patient had previously received a multi-drug chemotherapy regime (Minowada et al., 1972). After isolation of the cell line it was concluded that Molt4 is representative of a T-cell due to is ability to grow *in vitro* without an Epstein Barr virus (EBV)-genome, that it had no surface immunoglobulin or an Fc receptor, and also its ability to form direct rosettes with sheep red cells (Klein et al., 1974). Both Molt4 and CEM-CCRF cell lines are both T cell lines so they are not fully representative of B cell physiology in ALL and they are also from individual patients so lack the variability from patient samples across a full cohort. Nonetheless cell lines such as these provide useful model systems for the study of ALL.

2.13 Statistical analysis

Statistical analysis of the patient data was taken from the results of the human adipokine arrays and analysed in GraphPad Prism version 8.2.1. The data was first normalised to the average of the reference spots on each array so that adipokine levels from patient to patient could be compared together for particular groups of patients for example, do levels of adipokines change at day 29 between low risk and MRD risk patients? The selected groups of patients were then compared using an unpaired t-test where $p<0.05$ was significant, this was the most suitable way to compare the selected two groups i.e., MRD risk to low risk patients and weight gain to normal weight range in patients at the end of induction therapy . Statistical analysis of the cell counts, and trypan blue assays were performed using Graphpad. The effects of adding individual adipokines to cells in culture at increasing concentrations compared to the control was performed using 2-way ANOVA and Dunnett's multiple comparison test where $p<0.05$ was significant, using Dunnett's multiple comparison test each variable can be compared to the control. MTT results were processed using graph pad using 2-way ANOVA where $p<0.05$ was considered significant. The pilot study scatterplots were performed using linear regression to measure the r^2 value where the deviation from zero was

considered significant where $p<0.05$. Clinical variables by Liverpool university were analysed using R software by logistic regression where MRD risk was classed as the dependant categorical variable and selected clinical variables as the independent variables where $p<0.05$ was considered significant. Independent variables were BMI at diagnosis, age at diagnosis, sex (males), weight change percentile, BMI percentile at diagnosis, cytogenic risk and induction regimen. The work by The University of Liverpool was requested by service agreement and completed on patients 009- 040. After the final number of patient samples were collected and processed Graphpad was used to complete the data analysis, patients 009-057.

3.0 CHAPTER 3: 3T3-L1 Mouse adipose cell differentiation experiments

3.1 Differentiated 3T3-L1 cells produce an adipocyte-like phenotype

The major objective of this study was to profile circulating adipokine expression in paediatric ALL patients before and after induction treatment, however a number of initial experiments were performed in advance to validate the optimal conditions for the adipokine arrays, and to practice performing these arrays. One validation experiment was to perform an adipokine array using the 3T3-L1 cell line, in order to determine if biologically relevant changes in adipokine expression could be detected using this assay between conditioned media samples from pre-differentiated and differentiated adipocytes.

To confirm that the 3T3-L1 cells had fully differentiated and were producing adipokines three different methods were used; images were taken during the differentiation, oil red-o staining confirmed the presence of lipid droplets and mouse adipokine arrays verified the presence of adipokines in differentiated cell conditioned media.

Transmitted light micrographs were taken at weeks 0, 1, 2, 3, & 4 to confirm morphological changes during the differentiation process. Cell supernatant was taken at each weekly timepoint from designated differentiation wells before the differentiation medium was replaced, see figure 3.1a, c, e, g & h and from non-differentiation control wells, see figure 3.1b, d, f, h & j in which cells were refreshed with pre-adipocyte expansion medium not containing differentiation additives. At no point during the differentiation process or after were the cells passaged.

Figure 3.1a and b represents the cells at week 0 prior to the differentiation process and 1 day after the cells were initially seeded in the 24 well plate. There are no noticeable differences in phenotype between the control and differentiation wells. The cells appear as small crescent like shapes with no lipid droplets present. After one week of differentiation, figure 3.1c and d cells which were exposed to differentiation factors, appear to be larger in size and small lipid droplets are evident, the Week 1 control figure 3.1d shows the appearance of smaller lipid droplets with large gaps between the cells, all images were taken from the same experiment. Week 2 after differentiation, the number of lipid droplets appears to have

increased figure 3.1e, while the control well figure 3.1f appears to have comparably less droplet formation with large gaps between the cells. At this point, differentiated cell medium had a yellow colour and was viscous, the non-differentiated control well medium still had a pink colour similar to the medium when it is due to be refreshed when growing as a pre-adipocyte culture. There were no obvious phenotypic changes at week 3 compared with week 2, figure 3.1g and h, nor at week 4 figure 3.1i and j, although some lipid droplets are present in the un-differentiated control well after 4 weeks.

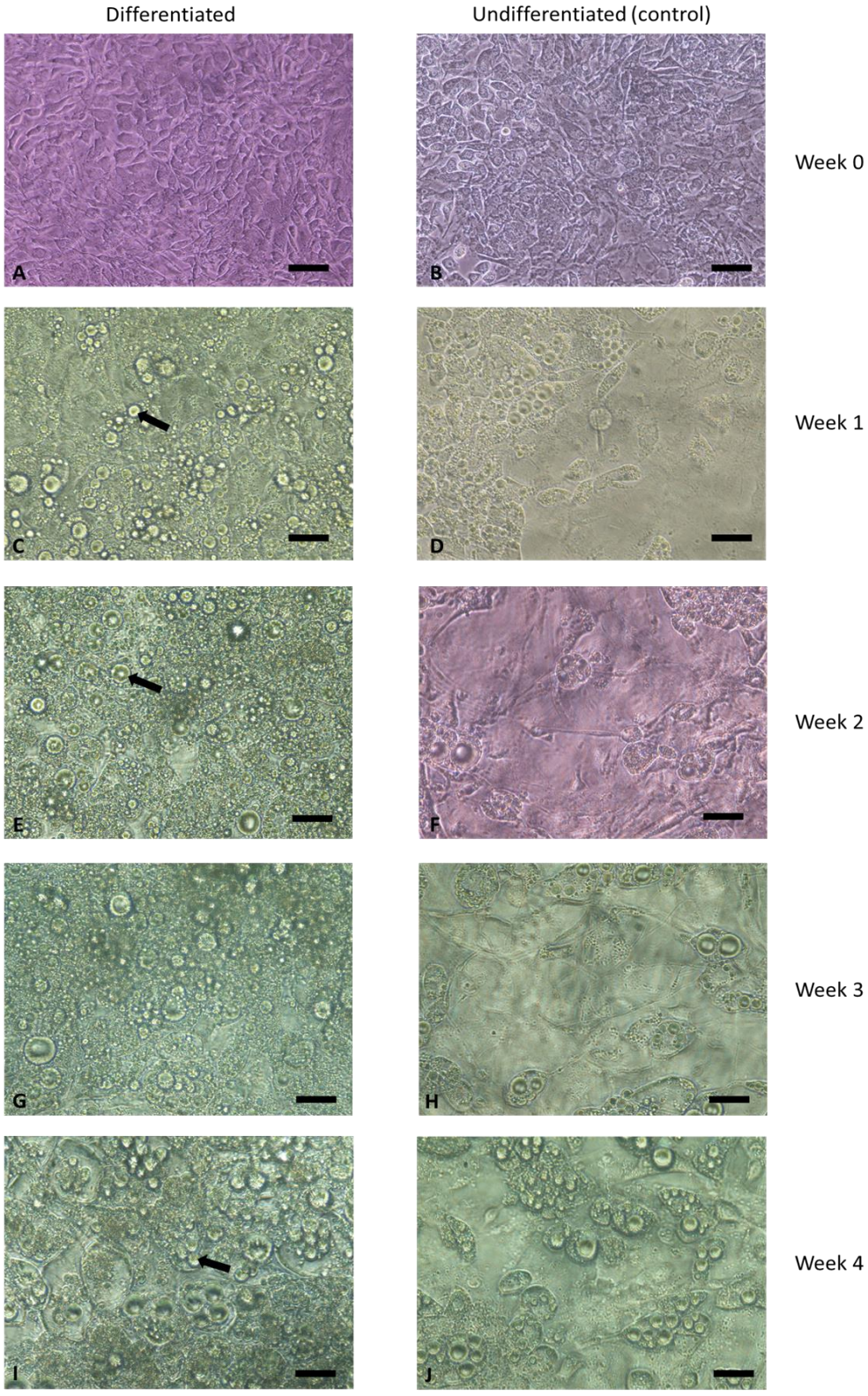

Figure 3.1: Differentiation of 3T3-L1 mouse adipocyte cells over 4 weeks. a, c, e, g, i, shows differentiation wells at weeks 0-4. b, d, f, h, j shows non-differentiation control wells where 3T3-L1 cells did not receive differentiation medium. Lipid droplet accumulation is apparent from week 1 (black arrows) and increases to week 4 within the differentiated wells, there are fewer fat droplets in the non-differentiated wells, Scale bars 25µM.

3.2 Oil red-O staining confirms the presence of lipids droplets in differentiated 3T3-L1 cells

Following 2 weeks differentiation the imaged cells were stained with the lysochrome diazo dye, Oil red-O solution, to confirm the presence of lipids in fixed cells (Proescher, 1927). Figure 3.2a shows 3T3-L1 mouse adipocytes after two weeks of differentiation and when lipid droplets are apparent within the cells. Cells were fixed and stained as described in the methods section 2.2 and imaged using light microscopy. Figure 3.2b shows the well stained with Oil red-O confirming the presence of lipids and red circular stained lipid droplets can be observed.

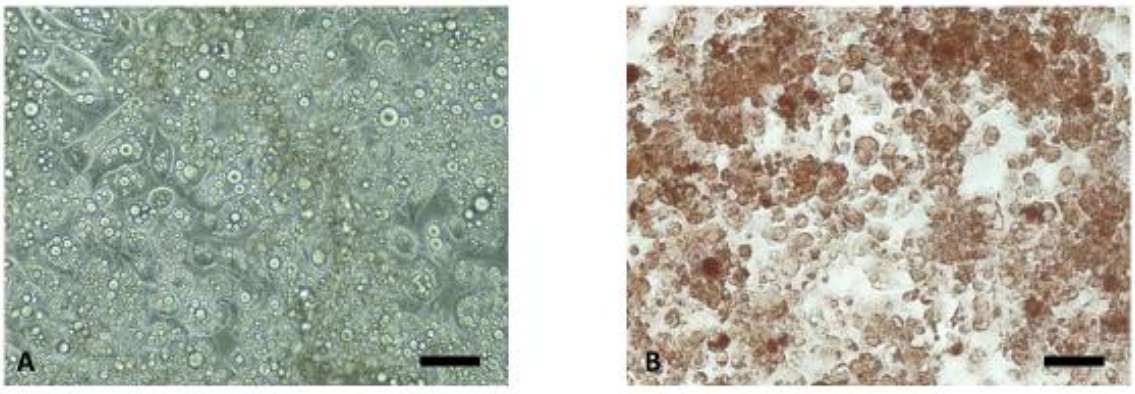

Figure 3.2: Light Microscopy to confirm lipid accumulation in differentiated 3T3-L1 cells at 2 weeks. Image a shows 3T3-L1 mouse adipocytes at week 2 after differentiation. Image b shows cells the cells stained with Oil red-O to highlight lipid droplets. Magnification x40, Scale bars 25µm.

3.3 Mouse adipokine arrays confirm the presence of adipokines in 3T3-L1 differentiated cell conditioned media

Supernatant from each differentiation time point was taken to determine which adipokines were produced by the differentiated and non-differentiated 3T3-L1 cells. Cell culture medium was collected 24 hours after seeding, before 100% confluence (when the cells were still in the pre-adipocyte stage) then at weeks 1, 2, 3, 4, from the differentiated wells and the undifferentiated control wells. Mouse adipokine arrays were run immediately as described in methods section 2.3, imaged, and analysed to obtain the mean pixel density for each adipokine. Results are displayed in a graph in appendix figure 7.1, this shows all adipokine expression grouped into corresponding weeks from each array processed with the overall

mean showing an increase from seeding at weeks 0, increasing in expression to week 3 and then decreasing in week 4. Results are also displayed as a heat map with expression values in appendix table 7.1 which helps to visualise the change in pixel density in relation to undifferentiated cells 24 hours after seeding. Results have been highlighted red when the mean pixel intensity decreases and green when the mean pixel density increases compared to control media taken 24 hours after cell seeding. These data demonstrate the biggest increases in expression level of each individual adipokine at week 3 of differentiation. This was defined as the optimum time point for medium collection to use in future fat conditioned media cell culture experiments. The undifferentiated control cells also show increases in adipokine expression at week 3. There are differences in expression between week 2 after differentiation and week 3, week 3 shows higher expression of the adipokines CRP, DPPIV, IL6, MCP-1, pentraxin-2, RAGE, serpin E1 and TIMP-1 in the differentiated cells compared with week 2 and medium collected 24 hours after seeding. Serpin E1 has still decreased overall compared to the levels recorded taken from media 24 hours after seeding. In some cases, adipokine expression was not detectable at the experimental time points and therefore it was not possible to calculate expression change. This observation has been captured within appendix table 7.1, the white boxes indicate where expression change could not be calculated.

The information in figure 3.3 and heat map in figure 3.4 shows the same set of data displayed as log2 fold change. Adipokine expression data for weeks 1, 2, 3 & 4 compared to week 0, which is the cell culture medium collected from pre-adipocytes 24 hours after seeding before differentiation. Presenting the data in this format make it clearer to see at week 3 all but two of the adipokines tested have increased in expression, the mean increase is higher for adipokine expression at week three in the differentiated and drops slightly by week 4. Also, the spot highlighted in dark grey is the fold change decrease for Serpin E1, this decrease is due to the high expression of Serpin E1 in the undifferentiated cells. The yellow spot highlights the expression levels of TIMP-1 which did not change compared to week 0, all remaining adipokines on the array increased in expression. By week 4 the results indicate that the overall mean for levels of expression decreased, this further confirmed the selection for week 3 adipocyte media as time for completing further experiments which the adipocytes and chemotherapy needed to be factored into the optimum collection point.

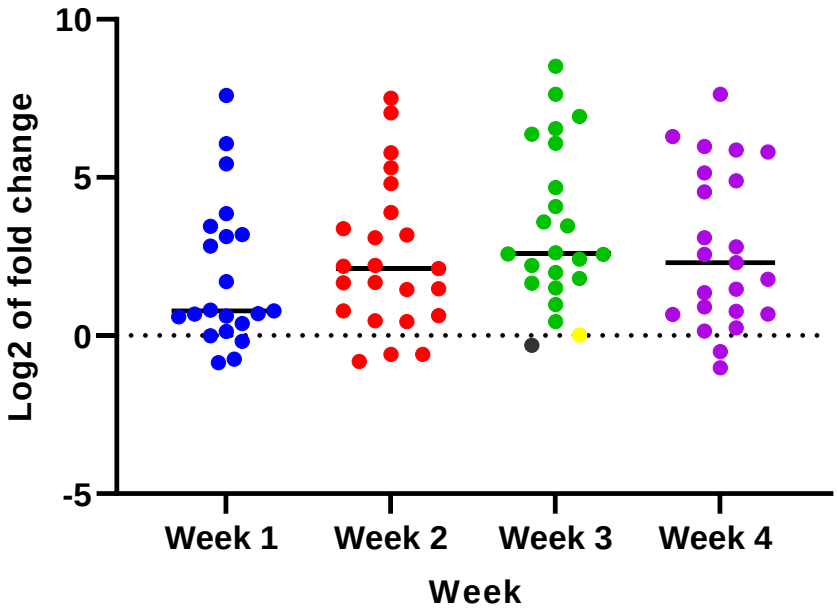

Figure 3.3: Mouse Adipokine array results and Log2 fold change data from 3T3-L1 differentiated and undifferentiated cells over the four-week differentiation period. Adipokine expression levels from each week 1, 2, 3 & 4 are displayed grouped to show the mean increases over the whole experiment compared to week 0. The dark grey spot is the result for Serpin E1 and the yellow spot TIMP-1, n=1.

The heat map displayed in figure 3.4 confirms log2 fold change expression levels, increasing shades of green correspond to increasing levels of the log2 fold change results for adipokines compared to undifferentiation cells 24 hours after seeding, indicated as zero on figure 3.3. Red indicates decreases on expression levels of adipokines and grey indicates no data available for that time point.

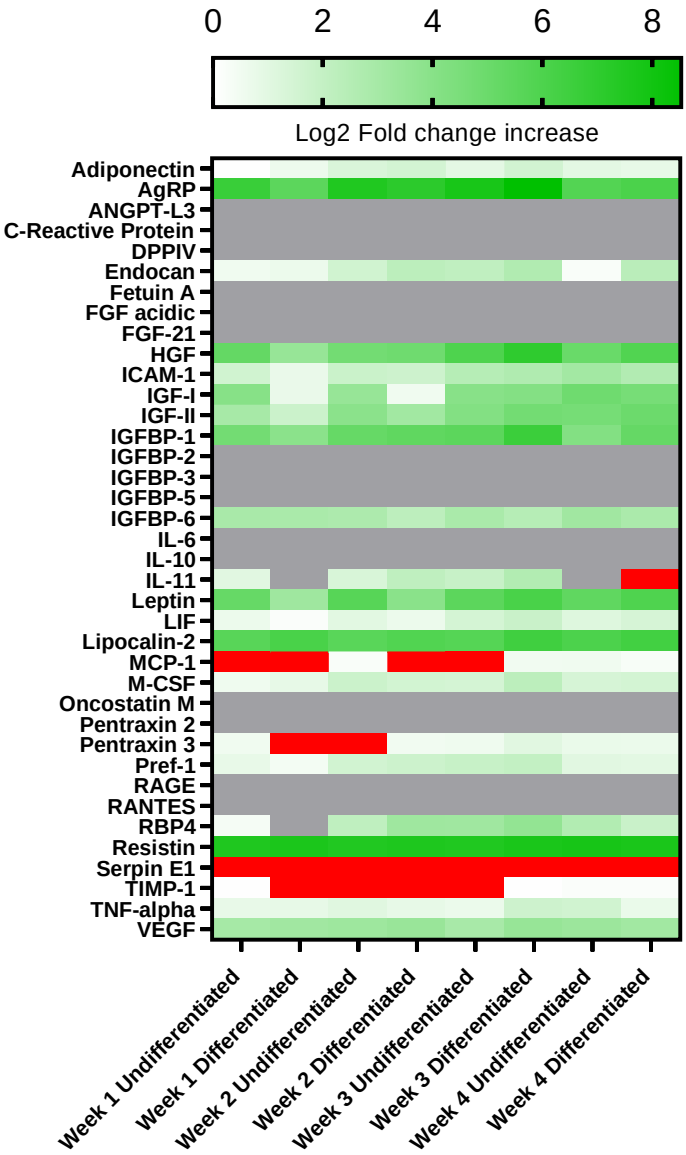

Figure 3.4: Mouse Adipokine array results and Log2 fold change data from 3T3-L1 differentiated compared to undifferentiated cells over a four-week differentiation period. The summary table indicates increases in log2 fold change (green) and decreases (red) of array pixel density for each corresponding adipokine. Results for differentiated cells and undifferentiated wells for weeks 1, 2, 3 & 4 compared to pre-adipocytes at 24 hours after seeding. Grey boxes indicate no data available, n=1.

3.4.1 MTT assay confirms Daunorubicin and Vincristine reduce cell viability of 3T3-L1 cells

Chemotherapy is known to have an impact on cell viability in many cells in the body not just the cancer cells being treated, a major aim in cancer treatment is to target cancer cells while avoiding damage to healthy cells (Cheok, 2012). The purpose of the following experiments

was to understand the impact of chemotherapy on the function of adipose tissue. It is well documented that daunorubicin is known to be toxic to cells, particularly cardiac cells (McGowan et al., 2017). To determine the viability of 3T3-L1 cells treated with chemotherapy, week 3 differentiated murine 3T3-L1 cells were treated with increasing two-fold concentrations of vincristine or daunorubicin from 0.019µM to 100µM. Viability was determined using an MTT assay as described in section 2.11. At concentrations above 1µM vincristine displayed less toxicity to 3T3-L1 cells than daunorubicin, Figure 3.5. Cell viability was significantly lower in cells treated with daunorubicin compared with vincristine at 25µM, 50µM and 100µM, $p=0.0001$, $p<0.0001$ and $p<0.0001$ respectively, n=3, calculated using 2-way ANOVA.

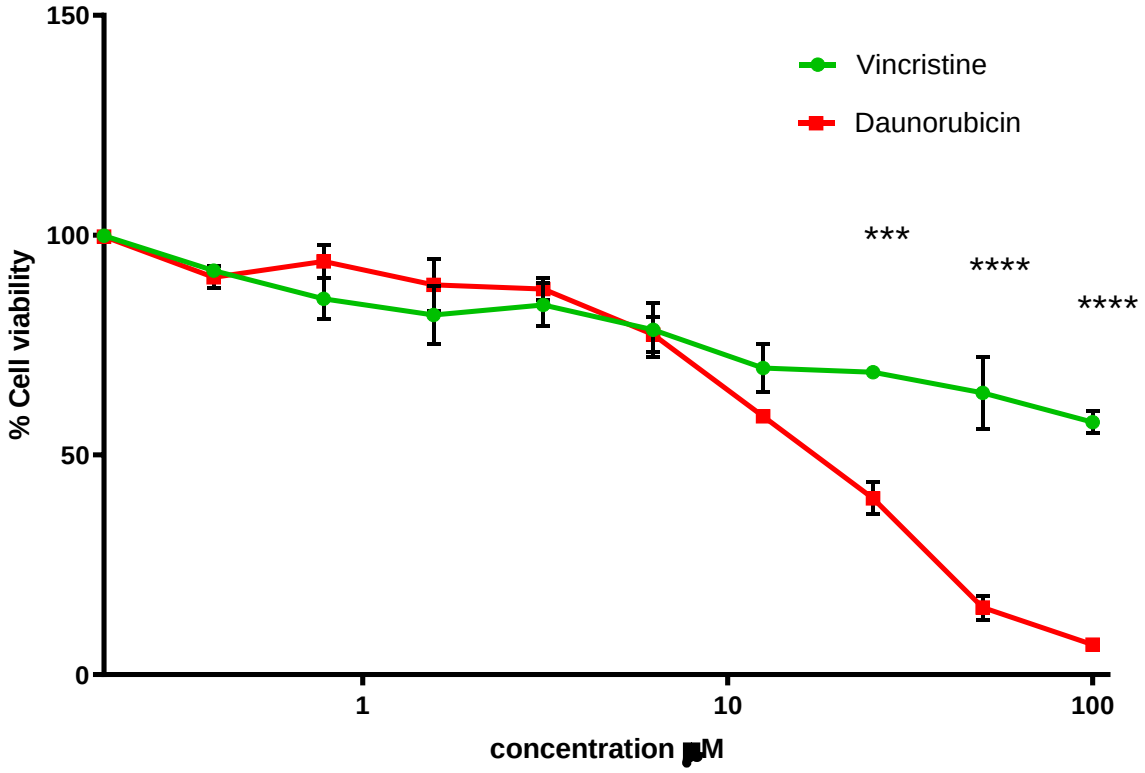

Figure 3.5: The effect of chemotherapy agents on the viability of differentiated 3T3-L1 cells. Viability of differentiated 3T3-L1 cells treated with Vincristine and Daunorubicin for 96 hours and were measured using an MTT assay. Concentration µM plotted against % cell viability. Significant changes were observed between cells treated with vincristine compared to daunorubicin at concentrations 25µM, 50µM and 100µM, denotes p values of $p=0.0001$, $p=<0.0001$ and $p=<0.0001$ respectively, n=3.

3.4.2 Oil red-O staining confirms changes in the levels of lipids droplets in differentiated 3T3-L1 cells treated with Vincristine and Daunorubicin

Experiments were performed to determine the effect of vincristine and daunorubicin on the accumulation of lipids and secretion of adipokines by the 3T3-L1 cells. A high concentration of the drug, where the viability was reduced, and a low concentration, which had no effect on cell viability, were chosen from MTT data, figure 3.6. Concentrations of 0.05µM and 5µM were chosen. Figure 3.6 displays the images taken of the differentiated 3T3-L1 cells after staining with Oil red-O. Lipid droplet accumulation appears to be negatively impacted by chemotherapy treatment and this effect was increased at higher concentrations of both vincristine, figure 3.6b and c and daunorubicin figure 3.6e and f compared with control well image 3T3-L1 cells without treatment, figure 3.6a and d. Daunorubicin treated cells have fewer visible lipid droplets at both concentrations compared to vincristine treated cells and untreated control cells.

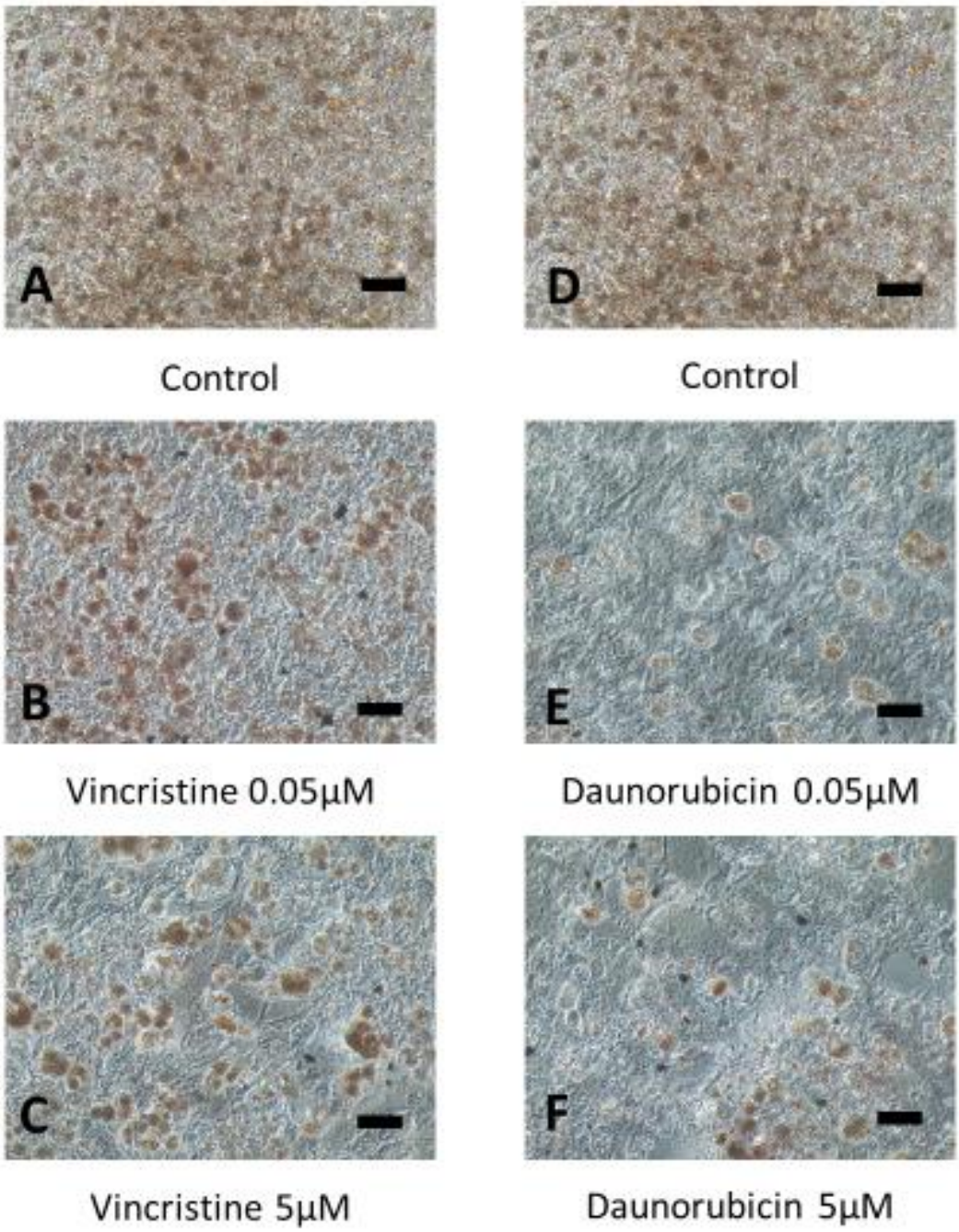

Figure 3.6: Images of 3T3-L1 cells after treatment with Vincristine or Daunorubicin. A and D show control wells without treatment. B shows cells treated with 0.05µM of Vincristine and C shows cell treated with 5µM of Vincristine. E shows cells treated with 0.05µM of Daunorubicin and F shows cells treated with 5µM Daunorubicin. Lipid droplet accumulation is apparent in control wells and decreases in both treatments with increasing concentrations of drug. Daunorubicin treated cells have less visible lipid droplets at both concentrations. Scale bars 25µM.

3.4.3 Mouse adipokine arrays confirm changes in adipokine expression in cells treated with Vincristine and Daunorubicin

To investigate the effect of vincristine and daunorubicin treatment on 3T3-L1 cells, mouse adipokine arrays were used to assess the changes in adipokine secretions. Secretions from the cells treated with 0.05µM and 5µM of Vincristine and Daunorubicin for 96 hours were tested and the fold change from the control untreated differentiated well was analysed. Results suggest that a relatively short treatment of cells to 96 hours of chemotherapy does not impact the ability of the cells to secrete adipokines, although the expression levels do differ in cells treated with vincristine and daunorubicin compared to non-treated cells. The green squares are the results from differentiated 3T3-L1 cells treated with 0.05µM of vincristine, the red circles are differentiated 3T3-L1 cells treated with 5µM of vincristine, shown in figure 3.7. Adipokines did not all follow the same patten of expression change following exposure to vincristine. The following adipokines show little difference when treated with 0.05µM or 5 µM of Vincristine; IGF-II, IGFBP-1, IGFBP-3, LIF, Lipocalin-2, MCP-1, M-CSF, Pentraxin-3, Pref-1, Serpin-E1, TNF-Alpha, VEGF, within this group of adipokines there are larger increases of IGF-II, IGFBP-1 and TNF-alpha compared to untreated control cells. There are differences in adiponectin expression; cells treated with 5µM of vincristine are similar to untreated cells but when treated with 0.05µM of vincristine expression of adiponectin is increased. The opposite pattern is seen in the following adipokines; ICAM-1, IGF-1, IGFBP-6, RANTES, resistin and TIMP-1 where treatment with vincristine decreases expression. The cells treated with 0.05µM of vincristine all show decreases in expression of adipokines compared to untreated control cells and when treated with 5µM of vincristine ICAM-1, IGF-1, IGFBP-6, RANTES and resistin all increase in comparison to untreated control cells.

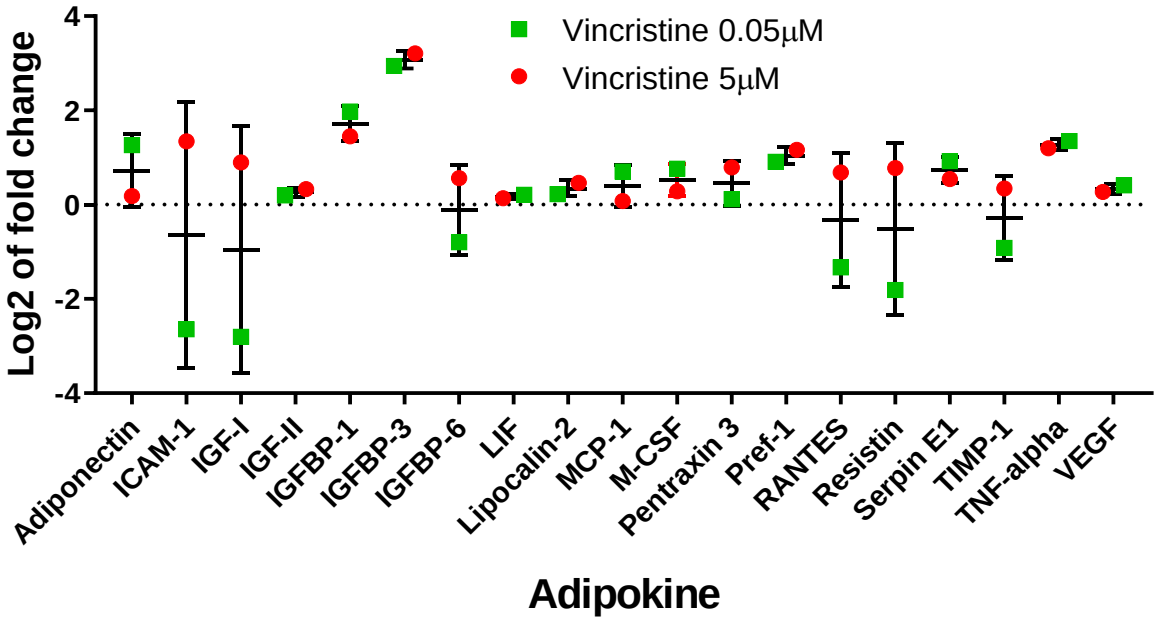

Figure 3.7: Mouse adipokine array results for 3T3-L1 cells treated with vincristine. Results displayed as Log2 of fold change from control well data represented as zero. The green squares are the fold change results for each adipokine for 0.05µM concentration of vincristine. The red circles are the fold change results for 5µm of vincristine. n=1.

Results in figure 3.8 show the same data for daunorubicin treated 3T3-L1 cells where the green squares are results from cells treated with 0.05µM of daunorubicin and the red circles cells treated with 5µM, n=1. Fold change data was plotted in comparison to untreated control cells. The following set of adipokines show little or no difference when treated with 0.05µM or 5µM of daunorubicin; adiponectin, ICAM-1, IGF-II, IGFBP-1, IGFBP-6, LIF, Lipocalin-2, MCP-1, M-CSF, pref-1, resistin, serpin-E1, TNF-alpha and VEGF. There are larger differences in expression levels of FGF-21, HGF, IGF-I, IGFBP-3, pentraxin-3, RANTES and TIMP-1, of these adipokines HGF and IGFBP-3 increase in both daunorubicin concentrations with 0.05µM displaying higher expression in comparison to untreated cells. FGF-21, pentraxin-3 and TIMP-1 also show differences in expression levels, when treated with 0.05µM of daunorubicin expression increased and when treated with 5µM of daunorubicin expression decreased. Expression levels of IGF-1 and RANTES decreased in both treatments of daunorubicin with the largest decreases in the cells treated with 5µM of daunorubicin compared to untreated control cells.

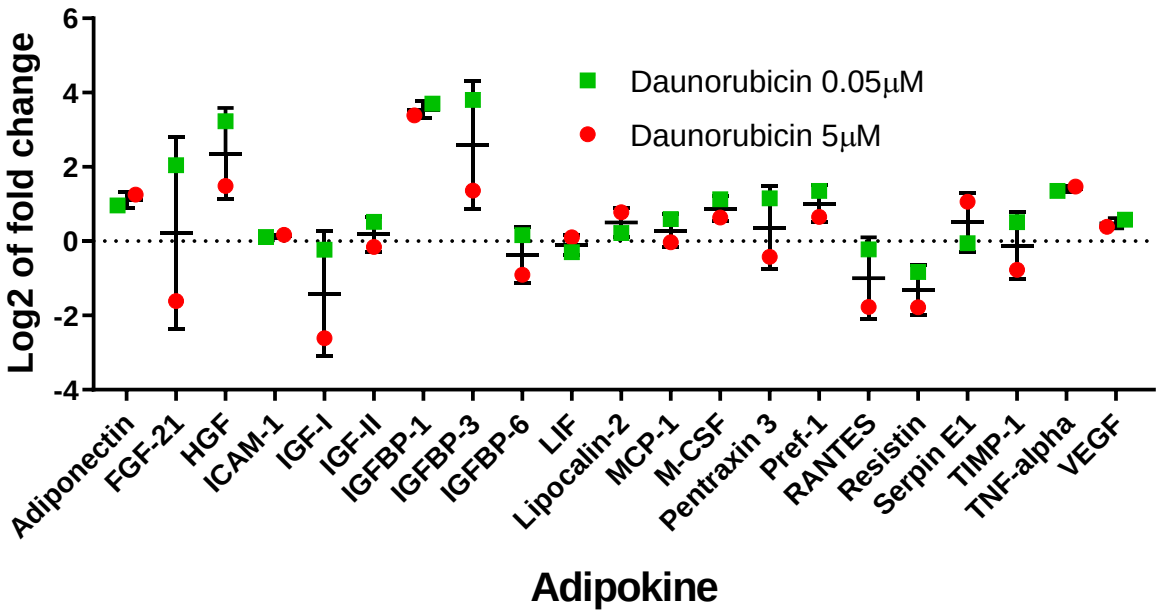

Figure 3.8: Mouse adipokine array results for 3T3-L1 cells treated with daunorubicin. Results displayed as Log2 of fold change from control well data represented as zero. The green squares are the fold change results for each adipokine for 0.05μM concentration of daunorubicin. The red circles are the fold change results for 5μm of daunorubicin, n=1.

3.5 CHAPTER 3 DISCUSSION

In this study the following outcomes have been achieved; successful differentiation of 3T3-L1 mouse adipocytes including measurement of adipokine expression using adipokine arrays. Differentiated cells were then treated with chemotherapy drugs and changes to cell secretions were measured using mouse adipokine arrays. Images of 3T3-L1 cells during differentiation have captured images of lipid droplets within the cells. Oil-red O staining has confirmed the presence of lipids in fat droplets and have also established an adipokine expression profile using mouse adipokine arrays for each week up to 4 weeks during the differentiation process. There are cell viability changes in 3T3-L1 cells treated with vincristine and daunorubicin, there is reduced cell viability shown in the MTT assay and also reduced oil red-O staining observed. Furthermore, cell viability was reduced further in cells treated with daunorubicin than vincristine. Oil red-O staining confirmed reduced levels of lipids present in response to treatment with both concentrations of vincristine and daunorubicin used and levels reduced further in daunorubicin treated cells. To finalise this set of experiments

adipokine secretions were tested from the treated cells to confirm the adipokines present, changes were apparent in this set of experiments, changes to adipokine profiles were observed in both concentrations of chemotherapy drugs tested.

Considering these results, it is likely that adipokine expression profile changes may occur in patients in response to chemotherapy during induction treatment for ALL. In other cancers such as Colorectal cancer, chemotherapy regimens have been shown to increase plasma levels of anti-inflammatory adipokines such as adiponectin and decrease plasma levels of pro inflammatory adipokines such as visfatin and resistin (Słomian et al., 2017), suggesting that baseline levels of these adipokines after treatment may serve as indicators of good or poor response to treatments. This could provide proof of principle that adipokine arrays or measurements could be used to test this hypothesis in our patient plasma samples.

3.5.1 Differentiated 3T3-L1 cells produce an adipocyte like phenotype

The 3T3-L1 cell line is widely used and has been used in more than 5000 published articles on adipocyte research (Poulos et al., 2010) making it a good choice of cell line for our study. 3T3-L1 cells can be notoriously difficult to differentiate and many studies have suggested modifications of standard protocols to maximise their differentiation potential (Sheng et al., 2014). For this study, the standard ATCC protocol with no amendments was used and it has produced expected results with changes in levels of adipokine expression after differentiation (Zebisch et al., 2012). We found that 3T3-L1 cells can partially differentiate when they reach confluence in the absence of differentiation media and produce adipokines, this is line with published observations (Cao et al., 2012) and has been described in the ATCC protocols; pre-adipocytes should not become contact inhibited when sub culturing stocks as this can lead to differentiation (Zhu et al., 2017). The importance of using the differentiation process may therefore be questioned.

Published literature is limited regarding the use of undifferentiated 3T3-L1 cells in experiments for our purpose. Pre-adipocytes in the human body only constitute a small percentage of total fat tissue and the differentiation step allows for synchronicity of the morphological changes required to differentiate the cells (Student et al., 1980). As differentiated cells do not divide it is more practical to grow the cells as a pre-adipocyte

culture at low passages and differentiate the cells when required, because allowing the cells to become confluent can inhibit a successful differentiation (Zhao et al., 2019). For the purpose of the chemotherapy experiments and its impact on the cell viability of 3T3-L1 cells, we required the expression of adipokines at the highest optimum levels to observe changes in experiments, our data indicate this happens at week 3 of differentiation. All adipokines, except serpin E1 were present in the cell culture media collected and at highest levels in week 3. Serpin E1 or Plasminogen Activator Inhibitor-1 (PAI-1) has been shown to inhibit adipocyte differentiation, which could explain its low expression during the differentiation process (Liang et al., 2006).

From these results it can be concluded that the differentiation period of time was the most suitable method for our purpose using cells 3 weeks after differentiation. At this timepoint a large number of adipokines showed the highest concentrations of the 4 weeks, and so would allow us to sensitively measure changes in adipokines produced and lipid droplet formation in response to chemotherapy. It was our intention to investigate the effects of fat conditioned media on ALL cell lines, however, a paper published during the time of this project found no protective effect of fat conditioned media from 3T3-L1 cells on leukaemia cells from daunorubicin alone (Sheng et al., 2016). Furthermore, it was only when Nalm6 ALL cells and 3T3 -L1 cells were co cultured to produce adipocyte and ALL cell conditioned media (ALCM) that this was protective of ALL cells in the presence of daunorubicin. Further experimental changes to our experiments with Molt4 and CEM-CCRF cells included a reduction in the FBS concentration 10% to 0.3% FBS for Molt4 and 1.25% FBS for CEM-CCRF. FBS has an abundance of unquantified growth factors and adipokines which could mask the signal from individual adipokines being tested (Rashid and Coombs, 2019). Fat conditioned media produced from 3T3-L1 cells are cultured in media containing 10% FCS/FBS which would be more difficult to reduce in percentage as the FCS/FBS is required for the high energy lipolysis activity in the 3T3-L1 cells (Kuri-Harcuch and Green, 1978). The main purpose of these experiments was to begin with mouse arrays for proof-of-principle and the testing of secretions from adipocytes and methodology for the future patient work using human arrays. Another question we could ask of the human arrays is, does the induction chemotherapy treatment impact the levels of adipokines present in the human plasma samples because it disrupts fat cell or tissue

function? The next section discusses the results of these experiments using the 3T3-L1 cell line.

3.5.2 Daunorubicin and Vincristine treatment reduces the cell viability and lipid production of 3T3-L1 cells

Experiments were performed to investigate the effect of chemotherapy drugs on adipocytes and their secretions. As chemotherapy is known to be toxic to cells, we wanted to explore the impact on adipocyte function. It is unclear how toxic chemotherapy is to adipocytes and how it may impact beneficial adipokines and their secretion when recovering. The following studies investigated the impact of doxorubicin (an anthracycline) on 3T3-L1 cell function. The studies focused on cancer in general and the weight loss associated with chemotherapy treatment and poor outcomes (Batatinha et al., 2014, Biondo et al., 2016). Both studies demonstrated the negative impact on adipose tissues homeostasis, with the latter confirming decreased lipolysis, lipogenesis, reduced glucose uptake and apoptosis of adipocytes. Another study in a rat model of colorectal cancer looked at the impact of the chemotherapy drugs Irinotecan and 5-fluorouracil (5-FU) (Ebadi et al., 2017). The study identified specific drug related mechanisms that contributed to adipose atrophy, as accelerated loss of adipose tissue is linked with shorter survival in cancer. The group discovered that chemotherapy decreased the size of adipocytes in rat periuterine fat tissue and reduced expression of several proteins associated with ATP generation, lipogenesis and β-oxidation. There is less research regarding how daunorubicin and vincristine treatment impacts adipocyte function. Other research confirms the protective and homing effect on ALL cells by 3T3-L1 cells (Pramanik et al., 2013a).

Our study confirmed reduced viability after 3T3-L1 cells were treated with vincristine and daunorubicin, with the greatest decrease in viability caused by daunorubicin. These findings could be explained by the way both drug treatments exert their effects on different cell types, both anthracyclines and vinca alkaloids have shown to exert cardiotoxicity in patients. Using cardiomyocytes as an example of a cell that does not divide, daunorubicin, an anthracycline, can exert irreversible damage to paediatric heart cells whereas damage from vincristine can be temporary and reversible over time (Thomas, 2017). Vincristine exploits cancer cell rapid division and binds to microtubules to prevent this (Bates and Eastman, 2017). Cardiomyocytes

divide less and administering vincristine along with doxorubicin has even been shown to be limit doxorubicin cardiotoxicity (Chatterjee et al., 2008).

Daunorubicin has multiple mechanisms of action including induction of apoptosis by upregulating death receptors (Zhao and Zhang, 2017), induction of ROS species which impact cardiomyocytes and damage cell membranes (Doroshow, 2019). Similar to cardiomyocytes, adipocytes do not undergo rapid division; during development there is a mixture of hypertrophy and hyperplasic mechanisms (Jo et al., 2009) so these cells may be less impacted by vincristine treatment as is the case with cardiomyocytes. Due to the many different mechanisms of cell death induced by daunorubicin treatment, this may explain the differences in our set of results and simply that the cells are experiencing decreased cell viability in daunorubicin treated cells. There is certainly a call for 'kinder' treatments, particularly in relation to preserving the potential protective effect of adipocyte function during treatment and later in life and understanding ways of preventing adipocyte damage and subsequent weight loss which impacts overall survival and event free survival in ALL (den Hoed et al., 2015)

3.5.3 Mouse adipokine arrays confirm changes in adipokine expression in cells treated with vincristine and daunorubicin

The results of the mouse adipokine arrays treated with daunorubicin and vincristine confirm that the 3T3-L1 still produce adipokines and that the treatments even though they look to be reducing the amount of lipid droplets present particularly in the daunorubicin treated cells, their ability to secrete adipokines does not seem to be severely impacted even though some are changed. In vincristine and daunorubicin treated cells the levels of pref-1 are increased. Increased pref-1 levels are an indication that adipocyte differentiation is inhibited (Wang et al., 2006) these findings may explain the reduced lipid droplets and the possibility that the adipocyte function is being inhibited by the chemotherapy treatments. The levels of IGF-1 are both decreased in the daunorubicin and vincristine incubations. IGF-1 is an important factor in adipocyte differentiation in 3T3-L1 cells and is essential to the process (Zhu et al., 2009), this may further explain the reduced presence of lipid droplets in figure 3.1. Another observation is the reduced levels of resistin present in the secretions though it does increase in the cells treated with 5µM of vincristine. This observation may be the first indicator that

chemotherapy impacts adipocyte secretions and this could have a positive or negative outcome on circulating adipokines in patients. The following study confirmed high resistin levels at diagnosis in patients with ALL which was reduced possibly due to the positive impact of chemotherapy which restores adipokines to normal functioning levels that do not impact the disease process (Moschovi et al., 2010a). Looking at these results it is possible that adipokine expression in patients changes in response to chemotherapy. In other cancers such as Colorectal cancer, chemotherapy regimens have been shown to increase plasma levels of anti-inflammatory adipokines and decrease plasma levels of adipokines such as resistin (Słomian et al., 2017). We do not know at present if these changes are long term and if there are negative impacts of adipokine secretion, for example does chemotherapy impact the ability to secrete beneficial adipokines from adipose tissue after successful treatment. Following this set of experiments, we are confident that the adipokine arrays are sensitive enough to detect changes in adipokine expression and will be suitable for our work planned with patient samples in the next chapter.

4.0 CHAPTER 4: Patient sample pilot study results

4.1 Patient sample pilot study confirms reproducibility of Human adipokine arrays

To confirm the reproducibility of the human adipokine arrays when incubated with human plasma, a pilot study was undertaken. Early trouble shooting of the clinical trial protocol meant some patient data were incomplete; patients 001 to 008 day 29 bloods and weight data for day 29 for patients 001 to 006 and 008 are missing. Data collection was resolved from patient 009 onwards. Early samples were used to complete this pilot study to check variability of adipokine profiles in day 0 bloods in a set of low MRD risk patients. The pilot study involved generating adipokine profiles using day 0 samples for patients 006 and 007 in triplicate, see table 4.1.

Table 4.1: Patient information and demographic for the patient pilot study. Patients highlighted in grey, 006-008 are not included in full study. Patients 006-007 are included in pilot study. Patients 006-011 are included to confirm the pre-treatment pattern of adipokine expression. Table shows patient ID, age at diagnosis, gender-male or female, BMI at diagnosis, BMI percentile at diagnosis, BMI at day 29, BMI percentile at day 29, cytogenetic risk and minimal residual disease risk, BMI percentiles in blue indicate the overweight category and the yellow category treatment acquired overweight.

ID	Age	M/F	Induction Treatment Category	BMI at Diagnosis (Kg/m2)	BMI Percentile diagnosis	BMI at Day 29	BMI Percentile Day 29	Cytogenetic risk	Day 29 MRD risk
006	2.6	F	B	15.3	39	N/A	N/A	Intermediate	Low risk
007	8.9	M	B	16.1	52	18.4	83	Intermediate	Low risk
008	6.3	M	A	14.9	33	N/A	N/A	Intermediate	Low risk
009	13.6	M	B	16.6	10	17.7	25	Good	Low risk
010	6.4	F	A	16.9	86	16.3	73	Good	Low risk
011	6.9	M	A	16	64	18.1	93	Intermediate	Low risk

Plasma samples were initially defrosted after collection from the hospital and replicate 1 performed immediately, with the remaining plasma frozen into 500µL aliquots. This meant that replicates 2 and 3 were exposed to an additional freeze-thaw cycle when compared with replicate 1. To check reproducibility between arrays, three separate arrays for the same patient samples were tested on three different days. Replicate arrays were performed for both patients on the same day (i.e. replicate 1 for patient 006 and 007 were performed

together). Figure 4.1 shows the full results for patient 006 and 007 plotted together with adipokine name on the x axis against the mean pixel density recorded from each array. Results show similarity between patients in the baseline expression levels of adipokines and show little variability between both patients of similar clinical characteristics in terms of BMI at diagnosis and MRD risk, results are displayed as mean pixel density with error bars showing standard deviation.

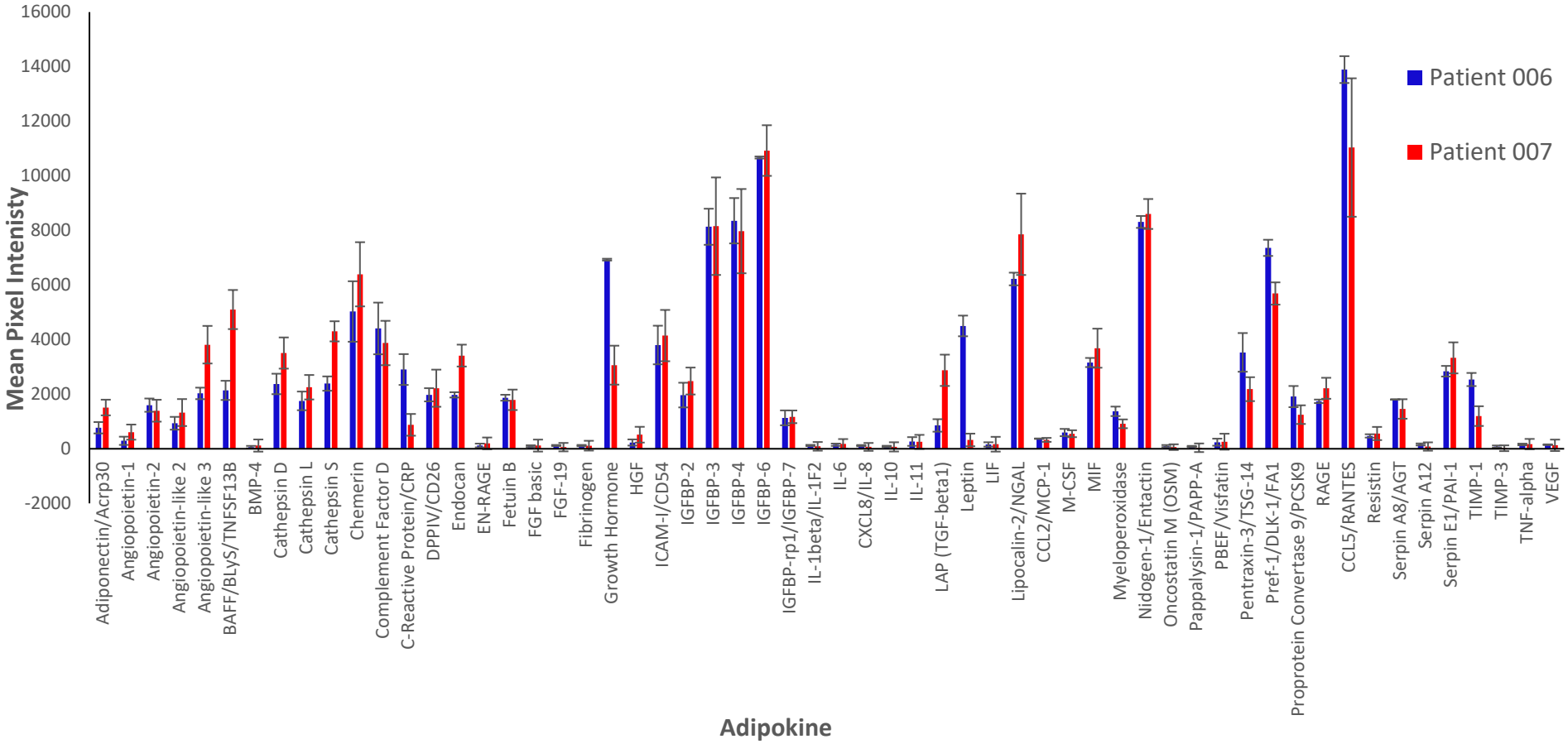

Figure 4.1: Pilot study data to show reproducibility of human arrays for patients 006 and 007. Adipokine expression plotted against arr ay mean pixel intensity. Larger differences are Growth hormone, leptin and BAFF, n=3, error bars shown as standard deviations.

To further confirm the apparently high similarity in adipokine expression profiles between patients 006 and 007, adipokine profiles were plotted against each other on an x-y scatter plot, using linear regression. A positive correlation was observed with r^2 value of 0.8493, the deviation from zero is statistically significant at <0.0001 using linear regression analysis, see figure 4.2. This figure highlights three adipokines as outliers from the line of best fit which are highlighted in red (Growth Hormone), green (Leptin) and grey (BAFF).

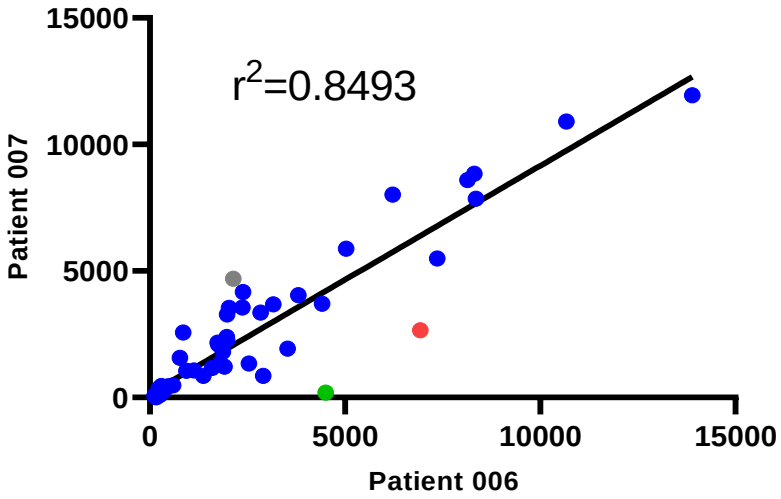

Figure 4.2: Pilot study results to show reproducibility of human arrays for patients 006 and 007. Scatterplot plotted with adipokine expression using linear regression with a positive correlation, r^2 value 0.8493, the deviation from zero was significant at <0.0001. The outliers are Growth hormone highlighted in green, Leptin highlighted in red and BAFF highlighted in grey, n=3.

To check if freezing and thawing caused differences in adipokine expression and to ensure array reproducibility between replicates 1 and replicates 2 and 3, adipokine expression profiles were compared for replicates 1 and 2, 1 and 3 and 2 and 3. Replicate 1 has been frozen and thawed once and replicate 2 has been frozen and thawed twice. Results are displayed in figure 4.3a to f. Scatterplots show adipokine expression for each replicate for patients 006 and 007. Figure 4.3a, b and c are comparisons for patient 006, a is replicates 1 versus 2, b is replicates 1 versus 3 and c replicates 2 versus3, with r^2 values of 0.9726, 0.9839 and 0.9810 respectively. Figure 4.3d, e and f show the same data for patients 007 with r^2 values of 0.9779 for d, 0.9668 for e and 0.9415 for f. The deviation from zero was significant for all replicate comparisons at <0.0001, n=3. These results gave confirmed that freezing the plasma has little impact on the quality of the sample also the variability between each replicate was low. These results gave more confidence in choosing one replicate for each

patient, this was also desirable to keep the overall cost lower so more patient samples could be tested.

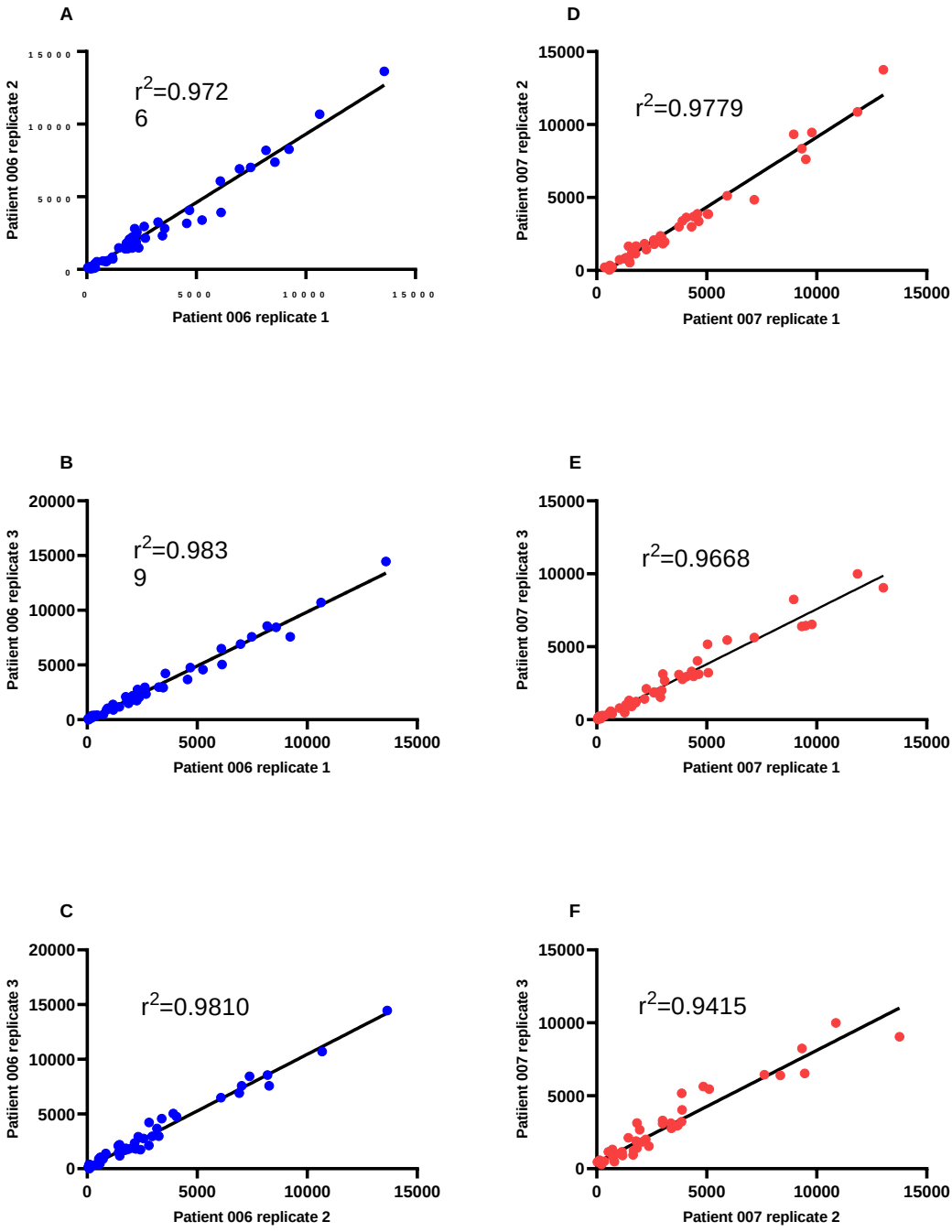

Figure 4.3: Pilot study results to show reproducibility of human arrays and potential impact of freezing and defrosting samples. Scatterplots show adipokine expression for each replicate for patients 006 and 007. A, B and C are comparisons for patient 006, A is replicates 1-2, B is replicates 1-3 and C replicates 2-3 with r^2 values of 0.9726, 0.9839 and 0.9810 respectively. D, E and F show the same data for patients 007 with r^2 values of 0.9779 for D, 0.9668 for E and 0.9415 for F. The deviation from zero was significant for all replicate comparisons at <0.0001, n=3.

4.2 Patient sample study confirms pre-treatment pattern of adipokine expression

To confirm the typical adipokine profile before treatment, an adipokine profile study using day 0 samples from patients 006, 007, 008, 009, 010, 011 who all have recorded normal to overweight range BMI and low MRD risk at day 29, see table 4.1 for clinical parameters. Mean pixel intensity for each adipokine from day 0 plasma for each patient was compared to determine what the pre-treatment pattern of adipokine expression was and how expression varied in a group of low MRD risk patients, figures 4.4, 4.5, 4.6. Results are plotted together with adipokine type on the x axis against the mean pixel intensity recorded for each array for each patient, they are displayed as mean pixel intensity for patients 006 and 007, n=3 and actual pixel intensity for patients 008, 009, 010 and 011, n=1. Figure 4.4 shows the results for adipokines adiponectin to fibrinogen; patients show differing levels in expression for each adipokine. The following adipokines show little variability between patients and are lower in expression compared to other adipokines in figure 4.4; angiopoietin-1, BMP-4, EN-RAGE, fetuin-B, FGF basic, FGF-19 and fibrinogen. It is important to point out that this is semi-quantitative array and prone to variation which is below the limit of detection for this assay, the adipokines are still expressed but at low levels but showing much smaller variations. The following adipokines display higher patient expression levels and show greater variability between patients and deviation around the mean; adiponectin, angiopoietin-2, cathepsin-D, cathepsin-L, cathepsin-S, complement factor D, DPPIV/DC26, endocan and fetuin b. Adiponectin expression levels are overall low and don't rise as much compared to other adipokine expression levels measured here on figure 4.4 and are also clustered together with the exception of patient 007 having higher levels than the others for adiponectin expression. This pattern is comparable for angiopoietin-2 but patient 010 has higher expression levels than the rest of the patients. Cathepsin-L and cathepsin-S intensity have a wider range and not clustered around the mean. C-reactive protein (CRP) and endocan are clustered with patient 006 having higher intensity than the rest of the group. The following adipokines have a broad distribution of pixel intensities across the group of patients ranging from low expression to high expression; angiopoietin-like 2, angiopoietin-like 3, BAFF, cathepsin-D and chemerin.

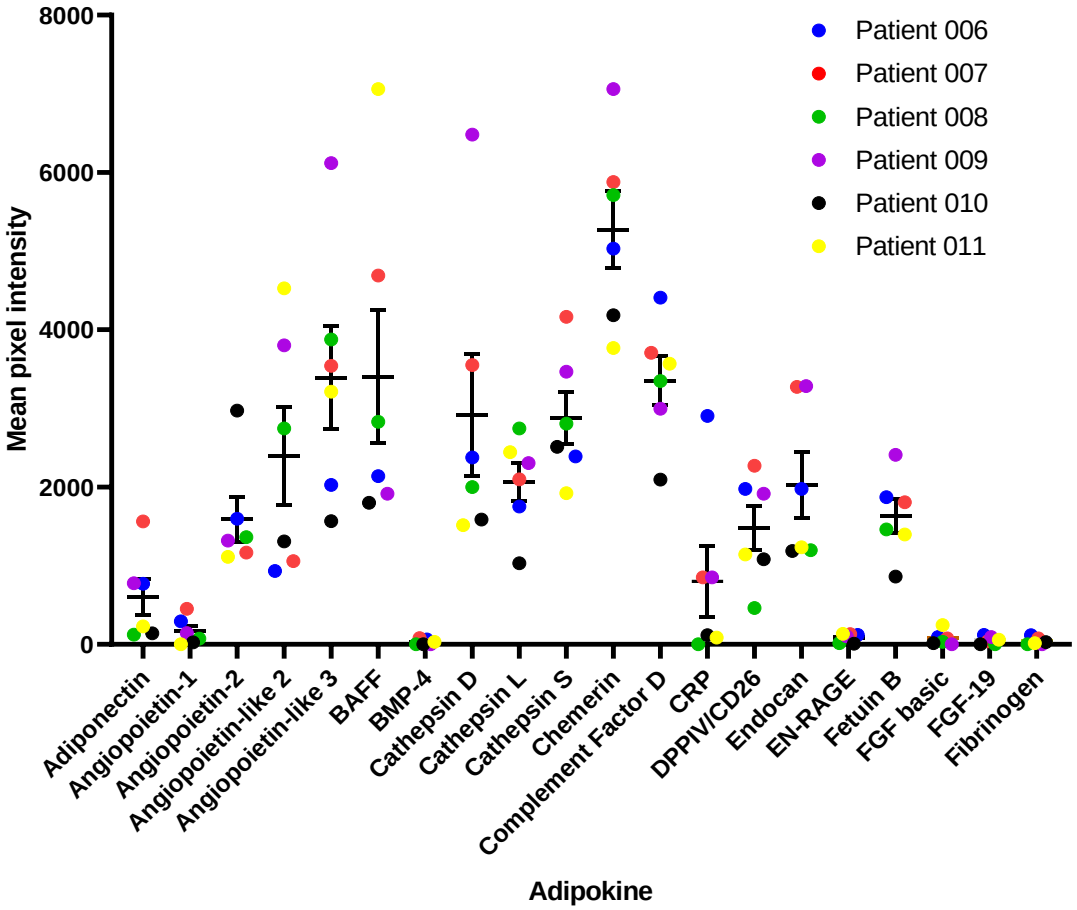

Figure 4.4: Pre-treatment pattern of adipokine expression in patient samples, adipokines adiponectin -fibrinogen. Patients results are grouped by adipokine with pixel intensity for each adipokine. Patient 006 is highlighted in blue, patient 007 in red, patient 008 in green, patient 009 in purple, patient 010 in black and patient 011 in yellow, error bars shown as standard error, n=1.

Figure 4.5 shows array expression levels for adipokines growth hormone to MIF. The following adipokines show little variability between patients and pixel intensities are clustered together at lower expression levels, below 5000 mean pixel intensity; HGF, ICAM-1/CD54, IGFBP-2, IGFBP-rp1/IGFBP-7, IL1-beta, IL-6, IL-8, IL-10, IL-11, LAP(TGF-beta1), LIF CCL2/MCP-1 and M-CSF. The following adipokines expression show more variability or with expression levels mainly clustered around the mean with one or two outliers from the mean: growth hormone, IGFBP-3, IGFBP-4, IGFBP-6, leptin, lipocalin-2, and MIF. Growth hormone, IGFBP-3, IGFBP-4, lipocalin-2 and MIF show larger variability in expression between patients from low to high levels. IGFBP-6 levels are mainly clustered, although pixel intensity is higher

for patient 009 and lower levels for patient 010 compared with the rest of the cohort. Patients 006 and 010 show higher levels of leptin compared to other patients.

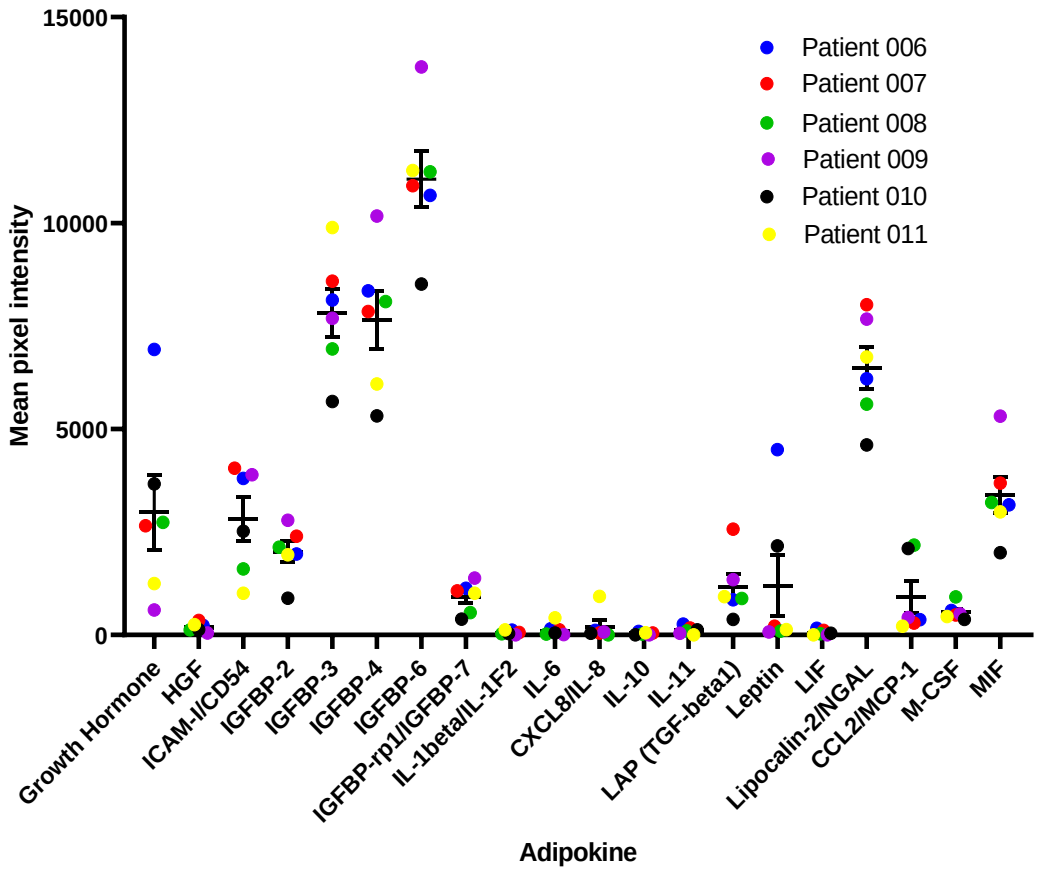

Figure 4.5: Pre-treatment pattern of adipokine expression in patient samples, adipokines growth hormone – MIF. Patients results are grouped by adipokine with pixel intensity for each adipokine. Patient 006 is highlighted in blue, patient 007 in red, patient 008 in green, patient 009 in purple, patient 010 in black and patient 011 in yellow, error bars shown as standard error, n=1.

Figure 4.6 shows the array expression levels for adipokines myeloperoxidase to VEGF. The following adipokines show very little variation between patients and are expressed at lower levels; myeloperoxidase, oncostatin-M, pappalysin-1, visfatin, pentraxin-3, proprotein convertase-9, RAGE, resistin, serpin A8, serpin A12, serpin E1, TIMP-1, TIMP-3, TNF-alpha and VEGF. Adipokines nidogen-1, pref-1 and CCL5/RANTES show greater variability between patients. The adipokine expression for pentraxin-3 are mainly grouped together with patient 006 showing higher levels than the rest of the patients.

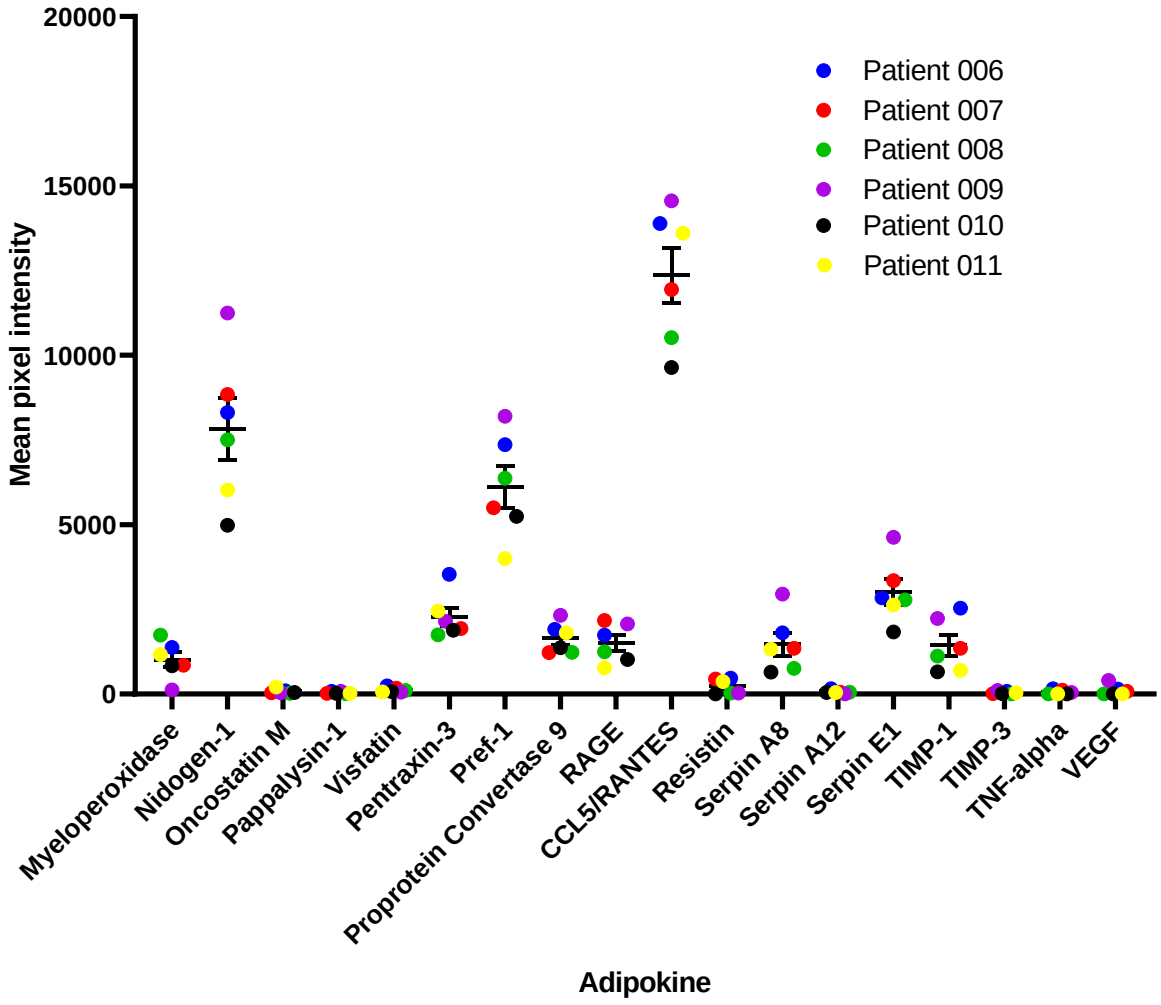

Figure 4.6: Pre-treatment pattern of adipokine expression in patient samples, adipokines myeloperoxidase - VEGF. Patient array results are grouped by adipokine with pixel intensity for each adipokine. Patient 006 is highlighted in blue, patient 007 in red, patient 008 in green, patient 009 in purple, patient 010 in black and patient 011 in yellow, error bars shown as standard error, n=1.

To conclude the following adipokines showed higher expression or greater variation between patients 006-011; adiponectin, angiopoietin-2, angiopoietin Like-2, angiopoietin Like-3, BAFF, cathepsin-D, cathepsin L, cathepsin S, chemerin, complement D Factor, CRP, DPPIV/CD26, endocan, growth hormone, IGFBP-3, IGFBP-4, IGFBP-6, leptin, lipocalin-2, MIF, nidogen-1, pentraxin-3, pref-1 and CCL5/RANTES. The other adipokines listed were expressed at lower levels with less variation between patient's expression levels at day 0; angiopoietin-1, BMP-4, EN-RAGE, fetuin-B, FGF-Basic, FGF-19, fibrinogen, HGF, ICAM/CD54, IGFBP-2, IGFBP-rp1/IGFBP-7, IL1-beta, IL-6, IL-8, IL-10, IL-11, LAP(TGF-beta), LIF, CCL2-MCP-1, M-CSF, myeloperoxidase, oncostatin-M, pappalysin-1, visfatin, proprotein convertase-9, RAGE,

resistin, serpin A8, serpin A12, serpin E1, TIMP-1, TIMP-3, TNF-alpha and VEGF. The aims from this section were to examine the accuracy and reproducibility of the arrays and before moving onto testing the patient samples, the clinical data and the individual patient array data will be set out in the next sections.

4.3 Summary of patient clinical data

Patients included in this study total 38, most patients diagnosed with ALL within the time period of 3 years from The Royal Manchester Children's Hospital were eligible for the study and have been recruited apart from patients diagnosed with B cell ALL which were Philadelphia Chromosome positive, these patients were not included as they are not included in the same risk stratification and treatments protocols in the UKALL2011 trial. The mean patient age is 6.5 ± 3.8 years, there were 14 female, and 24 male patients recruited. A total of 15 patients received induction treatment category A and 23 patients induction category B. The mean BMI at diagnosis is 16.6 ± 2.0 and the mean BMI after induction is 17.2 ± 3.3. There were 18 patients with low risk good cytogenic risk, 17 patients with intermediate risk, 1 patient with high risk genetics and 1 patient with high risk T-cell disease. The patients % weight changes ranged from -11.93 and +37.24 within the 29 day induction period, the mean 6.03 ± 11.30. Of the 38 patients recruited 22 patients were low risk at the end of induction and 15 patients were MRD risk, 1 patient did not have any MRD markers after testing, these data are summarised in methods table 2.2.

The information in table 4.2 shows all the patients information taken for analysis for this study, displayed are patient ID, age, sex, induction treatment category, BMI at diagnosis, BMI percentile at diagnosis, BMI at day 29, BMI percentile at day 29, cytogenic risk, MRD risk at day 29 and percentage weight change from diagnosis to day 29. Six patients were outside the normal weight range at diagnosis which were patients 010, 030, 040 and 044 were in the overweight category, patient 026 was underweight and patient 028 was in the obese category. By day 29 patient 010 has lost weight and moved in the normal category. Patient 026 moved from the underweight range to the normal range by day 29. Patients 028, 030, 040, 044 all stayed in the same overweight or obese category. Patient 021 moved from a normal weight category to being underweight at the end of induction treatment. Patients

011, 013 and 024 became overweight from a normal weight range by day 29 but did not go onto be MRD risk.

Six patients became obese after treatment, this has been classed as treatment acquired obesity, they are patients 015, 036, 045, 050, 051 and 053. Of the patients with treatment acquired obesity 2 of the patients, 015 and 050 were MRD risk by the end of induction treatment. The patients who gained weight, 15 gained more than 5% of their starting body weight, of these patients 12 patients gained more than 10% of their starting body weight and 5 patients gained more than 20% of their body weight in the 29 day induction period.

Table 4.2: Patient information, demographic and clinical parameters. Table shows patient ID, age at diagnosis, gender-male or female, BMI at diagnosis, BMI percentile at diagnosis, BMI at day 29, BMI percentile at day 29, cytogenetic risk and minimal residual disease risk, BMI percentiles in red indicate treatment acquired obesity, yellow indicates treatment acquired overweight, blue overweight, green underweight and MRD risk patients highlighted in purple, n=38

ID	Age	M/F	Induction treatment Category	BMI at diagnosis (Kg/m^2)	BMI Percentile diagnosis	BMI at Day 29	BMI Percentile Day 29	Cytogenetic risk	Day 29 MRD risk	% Weight change
009	13.6	M	B	16.6	10	17.7	25	Good	Low risk	6.96
010	6.4	F	A	16.9	86	16.3	73	Good	Low risk	-3.2
011	6.9	M	A	16	64	18.1	93	Intermediate	Low risk	13.23
012	1.8	M	A	17.8	75	17.3	70	Good	Low risk	-2.62
013	1.1	M	A	18.3	72	19.3	88	Good	Low risk	5.34
014	16	F	B	20.1	21	19.4	38	Intermediate	Low risk	-3.13
015	3.7	M	A	15.5	74	19	>95	Good	MRD risk	22.22
017	12	F	B	17.7	40	15.6	11	Intermediate	MRD risk	-11.93
018	11.8	F	B	16.2	18	15.8	13	Intermediate	MRD risk	-2.63
020	6.2	F	A	16.0	66	16.1	69	Good	Low risk	0.66
021	5.5	F	B	15.1	42	13.4	<5	Intermediate	Low risk	-11.56
023	6.7	M	A	14.3	16	16.8	79	intermediate	No MRD markers	17.27
024	3.3	M	A	17.0	75	17.5	86	Good	Low risk	3.33
025	6	F	A	16.2	70	15.2	45	Good	MRD risk	-6.1
026	4.2	M	A	13.2	<5	15.4	34	Intermediate	MRD risk	16.26
027	2.6	M	A	16.2	56	16.2	56	Good	Low risk	0
028	7.2	M	B	24.1	>95	24.5	>95	Good	Low risk	1.7
030	9.4	F	A	20.4	88	21.2	91	Intermediate	Low risk	4.05
031	12.7	M	B	17.1	25	17.4	30	T cell disease	MRD risk	1.66
032	11.5	M	B	16.7	29	17.5	43	Good	Low risk	4.5
034	1.8	M	A	15.9	31	16.4	45	Good	Low risk	2.67
035	12.1	F	B	15.5	9	16.1	16	Intermediate	MRD risk	3.68
036	2.7	M	A	15.5	31	19.8	>95	Intermediate	Low risk	27.36
037	4.6	F	A	14.6	25	17.1	86	Intermediate	MRD risk	16.77
038	3.2	M	B	15.3	29	15.4	31	Good	Low risk	0.42
040	2.9	F	A	17.8	90	17.4	86	Intermediate	Low risk	-2.01
041	2.8	M	A	16.1	49	16.7	67	Good	Low risk	3.72
044	10.3	F	B	21.3	90	20.9	88	Intermediate	Low risk	-2.06
045	4.1	M	A	16.6	79	20.6	>95	Good	Low risk	24.43
046	6.2	M	A	15.3	47	16.3	73	Intermediate	MRD risk	6.47
047	4.7	M	A	14.7	22	16.6	80	Intermediate	MRD risk	12.93
048	9	M	B	15.9	45	14.8	18	T cell disease	MRD risk	-7.14
049	4.8	F	A	16.2	77	15.5	59	Good	MRD risk	-4.73
050	6.6	M	A	14.7	29	21	>95	Intermediate	MRD risk	37.24
051	5.9	M	A	15.9	66	2.03	>95	Good	Low risk	27.25
053	2.8	F	B	17.1	81	19.7	>95	Intermediate	Low risk	14.84
055	2.9	M	B	15.8	40	17.4	84	Good	MRD risk	10.07
057	11.2	M	B	17.2	50	17.4	54	High	MRD risk	1.43

Figure 4.7 summaries the weight change data and shows the ratios of patient's weight change during induction. Figure 4.7a shows the patients BMI percentile at diagnosis, figure 4.7b shows the patients BMI percentile at the end of induction treatment. Figure 4.7c shows the percentage weight change of patients at day 29, the end of induction treatment.

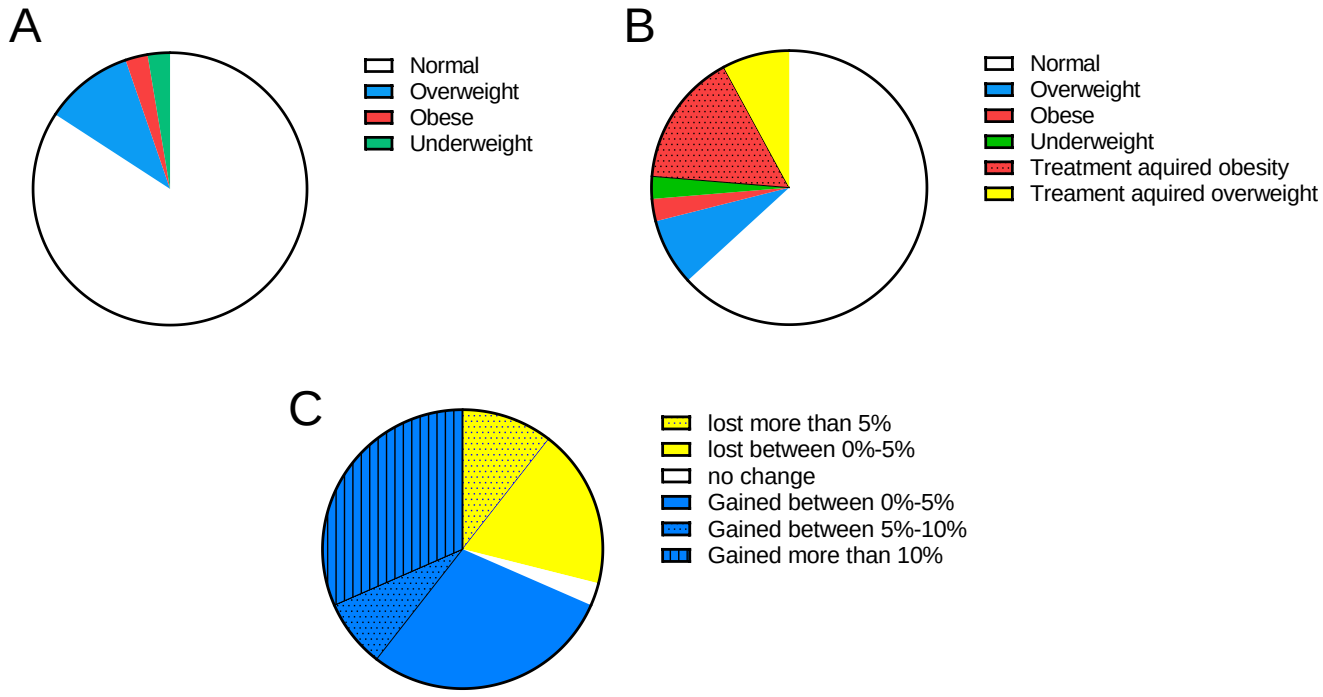

Figure 4.7: Summary of patient clinical weight data change during induction therapy. A; Pie chart illustrating categories of patient and BMI percentile at diagnosis. b; pie chart illustrating categories if patient and BMI percentile at day 29. c; Summary of percentage weight change for patients at day 29, n=38.

4.4 Identification of clinical variables as predictors of MRD risk

To predict if clinical variables are associated with MRD risk, logistic regression was performed in the R statistics package by the University of Liverpool by service agreement earlier in the patient data collection process. Further patient data was collected after the initial lockdown due to Covid 19, at this point it was unsure if any further patient data would be available. This initial set of data was to address if any of the following clinical markers could predict MRD risk; BMI at diagnosis, age at diagnosis, sex (males), weight change percentile, BMI percentile at diagnosis, intermediate cytogenic risk or the patient receiving induction regimen B, the

results are displayed in table 4.3. None of the clinical variables significantly corelated to the MRD risk outcome. The top three ranking variables are BMI at diagnosis, age at diagnosis and sex and in this case males, with p values of 0.126, 0.227 and 0.240 respectively, these figures are not statistically significant but do indicate a trend towards these variables being associated with MRD risk, the inconclusive results may be a reflection on the small population size. Less significant were weight change percentile, BMI percentile at diagnosis, intermediate cytogenic risk and patients receiving induction regimen B, all with a p value >0.402.

Table 4.3: Summary of clinical variables associated with MRD risk for patients 009-040. The data processed by The University of Liverpool up to patient 40 not including patients 041-057. The table shows logistic regression data produced in R statistics package, selected clinical variable as predictors of MRD risk, the standard error and the P value, n=26

Clinical Variable	STD Error	P value
BMI at diagnosis, Kg/m^2	1.323	0.126
Age at diagnosis	0.590	0.227
Sex (Males)	3.533	0.240
Weight change percentile	0.174	0.402
BMI percentile at diagnosis	0.064	0.516
Intermediate cytogenic risk	1.627	0.632
Induction regimen B	2.958	0.918

To further investigate these findings and using the full data set of patients 009 – 057 the data for BMI at diagnosis and BMI percentile at diagnosis were compared using t tests between low n=22 and MRD risk patients n=15, figure 4.8a & b and Figure 4.9a & b. Figure 4.8a confirms that a lower BMI at the beginning of induction, day 0 is significantly associated with MRD risk at day 29, p=0.0116, Low risk n=22 and MRD risk n=15. Figure 4.8b displays a similar finding that a lower BMI at Day 29 of induction therapy is significantly associated with MRD risk by the end of induction treatment, p=0.0379.

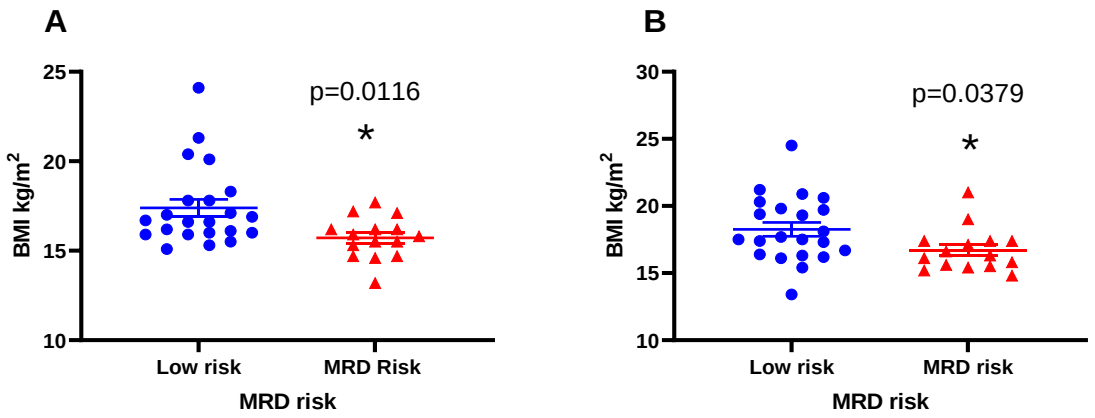

Figure 4.8: BMI at day 0 and day 29 as a predictor of MRD risk. A, patients BMI at diagnosis and at day 29 is a predictor of MRD risk at day 29, MRD risk status plotted against BMI Kg/m^{2,} n=22 for low risk patients, n=15 for MRD risk patients p=0.0116. B, patients BMI at diagnosis, MRD risk status plotted against BMI percentile, n=22 for low risk patients, n=15 for MRD risk at day 29 p=0.0379, error bars shown as standard error.

To investigate if BMI percentile at day 0 or day 29 is linked to MRD risk, BMI percentile at day 0 and day 29 were compared using t tests comparing low risk n=22 and MRD risk patients n=15 figure 4.9a & b. Figure 4.9a confirms that at a lower BMI percentile at the beginning of induction is significantly associated with MRD risk at day 29 day p=0.0120. Figure 4.9b shows the similar result for BMI percentile data at day 29 where patients with a range of BMI percentiles are MRD risk but overall compared to low risk patients a lower BMI percentile is associated with MRD risk at day 29 although not significant, p=0.947.

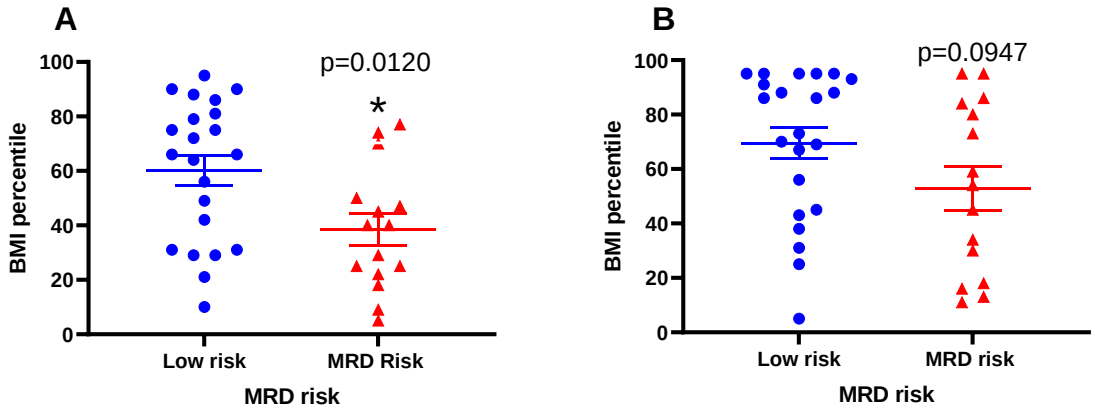

Figure 4.9: BMI percentile at day 0 and day 29 as a predictor of MRD risk. A, patients BMI percentile at day 29 and MRD risk, MRD risk status plotted against BMI Kg/m^{2,} n=22 for low risk patients, n=15 for MRD risk patients p=0.0120. B, patients BMI percentile at day 29 and MRD risk, MRD risk status plotted against BMI percentile, n=22 for low risk patients, n=15 for MRD risk at day 29 p=0.0947, error bars shown as standard error.

To determine the impact of age at diagnosis on MRD risk status at day 29, a t test was performed to compare low risk n=22 and high MRD risk n=15 patient status at day 29 and the patients age at day 0. The results in figure 4.10 suggest that increasing age at diagnosis is loosely associated with MRD risk but not significantly, p=0.2045.

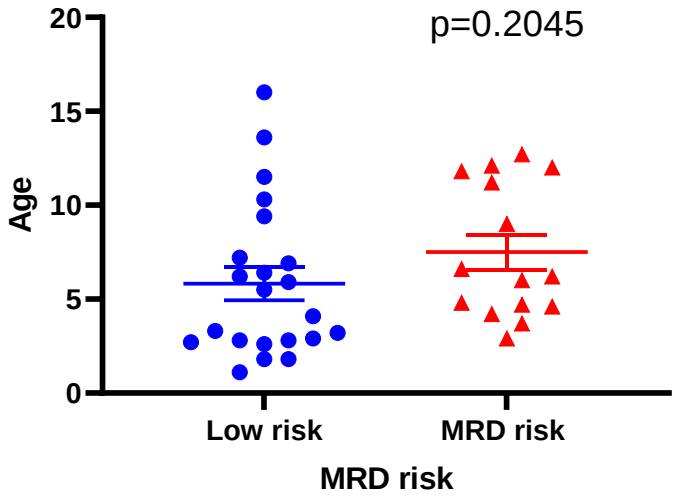

Figure 4.10: Patients age at diagnosis as a predictor of MRD risk at day 29. MRD risk status plotted against age n=22 for low risk patients, n=15 for MRD risk patients p=0.2045, error bars shown as standard error.

To summarise this results section, we have identified clinical variables associated but not significantly with MRD risk in patients up to and including patient 040 using logistic regression analysis. Using t-tests to compare MRD risk and BMI and BMI percentile results indicate that a lower BMI at day 0 and day 29 is associated with MRD risk, this was significant for BMI percentile. Increasing age at day 29 is associated but not significantly with MRD risk. The next section looks at each patients individual adipokine profile and how that changes from day 0 to day 29.

4.5 Identification of adipokine changes in patients before and after induction therapy

Adipokine arrays were completed for patients 009 - 057 using day 0 and day 29 plasma samples. Plasma for both day 0 and day 29 were thawed and arrays performed simultaneously. Results are shown for each patient in appendix figures 7.2 to 7.39, graphs are displayed as adipokine expression plotted against Log2 of the fold change of pixel

density from the day 0 array and day 29 array. Zero on the y axis as classed as day 0 and day 29 is shown as a Log2 fold change increase or decrease in adipokine expression, clinical data has also been added to each adipokine profile. The paragraphs in the appendix sections 7.1 to 7.38 describe further detail for individual adipokine expression profiles and clinical data for each patient.

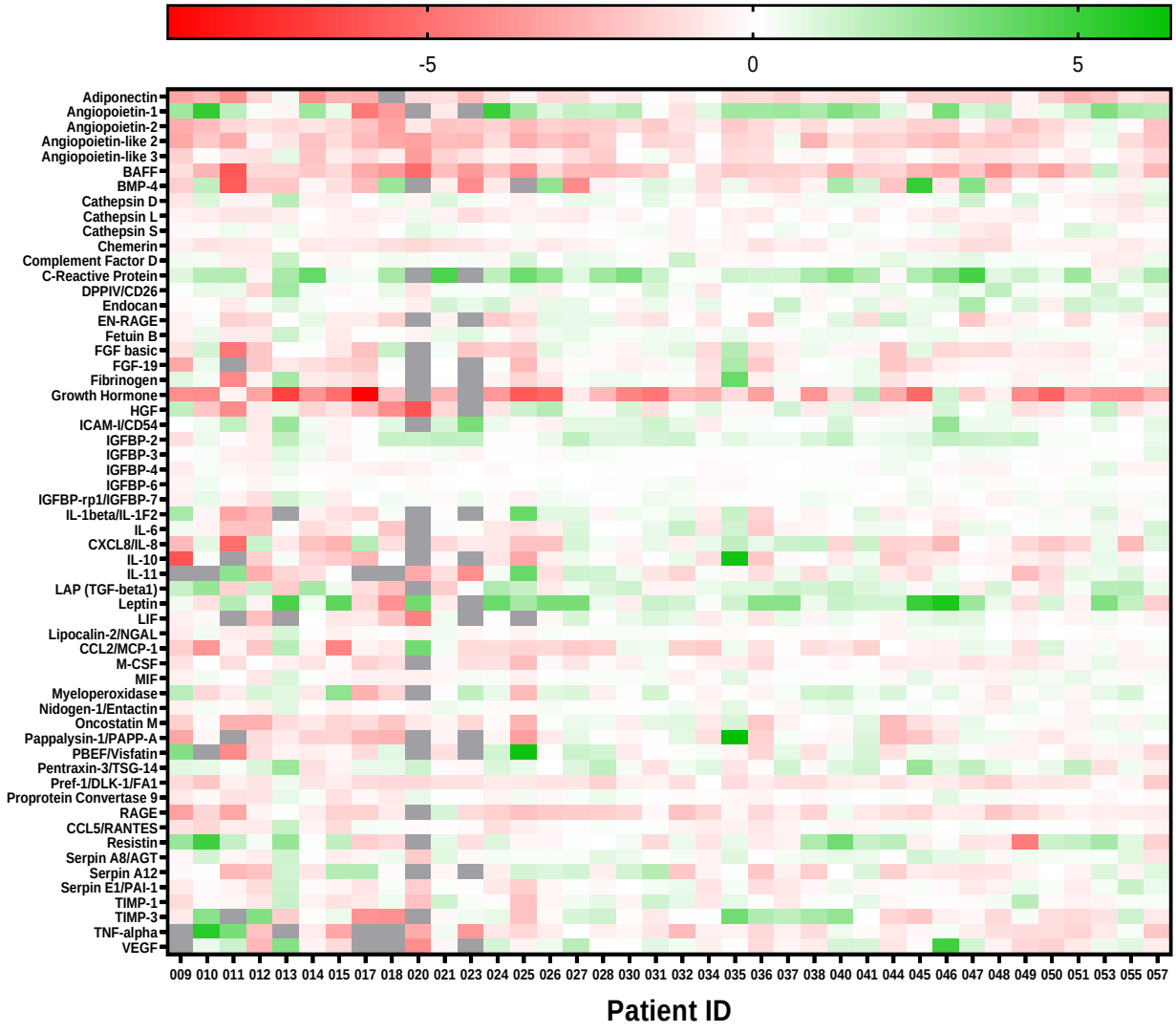

Figure 4.11: Heat map summary of adipokine expression changes in patients 009-057. Increasing shades of green represent increases in expression, increasing shades of red represent decreases in expression. Grey squares indicate no data available, white indicates no detectable change, n=38.

Figure 4.11 summaries all the individual adipokine patient data in a heat map, grey squares indicate no data available. The results for all the patients together display emerging similar

patterns for particular adipokines, i.e., Adiponectin expression is decreased overall in all patients at day 29. The Angiopoietin family of proteins are all decreased by day 29 apart from Angiopoietin-1. CRP is raised in most patients during induction therapy, whereas Growth Hormone expression decreases in most patients. The next sections will examine the overall trends in the adipokine profile data and also look if we can identify adipokines significantly associated with MRD risk patients and in patients who gain weight.

4.6 Identification of adipokine changes associated with weight gain

Using the data taken from the human adipokine arrays we have been able to identify a group of adipokines that change significantly from the start to the end of induction treatment in patients who gain weight. It would be useful in this early induction therapy to know which adipokines change in expression and if those adipokines go onto to impact the development of the disease moving onto the next stage of treatment. Results from the individual adipokine arrays were analysed to identify changes in adipokine levels from day 0 at the start of treatment to day 29, the end of induction when rapid weight gain can occur in patients. So that the individual results across patients could be compared, each array was normalised to the average of each array's three reference spots. To determine if particular adipokines were increased in patients who gained weight, the cohort was split into two groups. In the first group were the patients who stayed within the normal weight range percentile category >5 and <85 and patients who put on less than 5% of their starting body weight or lost weight, n=22, in the second group were patients who gained more than 5% of their starting body weight also patients who recorded a normal weight percentile at the start of induction and went onto become overweight >85, treatment acquired overweight (TAOW) or obese >95, treatment acquired obesity (TAO) by the end of induction, n=16. The results for the top five ranked adipokines associated with weight gain are displayed in figures 4.12-4.16.

4.6.1 Leptin levels increase in patients who gain weight

Leptin has been identified in our patient cohort as being significantly increased in patients who gain more than 5% of their body weight or become overweight or obese by the end of induction therapy, in addition to this there is a significant positive correlation to patients having a higher BMI at the end of induction therapy and higher circulating plasma levels of

leptin, this has been shown in other studies in children, although not specifically at the end of induction treatment as the results show in this study on day 29. Leptin concentration positively correlates with circulating leptin levels even in a normal settings without disease in obese paediatric patients (Soliman et al., 2012). Leptin has been shown to stimulate cancer growth in other cancers associated with obesity such as breast and ovarian cancer and contribute to chemoresistance (Gu et al., 2019b) (Gu et al., 2019a). More recently Lu *et al*. (2017) experimented with fasting protocols in mouse models to reduce circulating levels of Leptin which had a positive impact on ALL outcomes (Lu et al., 2017).

Here we show that leptin was significantly associated with weight gain, see figure 4.12a but not with MRD risk in our patient cohort, see figure 4.12b. Figures 4.12c and d display positive correlations between patient BMI and Leptin at day zero and at day 29, with a significant positive correlation between Leptin expression and BMI at day 29 in patients. In figure 4.12a zero on the *y* axis is classed as day 0 and then day 29 is plotted as an increase or decrease in normalised pixel intensity for leptin expression. Patients are grouped as normal weight range and patients who gained less than 5% of their starting body weight versus patients who gained more than 5% of their starting body weight or recorded a normal weight percentile at day 0 and became overweight or obese by day 29, in the weight gain group, n=22, leptin expression increased significantly compared to patients in the normal weight range, n=16, p=0.0001. Figure 4.12b shows the same set of results but for MRD risk status, here there is no significant difference in leptin expression between low risk patients, n=22 and MRD risk patients n=15, p=0.7643. To investigate whether there were correlations between starting BMI and leptin expression at day 29, BMI and Leptin expression were plotted against each other on an x-y scatter plot, see figure 4.12c and d using linear regression analysis there was a slight positive correlation and the r^2 value is represented at 0.01132, the deviation from zero was not significant at p=0.5309. Figure 4.12d shows the results for BMI at day 29 and leptin expression, a higher BMI is significantly correlated with higher leptin expression, the r^2 value is represented at 0.2059 and the deviation from zero was significant at p=0.0048. Statistical analysis for figures a and b was performed using an unpaired t test comparing both patients' groups where the results were considered significant at <p0.05.

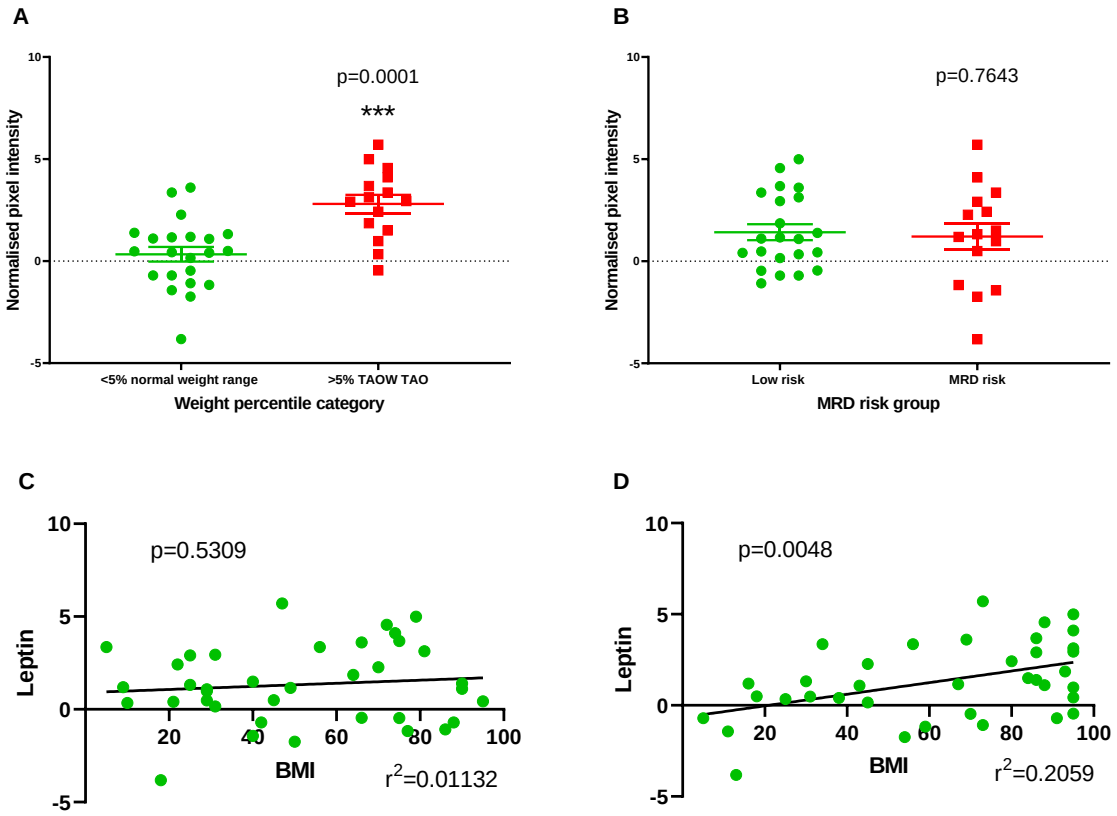

Figure 4.12: Patient leptin expression results. A, Leptin expression changes at day 29 in patients in the normal weight category, n=22 compared to weight gain, n=16, p=0.0001. B, Leptin expression at day 29 in patients with low risk, n=22, compared to MRD risk, n=15, p=0.7643, n=37. C, Linear regression analysis shows positive correlation between start BMI and leptin expression at day 29, p=0.5309, r^2=0.01132, n=30. D, Linear regression analysis shows a significant positive correlation between day 29 BMI and leptin expression, p=0.0048, r^2=0.2059, n=38, error bars shown as standard error.

4.6.2 IL-8 levels decrease in patients who gain weight

IL-8 is the second adipokine to be identified in our patient cohort to be significantly associated with weight gain, in this case patients in the weight gain category had decreased levels of IL-8 expression in day 29 plasma samples compared to patients in the normal weight category/patients who lost weight. IL-8 is a proinflammatory cytokine and was found to be circulating at higher levels at diagnosis of ALL in children compared to a healthy control group, this was indicative of a strong inflammatory response to disease process without underlying infection (Pérez-Figueroa et al., 2016). Studies investigating immature AML stem cells have shown that IL-8 is an adverse factor in the development of the disease (Schinke et al., 2015), less is known about the specific implications in ALL cells. Figures 4.13 a-d display the patients'

data for IL-8. Graph a shows the IL-8 expression levels in patients who gain weight compared to patients who lose weight or remain in the normal weight category at day 29. There is a significant decrease in IL-8 expression between patients in the weight gain group, n=22 compared to patients who lose weight or stay within the normal range, n=16, p=0.0084. The results in graph b show IL-8 expression changes in patients who are MRD risk, n=22 compared to patients who are low risk at day 29, n=15, there is no significant differences between IL-8 expression and the MRD risk groups, p=0.7947. Graphs c and d confirm the correlations between IL-8 expression and BMI scores at the start and end of induction therapy using linear regression analysis. Graph c displays a slight negative correlation with start BMI and IL-8 expression but not significant, p=0.5980, r^2=0008115. Graph d shows the same results for the end BMI, here there is a negative, but non-significant, correlation, r^2=0.04506, p=0.2072, n=38.

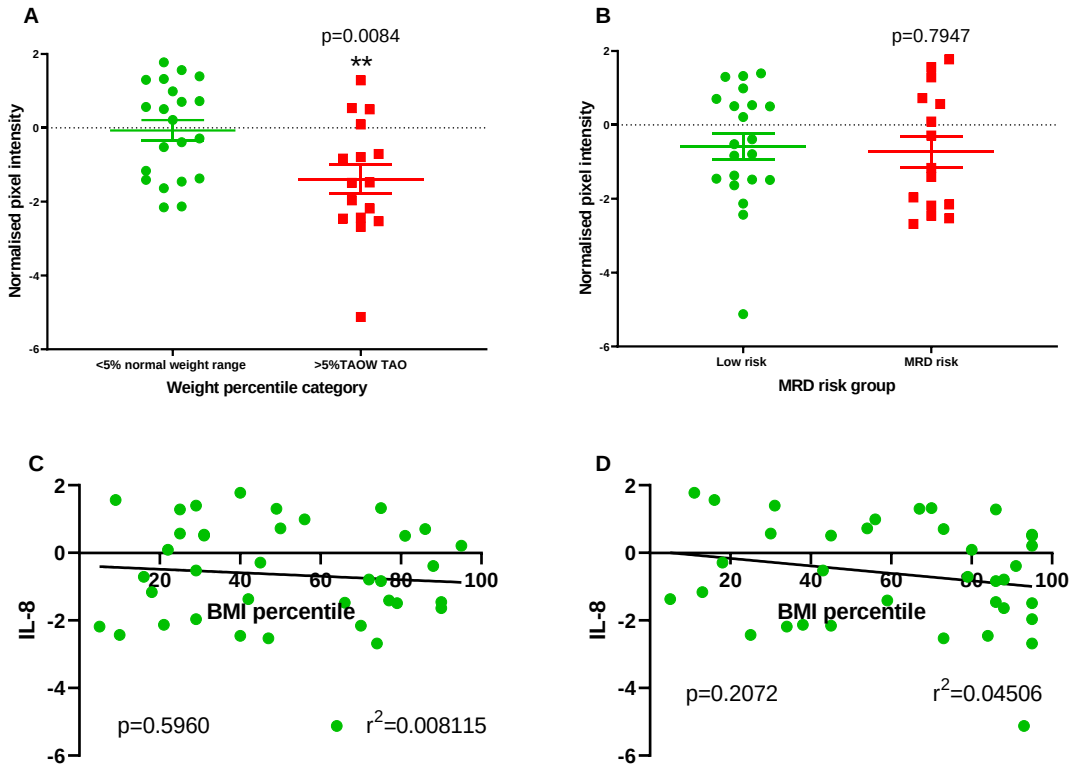

Figure 4.13: Patient IL-8 expression results. A, IL-8 expression changes at day 29 in patients in the normal weight category, n=22 compared to weight gain, n=16, p=0.0084, n=38. B, IL-8 expression at day 29 in patients with low risk, n=22, compared to MRD risk, n=15, p=0.7947, n=37 C, Linear regression analysis shows a negative correlation between start BMI and IL-8 expression at day 29, p=0.5980, r^2=0.008115. D, Linear regression analysis shows a negative correlation between day 29 BMI and IL-8 expression, p=0.2072, r^2=0.04506, n=38, error bars shown as standard error.

4.6.3 Endocan levels increase in patients who gain weight

Endocan has significantly increased in patients within the weight gain group. In ALL circulating endocan levels are associated with paediatric patients diagnosed with febrile neutropenia episodes and fever during the course of the disease (Kiral et al., 2016). Endocan is also highly expressed in untreated ALL and can act as a predictor for prognosis of the disease (Xu et al., 2014a). Less is known about the specific signalling pathways in ALL but in a study experimenting with AML cell lines knockdown of endocan signalling promoted apoptosis by reduced NF-kB activity (Sun et al., 2019).

Figures 4.14 a-d display the patients data results for endocan expression. Graph a shows endocan expression levels in patients who gain weight compared to patients who experience weight reduction or stay within the normal category from day 0 to day 29. There is a significant increase in endocan expression between patients in the weight gain group, n=22 compared to patients who lose weight or stay within the normal range, n=16, p=0.0197. The results in graph b show endocan expression changes in patients who are MRD risk, n=22 compared to patients who are low risk at day 29, n=15, there is slight increased but no significant difference between endocan expression and the MRD risk groups, p=0.4012. Graphs c and d confirm the correlations between endocan expression and BMI scores at the start and end of induction therapy measured using linear regression analysis. Graph c displays a slight negative correlation with start BMI and endocan expression but not significant, p=0.3428, the r^2=0.02503. Graph d shows the same results for the end BMI, here there is an increased positive correlation although not significant with the r^2=0.04313, p=0.01184, n=38, error bars shown standard error.

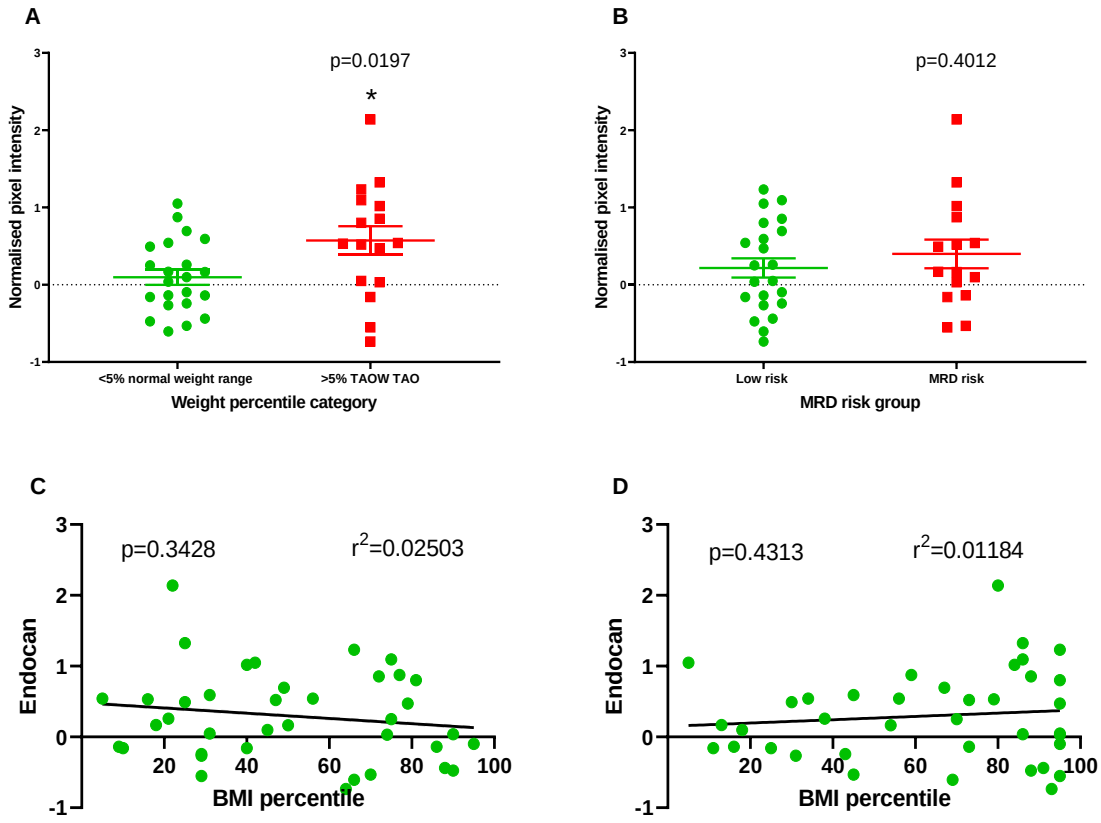

Figure 4.14: Patient Endocan expression results. A, Endocan expression changes at day 29 in patients in the normal weight category, n=22 compared to weight gain, n=16, p=0.0197. B, endocan expression at day 29 in patients with low risk, n=22, compared to MRD risk, n=15, p=0.4012, n=37. C, Linear regression analysis shows negative correlation between start BMI and endocan expression at day 29, p=0.3428, r^2=0.02503, n=38. D, Linear regression analysis shows a slight positive correlation between day 29 BMI and endocan expression, p=0.4313, r^2=0.01184, n=38, error bars shown as standard error.

4.6.4 Pref-1/DLK1/FA1 levels increase in patients who gain weight

Pref-1 has been identified as an adipokine that is increased significantly in the patents who gained weight. Pref-1 is described as a gatekeeper of adipogenesis and expression regulates this process by preventing adipocyte differentiation (Hudak and Sul, 2013). Studies in ALL show how exogenous DLK1 protein activates upregulation of the NOTCH1 receptor which in turn stimulates the proliferation of the ALL cell line CEM-CCRF, and activation of this receptor upregulated the downstream targets c-myc and NF-κB (Wei et al., 2013). NOTCH1 is known to interact with the PI3K/Akt/mTOR pathway by regulation of PTEN via HES1 and MYC (Efimenko et al., 2017), these processes may be exacerbated by the increased levels of pref-1. The results in figures 4.15 a-d display the patients data results for pref-1. Graph a shows

pref-1 expression levels in weight change patients. There is a significant increase in pref-1 expression between patients in the weight gain group, n=22 compared to patients who lose weight or stay within the normal range, n=16, p=0.0209. The results in graph b display pref-1 expression changes in patients who are MRD risk, n=22 compared to patients who are low risk at day 29, n=15, there is a small decrease in pref-1 expression levels between the MRD risk groups, although this is not significant p=0.2355. Graphs c and d confirm the correlations between pref-1 expression and BMI percentile results at the start and end of induction therapy using linear regression analysis. Graph c displays a slight positive correlation with start BMI and pref-1 expression, but this is not significant, p=0.4283, the r^2=01751. Graph d shows the same results for the end BMI, here there is an increased positive correlation which is significant, p=0.0319, r^2=0.1216, n=38, error bars shown standard error.

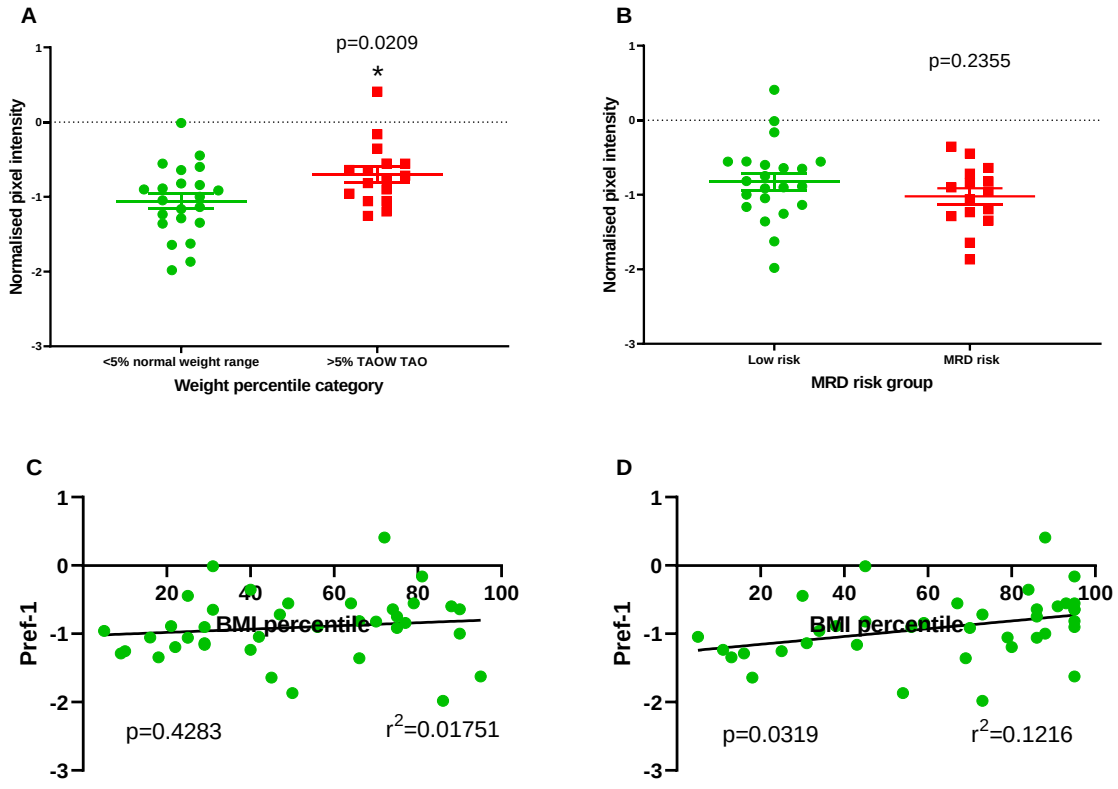

Figure 4.15: Patient Pref-1 expression results. A, Pref-1 expression changes at day 29 in patients in the normal weight category, n=22 compared to weight gain, n=16, p=0.0209. B, pref-1 expression at day 29 in patients with low risk, n=22, compared to MRD risk, n=15, p=0.2355, n=37. C, Linear regression analysis shows a small positive correlation between start BMI and pref-1 expression at day 29, p=0,4283 r^2=0.01751, n=38. D, Linear regression analysis shows a significant positive correlation between day 29 BMI and pref-1 expression, p=0.0319, r^2=0.1216, n=38, error bars shown as standard error.

4.6.5 Adiponectin levels decrease in patients who gain weight

Adiponectin has been highlighted as significant in our patient cohort and is shown at lower levels in the group of patients who have gained weight compared to the others. Adiponectin is known to be inversely correlated with obesity, diseased states and cancer progression including leukaemia (Ma et al., 2016). Studies describe reduced levels of adiponectin in patients diagnosed with ALL (Argyrou et al., 2019). There are less experimental studies looking into the impact of low adiponectin using all patient samples or ALL cells lines, the following study using a number of cell lines including Molt4 and CEM-CCRF found no inhibition of growth in these cell lines but when incubating patient derived $CD34^{+}$ cells adiponectin inhibited growth in these samples by downregulating Bcl-2 expression (Yokota et al., 2000). In non-diseased states higher adiponectin levels activate the AMPK pathway in addition to this inhibiting the PI3K/Akt and JAK/STAT signalling pathways, low circulating adiponectin levels attenuates this protective effect (Monks et al., 2019).

Figures 4.16 a-d display the patients data results for Adiponectin. Graph a shows the Adiponectin expression levels in weight gain patients compared to patients who lose weight or stay in the normal weight category after induction treatment. There is a significant decrease in adiponectin expression between patients in the weight gain group, n=22 compared to patients who lose weight or stay within the normal range, n=16, p=0.0212. The results in graph b show adiponectin expression changes in patients who are MRD risk, n=22 compared to patients who are low risk at day 29, n=15, there is no difference between adiponectin expression between the MRD risk groups, p=0.8810. Graphs c and d display the correlations between adiponectin expression and BMI percentile at the start and end of induction therapy using linear regression analysis. Graph c displays a small positive correlation with start BMI and adiponectin expression but not significant, p=0.2888, the r^2=0.03209, Graph d shows the same results for the end BMI, here there is no correlation between adiponectin and day 29 BMI, p=0.9389, r^2=0.0001701, n=38, error bars shown standard error.

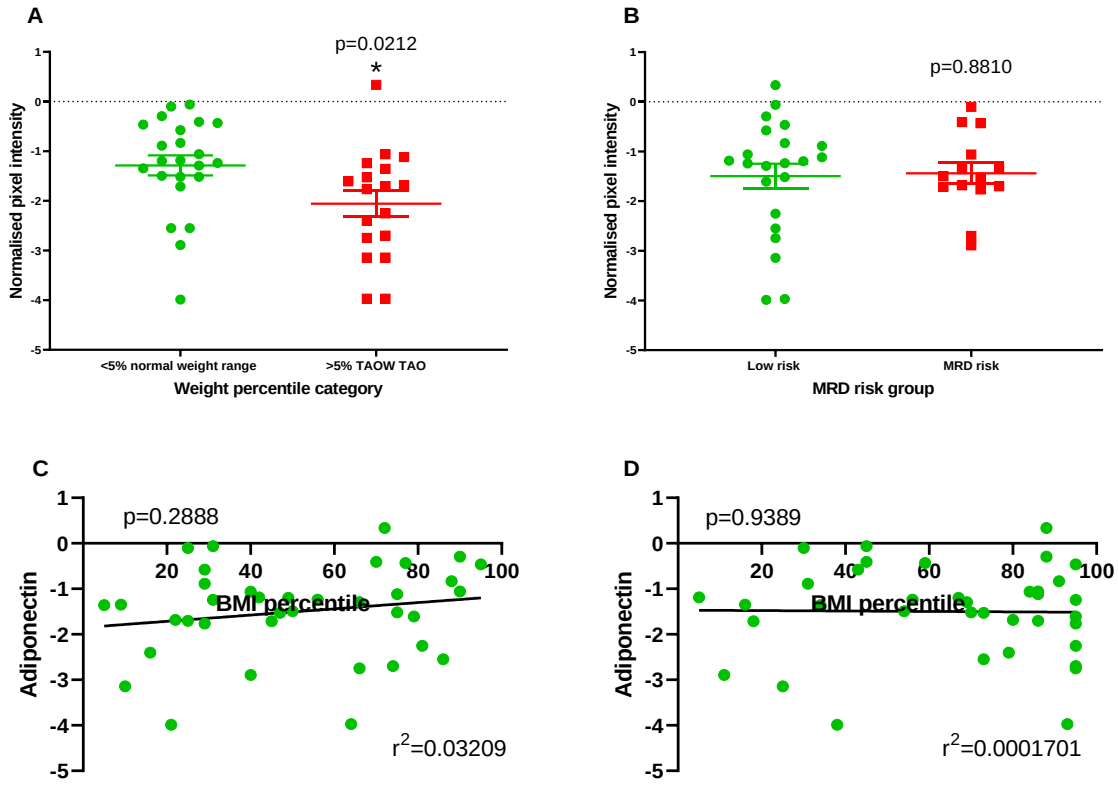

Figure 4.16: Patient adiponectin expression results. A, Adiponectin expression changes at day 29 in patients in the normal weight category, n=22 compared to weight gain, n=16, p=0.0212. B, Adiponectin expression at day 29 in patients with low risk, n=22, compared to MRD risk, n=15, p=0.8810, n=37. C, Linear regression analysis shows positive correlation between start BMI and adiponectin expression at day 29, p=0.2888, r^2=0.03209, n=30. D, Linear regression analysis shows no correlation between day 29 BMI and adiponectin expression, p=0.9389, r^2=0.0001701, n=38, error bars shown as standard error.

4.6.6 Adipokine summary data in patients who gained weight

The results in table 4.4 display all the adipokines tested in order of significance in relation to weight gain in our cohort. Adipokines are ranked by p value and then if the adipokine was increased or decreased in the weight gain group. Green indicates an increase in expression and red a decrease. The patients are grouped into those who lost weight or stayed within the normal weight category didn't gain more than 5% of their starting body weight compared to patients who gained more than 5% of their starting body weight, became overweight from a normal weight by day 29 or became obese with treatment from a normal weight range.

Table 4.4: Summary of all adipokines tested in relation to weight gain at day 29. Patients were grouped into those that didn't gain weight or lost weight and those that gained more than 5% of their starting body weight, became overweight or obese during induction, groups compared using an unpaired t-test. Adipokines ranked by p value significant to non-significant and indicated in green if the adipokine increased or red if decreased by day 29, n=38.

Adipokine	P value		Adipokine	P value	
Leptin	0.0001		Resistin	0.3482	
IL-8	0.0084		DPPIV/CD26	0.4073	
Endocan	0.0197		IL-1 beta	0.4404	
Pref-1	0.0209		BAFF/Blys	0.4539	
Adiponectin	0.0212		TIMP-1	0.4569	
Myeloperoxidase	0.0309		Serpin A8	0.4595	
IGFBP-2	0.0875		EN-RAGE	0.4600	
VEGF	0.0974		Chemerin	0.4701	
Cathepsin-L	0.1094		Pappalysin-1	0.4867	
Angiopoietin Like-3	0.1163		Fibrinogen	0.4988	
HGF	0.1357		RAGE	0.5453	
FGF-basic	0.1521		LAP(TGF-beta1)	0.5606	
Angiopoietin-2	0.1918		Growth hormone	0.5835	
Pentraxin-3	0.1927		TIMP-3	0.6203	
MIF	0.2004		Serpin A12	0.6486	
Angiopoietin-1	0.2302		IGFBP-6	0.6519	
Cathepsin-S	0.2320		Lipocalin-2	0.6573	
IL-10	0.2415		Visfatin	0.6953	
FGF-19	0.2570		BMP-4	0.7004	
Angiopoietin Like-2	0.2777		IGFBP-4	0.7135	
Complement D Factor	0.3065		CCL5/RANTES	0.7711	
ICAM/CD54	0.3085		Serpin E1/PAI-1	0.7751	
Cathepsin D	0.3248		Fetuin B	0.8119	
Nidogen-1	0.3293		TNF-alpha	0.8331	
LIF	0.3302		M-CSF	0.8419	
Oncostatin M	0.3317		CCL2/MCP-1	0.8533	
IL-6	0.3372		IL-11	0.8594	
Proprotein Convertase 9	0.3415		IGFBP-rp1/IGFBP-7	0.8637	
IGFBP-3	0.3480		CRP	0.9073	

The heat map in figure 4.17 summaries all the adipokines tested in association with weight gain. Each coloured square represents the log2 fold change figure of normalised pixel intensity from the array spots. Red squares indicate decreases in expression with the darkest red being the lowest expression, white squares have little no now change in expression and green squares indicate increases in expression from diagnosis with the darkest green having the highest expression. Patients are grouped into those who lost weight or stayed within the normal weight range and didn't put more than 5% of their starting body weight on during

induction and those who put more than 5% of their starting body weight or became overweight or obese with treatment.

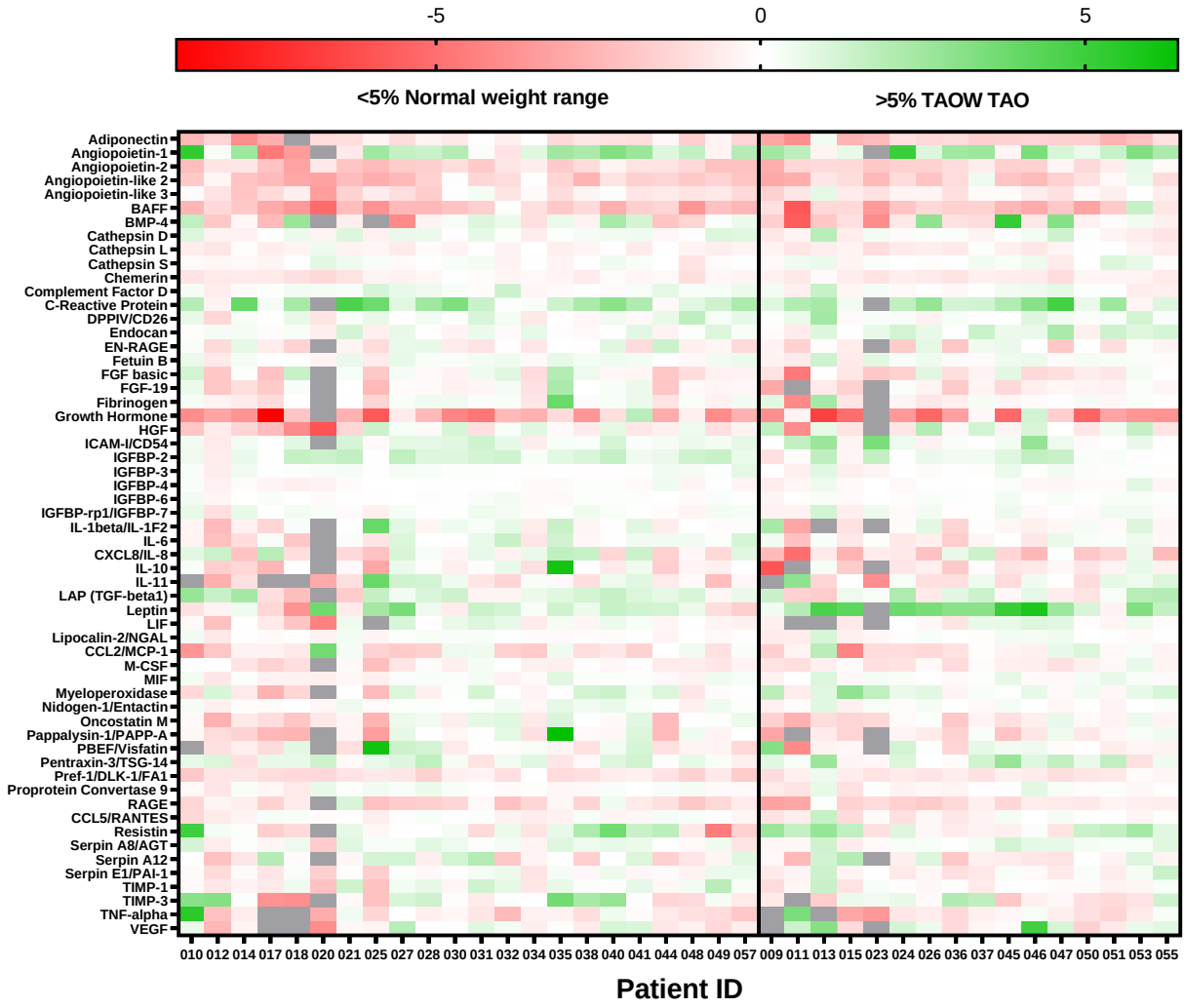

Figure 4.17: Heat map summary of all adipokines tested in association with weight gain. Comparison of patients who remained in the normal weight range of lost weight to patients who gained more than 5% of their starting body weight or became overweight or obese by the end of induction treatment. Red squares indicate decreases in expression from diagnosis, white squares little or no change and green squares increases in expression. Grey squares indicate no data available, n=38.

4.7 Identification of adipokine changes in patients associated with MRD risk

The data from the human adipokine arrays have also been used to identify adipokines which are associated with MRD risk at the end of induction treatment. There is very little previous data that looks to specifically link particular adipokines to MRD risk in ALL treatment at this

early stage. It would be useful in this early induction therapy to know which adipokines change in expression and if those adipokines go onto to impact the development of the disease moving through the next stages of treatment. Results from the individual adipokine arrays were analysed to identify changes in adipokine levels from day 0 at the start of treatment to day 29. So that the individual results across patients could be compared each array was normalised to the average of the arrays three reference spots. To determine if particular adipokines were increased in patients who were MRD risk the cohort was split into low risk n=22 and MRD risk groups n=15, patient 023 with no MRD markers has been excluded from the MRD risk analysis, total patients n=37, groups were then compared using an unpaired t-test. The results for the top five ranked adipokines associated with MRD risk are displayed in figures 4.18 – 4.22.

4.7.1 Resistin levels decrease in MRD risk patients

The patient results for Resistin show that reduced levels in plasma at day 29 are significantly associated with MRD risk category compared to low risk patients, p=0.0013, shown in figure 4.18a. There is a small non-significant increase in patients who gain compared to patients who remain in the normal weight category or lose weight at day 29, p=0.3482, see figure 4.18b, Resistin is often linked to weight gain and results do show a positive correlation with start BMI and Resistin expression although not significant, p=0.1706, r^2=0.09304 displayed in figure c. Figure d shows a positive correlation with day 29 BMI and Resistin expression although not significant p=0.0851, linear regression confirms the r^2=0.08232, n=37.

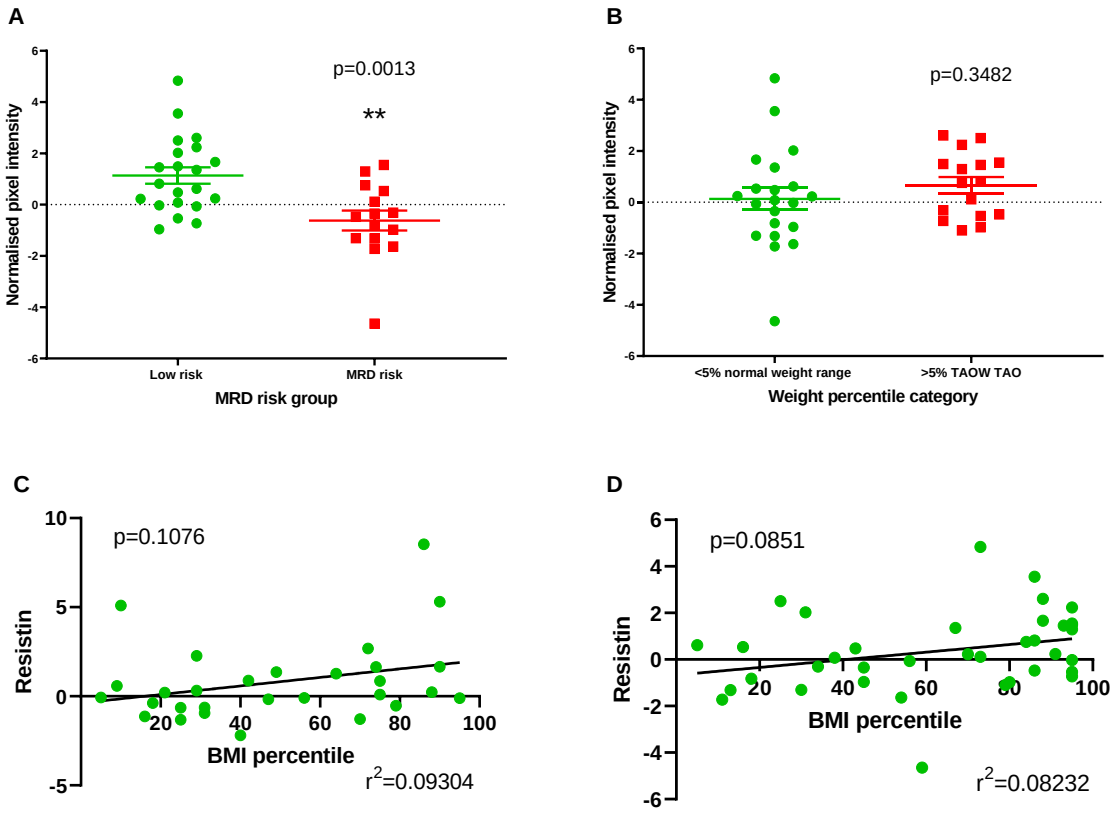

Figure 4.18: Patient resistin expression results. A, resistin expression at day 29 in patients with low risk, n=22, compared to MRD risk, n=15, p=0.0013. B, resistin expression changes at day 29 in patients in the normal weight category, n=22 compared to weight gain, n=16, p=0.3482. C, Linear regression analysis shows a positive correlation between start BMI and resistin expression at day 29, p=0.1076, r^2=0.093041, n=38. D, Linear regression analysis shows a positive correlation between day 29 BMI and resistin expression, p=0.0851, r^2=0.08232, n=37, error bars shown as standard error.

4.7.2 Serpin A12 levels increase in MRD risk patients

The results for serpin A12 expression shows that increased expression recorded on day 29 in patient samples is significantly associated with MRD risk, see figure 4.19a. Patients who were low risk were compared to MRD risk patients at day 29 and serpin A12 expression levels were significantly increased, p=0.0374, low risk patients, n=22 and MRD risk patients n=15, groups were compared using an unpaired t-test. Figure 4.19b shows serpin A12 expression in patients who gain weight compared to patients who stay in the normal weight range or lose weight at day 29, here there is decreased expression in patients who gain weight n=16, compared to patients in the normal weight range n=22 although not significant, p=0.6485. Figure 4.19c

displays no correlation between increasing BMI at diagnosis and serpin A12 expression, p=0.9998, r^2=0.00000000314, n=38. Figure 4.18d shows the same data for day 29 where we see a negative correlation between increasing BMI and decreased expression of serpin A12 although not significant, p=0.3946, here the deviation from zero is r^2=0.02140, n=37.

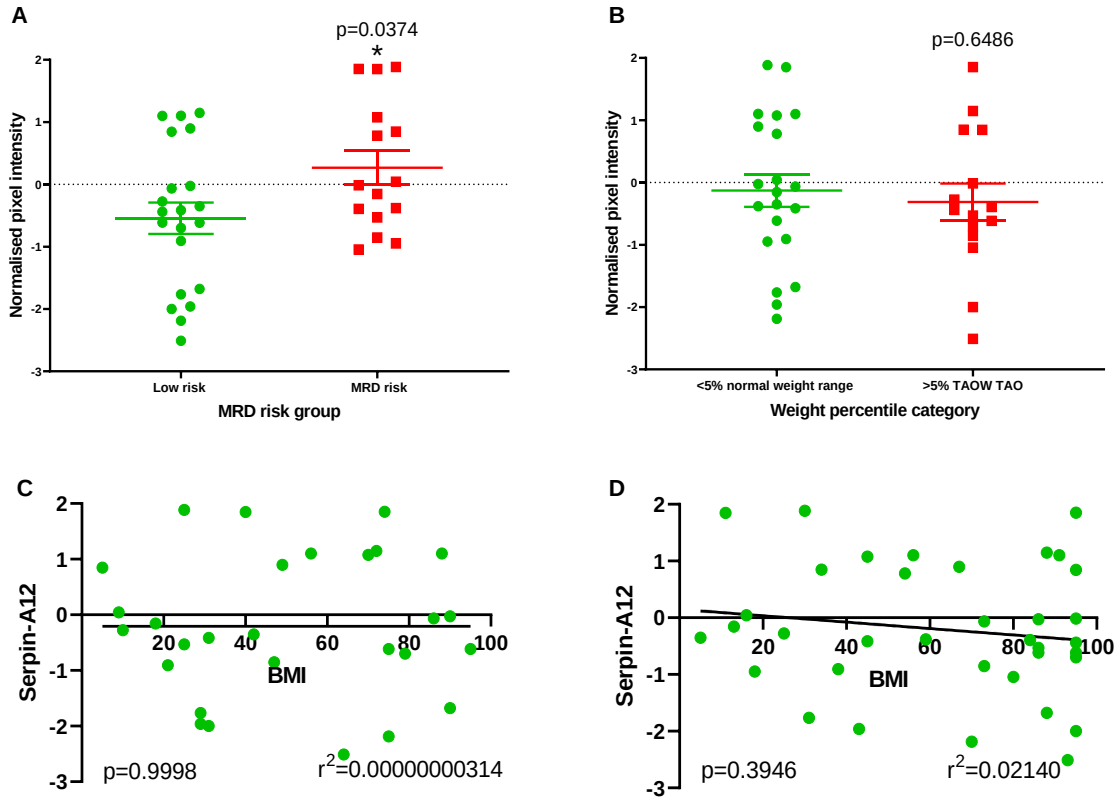

Figure 4.19: Patient serpin A12 expression results. A, serpin A12 expression at day 29 in patients with low risk, n=22, compared to MRD risk, n=15, p=0.0374. B, serpin A12 expression changes at day 29 in patients in the normal weight category, n=22 compared to weight gain, n=16, p=0.6486. C, Linear regression analysis shows a slight positive correlation between start BMI and serpin A12 expression at day 29, p=0.9998, r^2=0.00000000314, n=38. D, Linear regression analysis shows a negative correlation between day 29 BMI and serpin A12 expression, p=0.3946, r^2=0.02140, n=37, error bars shown as standard error.

4.7.3 Cathepsin-S levels decrease in MRD risk patients

Figures 4.20 a-d display the patients data results for cathepsin-S. The results in figure 4.20a show cathepsin-S expression changes in patients who are MRD risk, n=22 compared to patients who are low risk at day 29, n=15, there is a significant decrease in expression levels, p=0.0481. Figure 4.20b shows the cathepsin-S expression levels in weight gain patients. There is a small non-significant increase in cathepsin-S expression between patients in the weight

gain group, n=22 compared to patients who lose weight or stay within the normal range, n=16, p=0.2320. Figures c and d confirm the correlations between cathepsin-S expression and BMI scores at the start and end of induction therapy using linear regression analysis. Figure c displays a positive correlation with start BMI and cathepsin-S expression but not significant, p=0.1531, the r^2=0.07151. Figure d shows the same results for the end BMI, here there is a significant positive correlation, p=0.0202, r^2=0.1410, n=37, error bars shown standard error.

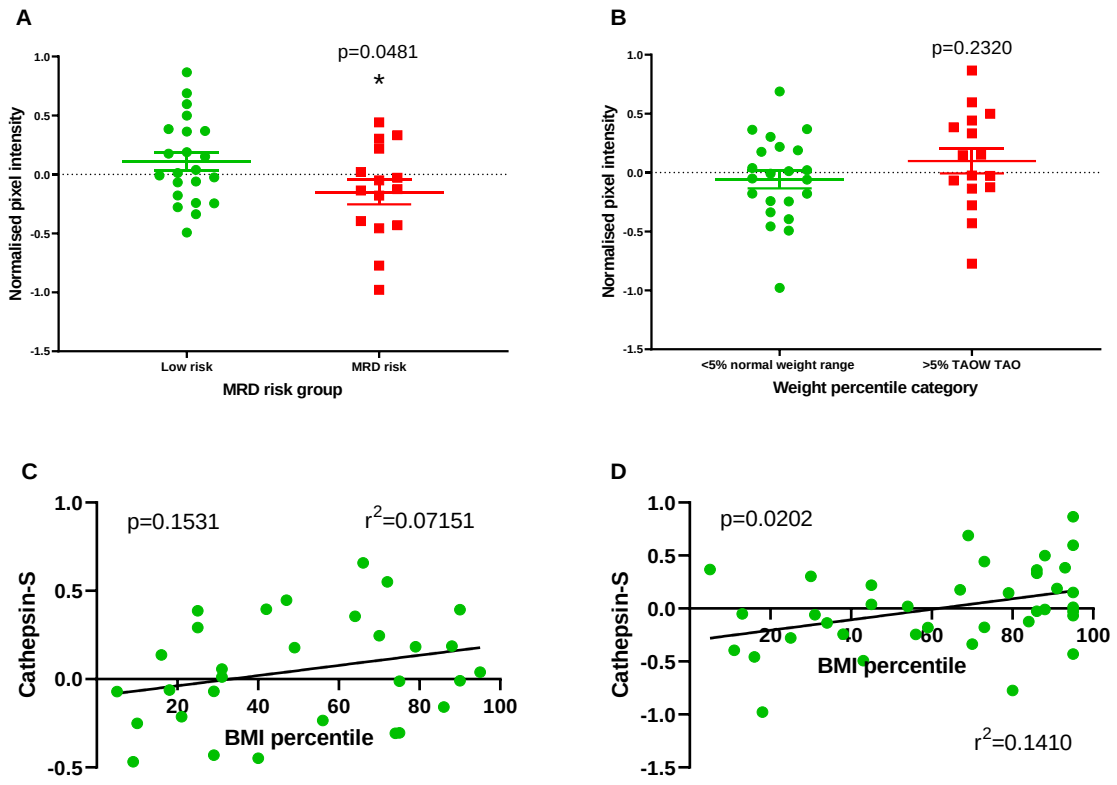

Figure 4.20: Patient cathepsin-S expression results. A, cathepsin-S expression at day 29 in patients with low risk, n=22, compared to MRD risk, n=15, p=0.0481. B, cathepsin-S expression changes at day 29 in patients in the normal weight category, n=22 compared to weight gain, n=16, p=0.2320. C, Linear regression analysis shows a positive correlation between start BMI and cathepsin-S expression at day 29, p=0.1531, r^2=0.07151, n=38. D, Linear regression analysis shows a significant positive correlation between day 29 BMI and cathepsin-S expression, p=0.0202, r^2=0.1410, n=37, error bars shown as standard error.

4.7.4 IGFBP-4 levels decrease in MRD risk patients

IGFBP-4 levels are shown to be reduced in MRD risk patients but not significantly in this study, this finding is in line with another study in children with ALL (Wex et al., 2005) Figures 4.21 a-d display the patients data results for IGFBP-4. Graph a shows the IGFBP-4 expression levels

in low risk patients compared to MRD risk patients at day 29 after induction treatment. There is a non-significant decrease in IGFBP-4 expression between patients in the MRD risk groups, n=22 for low risk patients and n=15 for MRD risk patients, p=0.1002. The results in figure b show IGFBP-4 expression changes in patients who gain weight n=16 compared to weight loss of no change in weight n=22 are MRD risk, there is no significant differences between IGFBP-4 expression in the weight change groups, p=7135. Figures c and d confirm the correlations between IGFBP-4 expression and BMI scores at the start and end of induction therapy using linear regression analysis. Figure c displays a positive correlation with start BMI and IGFBP-4 expression but not significant, p=0.0825, r^2=0.1038. Figure d shows the same results for the end BMI, here there is an increased positive correlation although not significant with the r^2=0.1257, p=0.0545, n=38, error bars shown standard error.

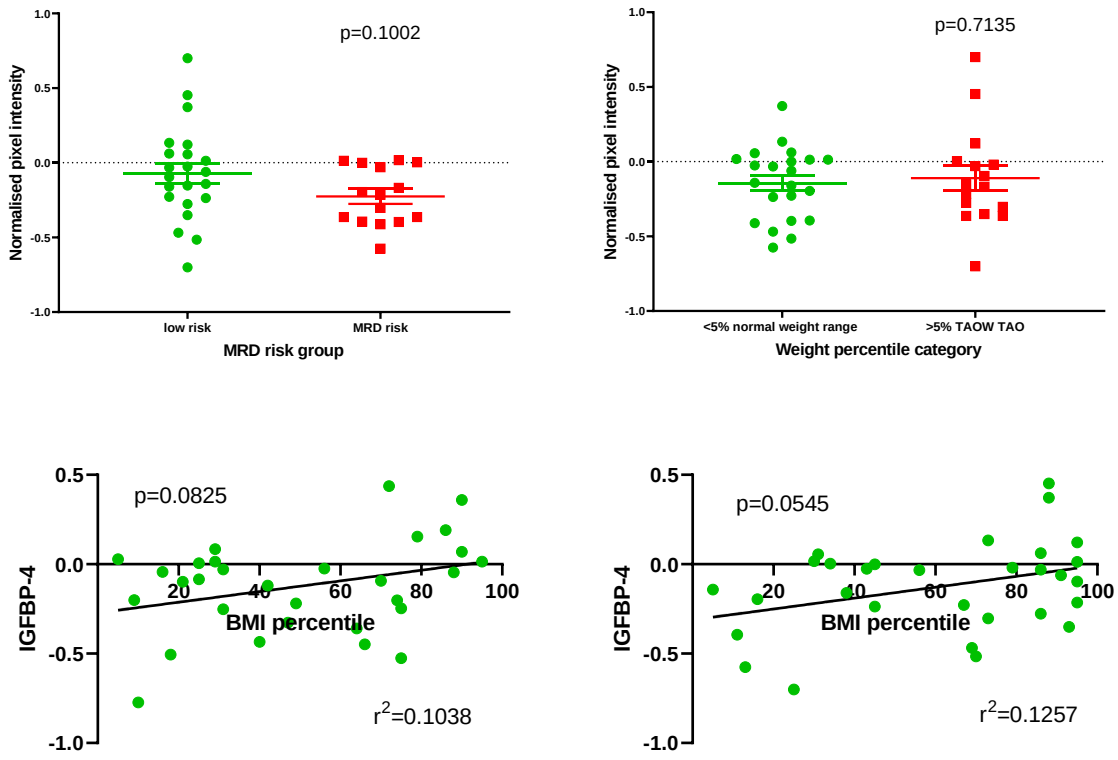

Figure 4.21: Patient IGFBP-4 expression results. A, IGFBP-4 expression at day 29 in patients with low risk, n=22, compared to MRD risk, n=15, p=0.1002. B, IGFBP-4 expression changes at day 29 in patients in the normal weight category, n=22 compared to weight gain, n=16, p=0.7135. C, Linear regression analysis shows a positive correlation between start BMI and IGFBP-4 expression at day 29, p=0.0825, r^2=0.10.8, n=30. D, Linear regression analysis shows a significant positive correlation between day 29 BMI and IGFBP-4 expression, p=0.0545, r^2=0.1257, n=38, error bars shown as standard error.

4.7.5 Angiopoietin-2 levels decrease in MRD risk patients

The patient results for angiopoietin-2 confirm a non-significant decrease in expression in patients at day 29 who are classed as MRD risk compared to low risk patients, p=0.1205, see figure 4.22a. Figure 4.22b shows angiopoietin-2 expression is increased in patients who gain weight compared to patients who stay in the normal weight range or lose weight at day 29, although not significant, p=0.1918. Figure 4.22c displays a positive correlation between increasing BMI at diagnosis and angiopoietin-2 expression, r^2=0.03392, the deviation from zero was not significant, p=0.2683. Figure 4.22d displays the same data for day 29, here there is a positive correlation between increasing BMI and increased expression of angiopoietin-2 and significant, p=0.0016, linear regression confirms the r^2=0.2451, n=38.

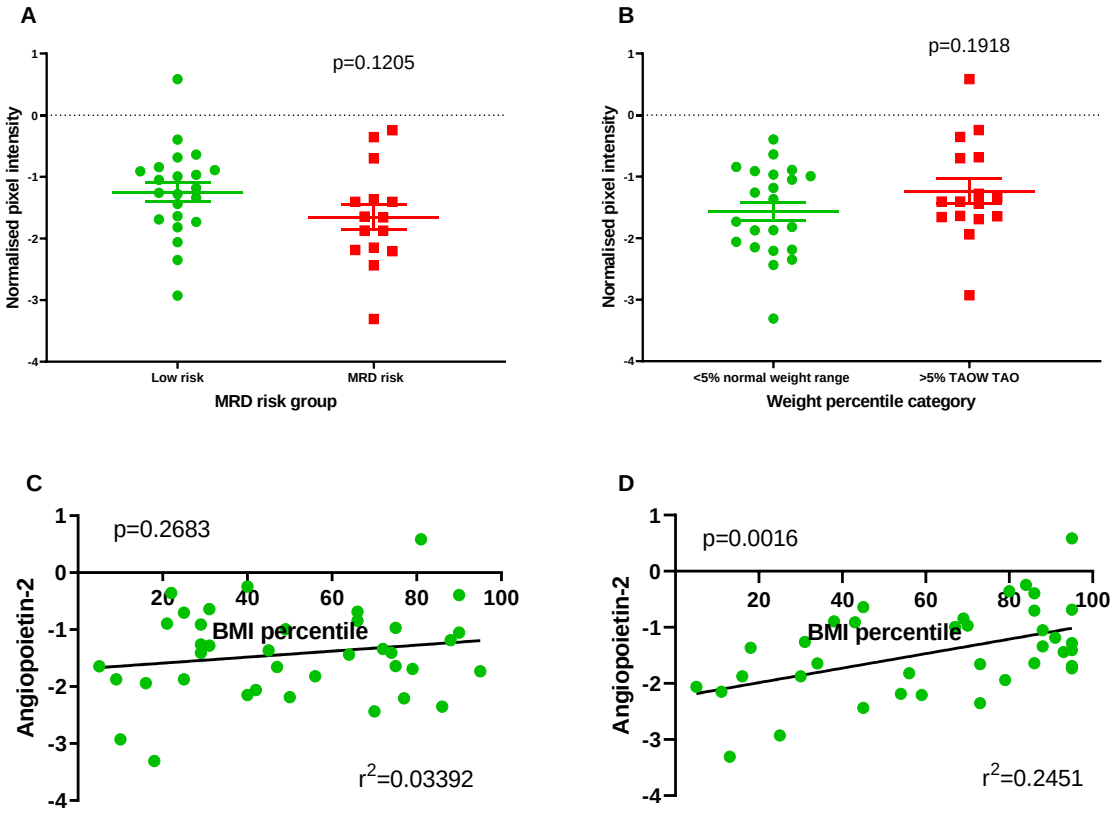

Figure 4.22: Patient angiopoietin-2 expression results. A, angiopoietin-2 expression at day 29 in patients with low risk, n=22, compared to MRD risk, n=15, p=0.1205. B, angiopoietin-2 expression changes at day 29 in patients in the normal weight category, n=22 compared to weight gain, n=16, p=0.1918. C, Linear regression analysis shows a positive correlation between start BMI and angiopoietin-2 expression at day 29, p=0.2683, r^2=0.03392, n=38. D, Linear regression analysis shows a significant positive correlation between day 29 BMI and angiopoietin-2 expression, p=0.0016, r^2=0.2451, n=38, error bars shown as standard error.

4.7.6 Adipokine summary data MRD risk patients

The results in table 4.5 display all the adipokines tested in order of significance in relation to MRD risk in our cohort. Adipokines are ranked with the p value and then if the adipokine was increased highlighted in green, or decreased which are highlighted in red. The patients are grouped into those that were MRD risk at the end of induction therapy and those that were low risk at the end of induction.

Table 4.5: Summary of all adipokines tested in relation to MRD risk at day 29. Patients were grouped low risk and MRD risk at the end of induction treatment and the two groups compared using an unpaired t-test. Adipokines ranked by p value significant to non-significant and indicated in green if the adipokine is increased or in red if decreased by day 29, n=37.

Adipokine	P value		Adipokine	P value	
Resistin	0.0013		IL-11	0.5858	
Serpin A12	0.0374		Growth hormone	0.5863	
Cathepsin-S	0.0481		Serpin E1	0.5949	
IGFBP-4	0.1002		MIF	0.6033	
Angiopoietin-2	0.1205		Angiopoietin Like-3	0.6205	
Angiopoietin-1	0.1368		ICAM-1/CD54	0.6276	
TIMP-3	0.1468		LIF	0.6321	
LAP(TGF-beta1)	0.1834		IL-6	0.6360	
Pentraxin-3	0.1897		FGF-19	0.6750	
M-CSF	0.2162		Fibrinogen	0.6950	
Pref-1	0.2355		Cathepsin D	0.6957	
TNF-alpha	0.2726		RAGE	0.7003	
IL-1beta	0.2763		CCL2/MCP-1	0.7016	
BMP-4	0.3105		IGFBP-3	0.7133	
BAFF	0.3736		VEGF	0.7259	
IGFBP-rp1/IGFBP-7	0.3995		FGF basic	0.7362	
Oncostatin M	0.4005		IGFBP-2	0.7415	
Endocan	0.4012		Leptin	0.7643	
DPPIV/CD26	0.4086		HGF	0.7798	
Myeloperoxidase	0.4123		IL-8	0.7947	
EN-RAGE	0.4226		TIMP-1	0.7986	
Visfatin	0.4458		Proprotein Convertase 9	0.8009	
Chemerin	0.4883		Serpin A8	0.8188	
Fetuin B	0.5050		Cathepsin L	0.8567	
IL-10	0.5174		CRP	0.8630	
Angiopoietin Like-2	0.5222		Adiponectin	0.8810	
Pappalysin-1	0.5451		Lipocalin-2	0.9070	
CCL5/RANTES	0.5461		IGFBP-6	0.9855	
Nidogen-1	0.5514		Complement D factor	0.9992	

The heat map in figure 4.23 summaries all the adipokines tested in association with MRD risk. Each coloured square represents the log2 fold change figure of normalised pixel intensity from the array spots. Patients are grouped into MRD risk and low risk. Increases in expression are highlighted in green, decreases in expression are highlighted in red, the darker of each colour reflecting the largest change in expression. White squares indicate no change or undetectable expression and grey squares indicate no data available. n=37.

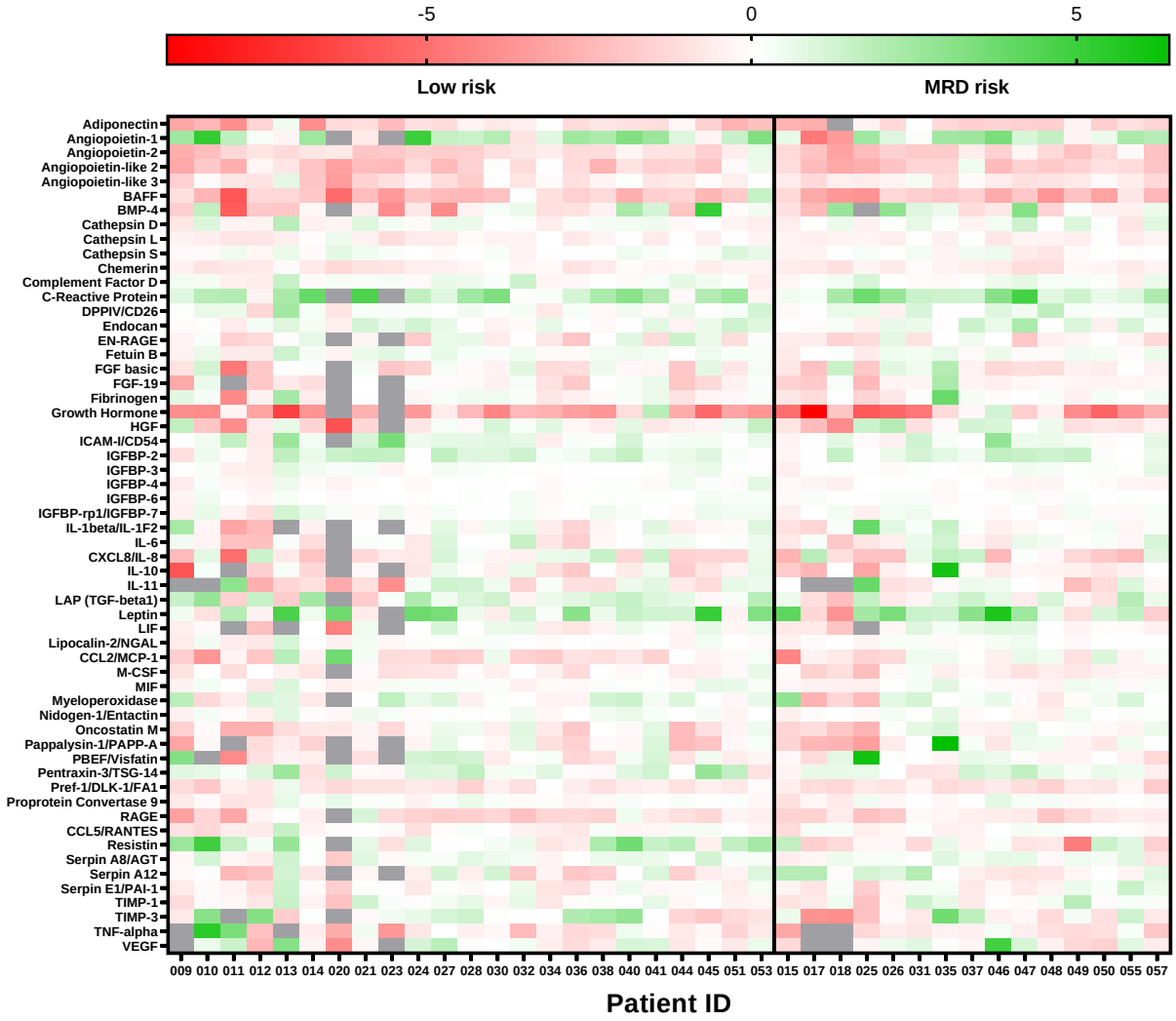

Figure 4.23: Heat map summary of all adipokines tested in association with MRD risk. Comparison of patients who were low risk or MRD risk by the end of induction treatment at day 29. Increases in expression are highlighted in green, decreases in expression are highlighted in red, the darker of each colour reflecting the largest change. White squares no change or undetectable, grey squares indicate no data available. n=37.

4.8 CHAPTER 4 DISCUSSION

4.8.1 Patient sample pilot study confirms reproducibility of Human adipokine arrays

A pilot study was performed using the human arrays to check the reproducibility of arrays and also to determine if the freezing and defrosting of samples impacted sample quality. The human arrays showed a high level of similarity between the pre-treatment plasma adipokine profiles of patients 006 and 007 figure 4.1 and 4.2. There are two adipokines that appear not to be correlated between the two patients which are growth hormone and leptin. From the clinical characteristics, patient 006 is female, age 2.6 years with a BMI of 16.1, patient 007 is male, age 8.9 with a BMI of 14.9, the differences being age, gender and BMI. Growth hormone can fluctuate in childhood and can even fluctuate throughout the day in a pulsatile manner (Gill et al., 1999). Growth hormone can also decline with increasing age and patient 007 is older (Garcia et al., 2019). It can also be attributed to a growth spurt and not necessarily due to the diagnosis of ALL; growth hormone levels have been shown to be impacted by chemotherapy later in therapy where they decline and can lead to short stature in adulthood (Haddy et al., 2006), however, we do not have data for these patients after induction treatment.

Leptin is associated with obesity and with increasing fat mass, leptin levels raise exponentially and are a reflection of the amount of stored fat in the body (Ekmen et al., 2016). Patient 007 did put weight on during induction therapy, starting at a BMI percentile of 52 and raising to 83 by the end of induction therapy. This is in comparison to patient 006 who recorded at BMI percentile of 39 at diagnosis which is lower than patients 007 at 52. There are also differences in secretion of BAFF between patients 006 and 007, BAFF has been linked to patient's poor treatment response but by the end of therapy patients did not have a poorer outcome (Zidan, 2020).

In previous research it has been shown that storage conditions and freezing and thawing can affect the sample quality and in the following study (Mitchell et al., 2005) it was found that long term storage of samples at -80 has little impact on sample quality but it was important to limit the number of freeze thaw cycles which has been taken into account for this study.

To complete the pilot study each replicate was compared to check if there was any impact from freezing the samples on array results and also to check the variability between each of the three replicates, replicate 1 and 2 was compared, 1 and 3 and finally 2 and 3, this is displayed in figure 4.3. There was a high level of reproducibility between samples exposed to one freeze-thaw cycle and samples exposed to two freeze-thaw samples, the three replicate arrays were very similar, this increases reliance on this technology to be sensitive to changes in adipokine expression between patients. From this set of results, we confirmed that the freezing and thawing process of the plasma samples did not adversely impact on the quality of the patient samples as adipokine concentrations remained consistent. Due to the high level of reproducibility between the replicate expression profiles this suggests that the human adipokine arrays were highly accurate and reliable, also there may be little benefit from repeating these arrays for each sample. Performing only one replicate per sample would allow more patients to the included in the study, as there were financial limitations associated with the number of human adipokine array kits available to us. The decision was made to include more patients and perform a single replicate for each sample, as this will allow a larger dataset to be collected, from which meaningful adipokine expression patterns linked to patient characteristics might be derived.

4.8.2 Patient sample study confirms pre-treatment pattern of adipokine expression

To establish if there was a pre-treatment pattern of adipokine expression plasma from day 0 was taken from patients 006-011 who all went onto be low MRD risk at the end of induction therapy. Adipokines displayed varying levels of expression between patients, figures 4.4 to 4.6. The following adipokines showed lower expression and little variability between patients, the mean pixel intensity was below 5000 for each adipokine reading across patients 006-011 or the recorded spot intensities were grouped closely to the mean pixel intensity for each adipokine; angiopoietin-1, BMP-4, EN-RAGE, fetuin B, FGF-basic, FGF-19, fibrinogen, HGF, ICAM1/CD54, IGFBP-2, IGFBP-rp1/IGFBP-7, IL-1 beta, IL-6, IL-8, IL-10, IL-11, LAP(TGF-bet1), LIF, CCL2/MCP-1, M-CSF, myeloperoxidase, oncostatin-M, pappalysin-1, visfatin, proprotein convertase 9, RAGE, resistin, serpin A8, serpin A12, serpin E1, TIMP-1, TIMP-3, TNF-alpha and VEGF. As there is little variability or low detection levels these adipokines have not been

discussed in this section. The next group of adipokines show greater variability between patients with one or two outliers from the mean or are expressed at much higher levels across figures 4.4,4.5 and 4.6.

4.8.2.1 Adiponectin

Low adiponectin levels are associated with obese leukaemia patients (Srivastava et al., 2015a) and are inversely correlated with leukaemia diagnosis in general in meta-analysis studies (Ma et al., 2016). Low adiponectin levels at diagnosis in paediatric leukaemia patients has been observed previously (Moschovi et al., 2010b), this is in support of our findings. However, patient 007 did have higher levels but it impossible to stay at this stage if that was a positive factor in the patient's outcome, lower levels could indicate poorer outcome but we do not have normal controls to confirm this hypothesis.

4.8.2.2 Angiopoietin-2

Angiopoietin-2 plays a role in angiogenesis; it increases vascular permeability and stimulates stromal derived factor 1 alpha (SDF1a) which stimulates angiogenesis in ALL (Karakurt et al., 2016). In the Karakurt study angiopoietin-2 levels were high and angiopoietin-1 low, this finding may be associated with increased homing and mobilisation of leukemic blasts, remission reversed this pattern. In our study patient 010 displayed higher levels of angiopoietin-2 and also was in the overweight percentile at diagnosis, angiopoietin-2 is associated with obesity related inflammation and has been shown to reduce survival in other cancers and can be a predictive marker in poorer survival (Volkova et al., 2011).

4.8.2.3 Angiopoietin-Like 2,3

The angiopoietin-Like 2 protein is capable of increasing NOTCH activation which leads to activation of c-myc targets and expansion of human hematopoietic stem and progenitor cells (HSPCs) (Lin et al., 2015). In a study using angiopoietin-Like 3-null mice, hematopoietic stem cells (HSCs) were reduced in number and quiescence as angiopoietin-3 supported stemness in the bone marrow niche (Zheng et al., 2011). Patient levels of angiopoietin-Like 2 and 3 show larger variations with patient 009 having the highest levels this could be a predictor of outcome due to its link with inflammation and poorer outcome in other cancers (Thorin-

Trescases and Thorin, 2017). None of the angiopoietin-Like adipokine data can be linked to the clinical parameters in this dataset.

4.8.2.4 BAFF

Abnormal levels of BAFF in patients have been associated with the development and progression of ALL in children due to a positive correlation with white blood cell count. Tracking this may lead to the development of BAFF as a diagnostic biomarker, this was found in the following study using plasma to test for BAFF levels (Bienertova-Vasku et al., 2012). BAFF levels are variable from low to high levels in our study and were not correlated with any clinical parameters at this early stage of testing adipokine expression and the patients in this sub-set being low MRD risk.

4.8.2.5 Cathepsin proteins D, L, S,

Cathepsin-L and cathepsin-S are a family of proteases that can modulate the function of the extracellular matrix, promote tumour dispersal and be a marker of tumour progression (Gormley et al., 2011). A study in ALL showed the cathepsins L and D have been implicated in poor prognosis when expressed at high levels (Enzenmueller et al., 2016). In a study investigating cathepsin levels in Acute Myeloid leukaemia (AML), high expression levels of cathepsins was correlated with a poorer outcome (Pandey et al., 2018). Cathepsin-D has been discussed in the previous section, with higher levels associated with poor prognosis in ALL; patient 009 in this study has higher levels compared to the other patients but they were not MRD risk at the end of induction. The cathepsin family could be used as early biomarkers for outcome, patient 007 and 009 have increased levels of cathepsin-D at diagnosis in our study and higher levels of cathepsin-S more than the overall levels of cathepsin-L.

4.8.2.6 Chemerin

Chemerin's role in cancer is mixed (Shin et al., 2018), there is little evidence on its specific role in ALL, however, in AML reduced levels have been correlated with poor outcome (Zhang et al., 2017), whereas in multiple myeloma increased levels are associated with poor outcome (Westhrin et al., 2018). This small study shows higher expression of Chemerin in patients with no obvious links to clinical characteristics.

4.8.2.7 Complement Factor D

Studies which involve complement factor D (adipsin) are limited in the literature. Research undertaken in mouse models found raised adipsin levels in a mouse model of ALL was linked to expression from gut associated lymphoid tissue (GALT) in response to bacterial translocation in mice due to intestinal permeability. Along with Lyzosome1 and defensin they play in important role in mucosal immunity (Song and Gyarmati, 2019). Raised adipsin may constitute another marker for bacterial infection in ALL. The expression of complement factor D is varied between patients in this pilot study and there are no obvious links to the clinical data although there could be a role for bacterial infection in patients.

4.8.2.8 CRP

CRP in paediatric ALL is associated with dysregulated immune function and lower levels of CRP before developing ALL (Søegaard et al., 2018). All patients bar patient 006, had low levels of CRP at diagnosis. As higher levels of CRP in ALL can indicate infection (Karppinen et al., 2019), this may have been the case with patient 006. Literature indicates that infection can be common during the intensive induction phase and more common with a four drug induction regimen which patient 006 received (Afzal et al., 2009). The plasma for this small study was tested at day 0 so the patient may have contracted a virus or infection unrelated to ALL or treatment. Previous study suggest a link to elevated CRP to poor outcome due to raised levels (Peltola et al., 1983), this has since been disputed where the was no significant difference in CRP levels between after treatment and healthy children (Hamodat et al., 2020).

4.8.2.9 DPPIV/CD26

The role of DPPIV/CD26 in ALL is limited, although it has been studied in its role in regulating haematopoiesis and, in B-ALL and T-ALL, its activity is significantly higher compared to non-leukaemia subjects (De Andrade et al., 2009). In a further study it was found that DPPIV/CD26 was increased on cell membranes of the ALL cell lines CEM-CCRF and Molt3 in response to chemotherapy and has been suggested as a target for its potential role in chemotherapy resistance (Dourado et al., 2007), it this stage is it not possible to link the varied expression of DPPIV/CD26 to the clinical outcomes of this study.

4.8.2.10 Endocan

Endocan levels in patients in patients 006, 008, 010 and 011 display similar expression, whereas patients 007 and 009 both have increased levels. Endocan has been indicated as a potential biomarker in acute leukaemia's and is highly expressed in untreated leukaemia and is associated with the stage of disease (Xu et al., 2014a). Another study linked endocan to the development of febrile neutropenia (FN), a complication of ALL; significantly higher levels of endocan were observed in the children with FN (Kiral et al., 2016). There are no clinical findings at this stage in our study that could link to them having increased endocan levels.

4.8.2.11 Growth hormone

Growth hormone levels are varied in patients, as explained in the discussion section 4.1, when discussing patients 006 and 007. This can fluctuate during the day or in growth spurts and is known to be changeable. Growth hormone levels have been shown to be impacted from chemotherapy later in therapy where they decline and can lead to short stature in adulthood (Haddy et al., 2006).

4.8.2.12 IGFBP proteins

In a study involving IGFBP proteins IGFBP-3 was significantly lower at ALL diagnosis than IGFBP-1 and 2 (Zakhary et al., 2012), our patients show increased levels in comparison to IGFBP-2, IGFBP-3 in other cancers is linked to increased pathogenesis of cancer (Natsuizaka et al., 2014). There is one study regarding IGFBP-6 in ALL which were all within normal ranges with no correlations with disease progression at high or low levels (Doorn, 1999), the patients in this study show patients 006, 007, 008 and 011 expression around the mean with patient 009 with high levels and patient 010 with low levels with no obvious correlations with the clinical data.

4.8.2.13 Leptin

Leptin is showing variation in levels between patients, this could be an early marker for predicting BMI increases or increases in body fat percentage and poorer outcome as discussed previously with patient 006 day 1 leptin expression. Patient 010 also shows higher

levels of leptin, this may be due to the patients BMI percentile at diagnosis which is 86 in the overweight category.

4.8.2.14 Lipocalin-2/NGAL

Lipocalin-2/NGAL is an acute phase inflammatory protein and has been associated with kidney injury and chronic kidney disease in ALL survivors Lipocalin-2 levels were significantly higher on a five year follow up compared with healthy controls (Latoch et al., 2020). Differences in patients may be in indicator of inflammation present at diagnosis.

4.8.2.15 MIF

Macrophage migration inhibitory factor (MIF) is a pro inflammatory cytokine and has been implicated in the prognosis of ALL. Patients who expressed higher levels displayed lower incidence of complete remission and significantly shorter event free survival than patients who expressed MIF at lower levels (Sharaf-Eldein et al., 2018).

4.8.2.16 Nidogen-1

There is very little information regarding nidogen-1 and ALL, in a study on ovarian cancer it showed promise as a candidate biomarker and could reflect tumour burden (Li et al., 2015). Nidogen-1 levels are increased in patients with different levels of expression for each patient.

4.8.2.17 Pentraxin-3

Pentraxin-3 levels in ALL have been associated with invasive mold infections, sepsis and increased mortality (Brunel et al., 2018). Levels in our small pilot study show patients 006 with slightly increased levels compared to the other patients this still may not reflect the patient has an infection but interestingly patient 006 also has higher levels of CRP which is also an indicator of infection, in this study (Elghandour et al., 2015) pentraxin-3 is seen as a superior marker in acute leukaemia's in prediction of septic shock, coagulation impairment and mortality.

4.8.2.18 Pref-1/DLK1

Information is lacking in the role of pref-1/DLK-1 and ALL, in CML pref-1/DLK-1 was higher in patients compared to controls but displayed no correlation with outcome of clinical characteristics (Liang et al., 2006). Studies in ALL show how exogenous DLK1 protein activates upregulation of the NOTCH1 receptor which in turn stimulates the proliferation of the ALL cell line CEM-CCRF, activation of this receptor upregulated the downstream targets c-myc and NF-κB (Wei et al., 2013). At this stage pref-1/DLK1 cannot be linked to the clinical parameters.

4.8.2.19 CCL5/RANTES

Studies into CCL5/RANTES expression in ALL are lacking, in triple negative breast cancer CCL5 has been proposed as a candidate immunotherapeutic target as it is strongly associated with disease progression (Lv et al., 2013). There is no obvious correlation between nidogen-1, pref-1 and CCL5/RANTES expression levels and our clinical parameters in this study from this group of adipokines.

The main purpose of this experiment was to establish if there were consistent adipokine expression patterns in a selected group of low risk patients. It also begins to answer questions about how reproducible the arrays are and which adipokines are likely to be of interest, for example if all of the adipokines which were not detectable were consistently not detected in all the patients, this demonstrates reproducibility and suggests these adipokines are either expressed at very low levels, below the limit of detection, or are not found in the bloodstream. All of the adipokines which were expressed at relatively high levels are relatively high in all patients, this is often the case when adipokines are variable between patients. The results from this small pilot study gave us indications to why certain adipokines may be increased or decreased at diagnosis. Many of the adipokines show little or no variation from patient to patient at diagnosis in this selection of patients suggesting a reproducible pattern of expression, the adipokines that do vary between patients could be something to consider in the detection of biomarkers or if particular adipokines begin to show obvious correlations with BMI and MRD risk at day 0 or day 29.

4.9 Identification of clinical variables as predictors of MRD risk

The next sections discuss the results obtained from the patient clinical data from the 38 patients recruited from the study with a full clinical data set and also two adipokine profiles at induction and after induction treatment at day 29. We are working with two data sets, the first covering patients 009 – 040 and was completed by colleagues at The University of Liverpool and further analysis including patients 009 – 057 which is the complete data set of all patients.

The University of Liverpool analysis confirmed the well-established risk factor contributing to poor outcome in paediatric ALL is age at diagnosis (Lee and Cho, 2017), with increasing higher age within childhood leukaemia implicated in poorer overall survival and MRD risk (Meraj et al., 2020). Induction regimes take this into account and all children diagnosed with ALL over ten years old automatically receive induction therapy B which mitigates this increased risk. The results of the Liverpool analysis also demonstrate that increased age is associated with MRD risk although not significantly, this observation was comparable to that with the full data set of patients 009-057.

Another known risk factor is the child's sex, with males having a slight increased incidence and a poorer outcome in patient cohorts (Gustafsson and Kreuger, 1983). In large population cohorts, females are reported to fair better than their male counterparts even after controlling for other prognostic factors such as age, race and cell counts at diagnosis (Holmes et al., 2012). Within our cohort there are more males than females diagnosed with ALL, with males linked to MRD risk although this is not significant. This may become significant with a higher number of patients within the cohort, which is an important limitation of this study.

Drug treatment regimens differ between patients depending in starting risk factors such as age and white cell count at induction, with a white cell count > 10,000 cells. Having this higher level of cells at diagnosis is linked to significantly lower survival rates (Vaitkeviciene et al., 2013). We wanted to know if receiving drug regimen B, which is a four drug treatment containing daunorubicin, was linked to increased MRD risk. Published literature is limited regarding this. This study confirmed that there is no increased MRD risk when receiving regimen B versus regimen A during induction therapy in our patient cohort. Due to the lower numbers of patients we cannot say for sure if this would be the case in a larger cohort. We

had 3 patients of whom 9 patients were MRD risk and of them 4 patients received induction therapy B. Although it did not add to MRD risk at the end of induction treatment, we cannot say in this study if receiving daunorubicin as a treatment option contributes to the known issues like cardiotoxicity which can add to complications during the remaining treatment and long after treatment has ended or MRD risk when tested at a later stage of treatment. This may be better represented with a follow up study to test adipokine profiles after treatment and full recovery.

One of the main questions of this study was to determine if increased weight during induction therapy was linked to MRD risk. This would include children that became overweight or obese with treatment or who were overweight or obese at diagnosis before starting treatment. In our complete study which includes patients 009-057, we can conclude that weight gain in induction therapy is not associated with MRD risk although more patient data would be needed to statistically confirm this data.

Of the 15 patients who were recorded as MRD risk, six patients put on more than 10% of their starting body weight and, of those, two became obese during treatment from being in the normal BMI percentile category at diagnosis. Our data supports the opposite of our hypothesis; we found that a lower BMI at the start and end of induction, weight loss or a lower weight at induction and day 29, were significantly associated with high MRD risk at day 29, figure 4.8. Similarly, BMI percentile where a lower BMI percentile at diagnosis significantly predicted MRD risk at day 29 and a lower BMI percentile at day 29 was associated with MRD risk but not significantly, figure 4.9.

We are not the first study to show that reduced weight is linked to MRD risk and poorer outcome in ALL, in a study by the Orgel group, weight extremes i.e., gaining and losing large amounts of weight, contributed to MRD risk, EFS and morbidity in paediatric ALL (Orgel et al., 2014a). A further study including 238 patients also found this to be the case and maintaining weight or even gaining weight was positive for reduced MRD risk, with obesity impacting overall survival by the end of treatment (Ethier et al., 2012). Weight loss early in induction therapy, which is the period of time in treatment that we are concentrating on has been associated with decreased survival (den Hoed et al., 2015). These findings do not suggest that weight gain is not a risk factor in ALL. A study by the Orgel group found findings different to ours where children starting in a normal weight category who progress to overweight or

obese at the end of induction, were associated with MRD risk, and being overweight or obese was linked to EFS and OS throughout the course of treatment and after (Orgel et al., 2014b).

Another study found that obesity at diagnosis has no association with MRD risk at days 19 or 46, nor did weight change in induction constitute MRD risk. However, it did indicate that patients who became obese during treatment have poorer OS due to treatment related mortality (Eissa et al., 2017a). The Butturini study concluded that obesity predicts the likelihood of relapse and chances of being cured in preteenagers and adolescents (Butturini et al., 2007). Children who were obese during induction have increased risk of hypertension, hyperglycaemia and hospital admission for febrile neutropenia (Meenan et al., 2019). Also gaining weight early in treatment is predictive of the child still being obese or overweight by the end of treatment and increases the child's risk of continuing with the excess weight and risks when recovered (Withycombe et al., 2015) which contributes to the number of disease associated with being obese such as cardiovascular disease and further cancers.

In this study, weight gain in the short induction period is not linked to MRD risk. Our study does not consider the risk of being overweight or obese in the longer consolidation therapy and maintenance therapy which can last for up to 2 years or how this rapid weight gain early in treatment adds to risk moving into the later phases of treatment. We also have a very small sample size and would need more patients to confirm if our findings would be comparable to published literature. Weight gain and its impact on event free and overall survival is studied more over the whole course of treatment and can be linked to events that occur after treatment has ended and the children are older. In recent research, there is a call for weight management as a therapeutic strategy in paediatric ALL (Tucci et al., 2018) and also for childhood cancer survivors to negate the risks of ongoing obesity (Zhang and Parsons, 2015). There is a lot of research investigating why obesity contributes to poorer outcomes as discussed in the introduction such, for example the protective environment fat tissue gives to ALL cells which may reduce chemotherapy efficacy further adding to risk long term after induction therapy.

4.10 Identification of adipokine expression changes in patients before and after induction therapy

Using human adipokine arrays to test our patients at the beginning of induction therapy and at the end on day 29 has given an extensive adipokine profile for each patient in this intensive early phase of treatment, see summary figure 4.11. The appendix sections 7.1.1 – 7.1.38 and figures 7.2 – 7.39 has individual descriptions and patient graphs. Sections 7.2 – 7.2.16 also includes a more in-depth discussion of individual patient data. The adipokine arrays have been a useful tool to begin to identify interesting patterns in profiles and to also ask questions of the data to support our aims. One aim of this study is to establish if a particular adipokine expression pattern is associated with MRD risk or weight gain. The previous section has discussed how the clinical dataset may contribute to MRD risk and highlights many factors that increase risk, this section will add to that complexity by discussing how the individual adipokine profiles may further increase this risk in MRD risk, weight gain or both.

As mentioned throughout the literature review and in the results sections, there are many factors that contribute to poor outcome or, in the case of this study, specifically MRD risk as we are dealing with induction therapy and this is the first milestone once treatment has commenced that will dictate the later, longer phases of treatment (Jastaniah et al., 2018). It is also important to point out that this study is the first comprehensive investigation of circulating adipokine expression in ALL. We have tested 58 adipokines at diagnosis and after induction treatment which will shed light on adipokines associated with MRD and weight gain at this critical early stage of treatment. This will be the first study that has identified particular adipokines which are linked to MRD risk.

This study also supports evidence from previous observations linked to adipokines associated with obesity which change in ALL (Siviero-Miachon et al., 2020). Our data shows expected patterns in studied adipokines associated with ALL and also with obesity; adiponectin is decreased overall in our patients which confirms findings in a previous study (El-Baz et al., 2013b), leptin and resistin increased which has been shown previously (Shahramian et al., 2016) (El-Baz et al., 2013b). Work by Lu et al demonstrated that fasting patients during treatment improves outcomes and have suggested that this is due to decreased leptin levels circulating in the blood (Lu et al., 2017). Whilst this is a valid conclusion and leptin may be a

good candidate to target in cancer treatment and ALL, targeting one adipokine may not be the ideal course of action as it is more likely to be a range of adipokines activating a number of downstream cellular pathways that contributes to MRD risk and/or weight gain . The next section discusses the overall trends in adipokine expression between weight gain and MRD risk patients.

4.10.1 Patient adipokine summary

In this chapter we have begun to unravel the adipokine profiles for the patients who became obese or overweight at the end of induction, also those who are MRD risk or both, this is summarised for all patients in figure 4.11. We have shown what a typical profile is for patients who gain weight, but what are the general patterns and trends and what could this mean for the patients risk during induction and beyond into consolidation treatment. As discussed previously in the introduction obesity is significantly linked to MRD risk in the orgel study (Orgel et al., 2014b). Being obese increases risk throughout treatment and beyond after successful treatment due to late effects and decreased overall survival. We have observed the opposite in this study with reduced weight at the end of induction linked to MRD risk, does this mean that an obese adipokine profile does not contribute to worse outcomes ? and what can it tell us about obesity and ALL. Higher leptin levels in obese children correlate with higher insulin levels in the blood (Tahir et al., 2014). Increased leptin is also indicative of insulin resistance and metabolic syndrome (Chu et al., 2002) and higher blood sugar levels in obese patients with ALL compared to non-obese patients (Zareifar et al., 2015). There are other indicators of obesity, insulin resistance and metabolic syndrome in the adipokine profiles of weight gain patients. Endocan is significantly increased in our weight gain patients and in a study of ALL survivors their lipid profiles and post prandial glucose levels were significantly increased, the study concluded that the survivors were at risk of developing premature atherosclerosis (Sherief et al., 2021). Low adiponectin is inversely correlated with obesity in children (Nascimento et al., 2014) and also linked to insulin resistance (Körner et al., 2007). Higher circulating adiponectin has an insulin sensitising affect (Calcaterra et al., 2009). This can improve insulin resistance and in turn lower blood sugar then insulin levels which further reduce IGF1 levels which both feed cancer cell growth (Biondani and Peyron, 2018). This one of the mechanisms suggested behind the use of the drug metformin in cancer

and leukaemia the other mechanism is discussed in the introduction as an AMPK activator. Further indicators of obesity markers and insulin resistance shown in the obese profiles are increased levels myeloperoxidase (Qaddoumi et al., 2020), increased angiopoietin like-3 (Arab Sadeghabadi et al., 2021), raised resistin (Simões et al., 2018) and Lipocalin-2 levels (Yan et al., 2007), all have been shown to be raised in obesity and positively correlate with insulin resistance and low grade inflammation. Another factor that contributes to insulin resistance are glucocorticoids, although steroids are an important part of treatment protocols at present, they do contribute to rapid weight gain and may be a driver of this phenotype and adipokine profile (Chow et al., 2013). In MRD risk patients on the whole the opposite is observed in adipokines associated with obesity and metabolic syndrome, leptin is reduced, adiponectin is slightly increased but not significant, myeloperoxidase is decreased, angiopoietin like-3 and Lipocalin-2 are slightly increased but not to the level of the weight gain patients and resistin in significantly decreased in MRD risk, this may indicate that MRD risk patients do not have the same risk associated with weight gain and insulin resistance. At this stage in treatment, we may not know the full impact of obesity as this study looked at early treatment and not the whole course of treatment and how being obese adipokine profiles could impact event free survival and overall health later in life. In a recent study which was part of the IDEAL trial, the group identified the PI3K/Akt pathway involved the cellular insulin glucose response which is implicated in chemoresistance. Rather than targeting the pathway directly calorie restriction was introduced in patients which improved MRD risk status in the trial by lowering insulin levels in the blood (Orgel et al., 2021). Calorie restriction and the targeting of insulin pathways in acute lymphoblastic leukemic does show promise in cancer cells and ALL cells. It has been shown that restoring normal glucose metabolism throughout the body can be an effective strategy in treating ALL (Ye et al., 2018). There are differences in markers of inflammation in weight gain compared to MRD risk patients, in weight gain patients the inflammation markers are lower grade more associated with metabolic syndrome. The MRD risk patients have non-significant increased levels of the acute inflammatory markers IL-6 and CRP compared to decreased levels in weight gain patients. This may be an underlying clue to the adipokine profiles of MRD risk patients and what is contributing to increased risk. Infection in acute leukaemia's is common and patients are at an increased risk of mortality, sepsis, bacterial, fungal infections and also febrile neutropenia (Logan et al., 2020). In ALL increased IL-6 is linked with increased levels of infection during

treatment, the following study observed increased levels of IL-6 in newly diagnosed ALL patients with pneumonia (Zhou et al., 2021). Further experiments in the Zhou study using mice and inhibiting the IL-6/STAT3 pathway reduced expression of renal multidrug resistance proteins which lessened the severity disease severity. Raised CRP is associated with poor outcome and bacterial sepsis in children with ALL (Peltola et al., 1983). This is in line with the expression of other chemokines associated with acute phase inflammatory proteins. CCL2 has raised expression in MRD risk patients at day 29 and reduced expression in weight gain patients. CCL2 has been shown to boost ALL cell survival indirectly by supporting the adhesion to BMMSC cells (de Vasconcellos et al., 2011). IL-8 although decreased in both groups of patients was decreased significantly in weight gain patients but not significantly in MRD risk patients, IL-8 in a range of concentrations has been shown to be protective of Molt4 cells in the final chapter of this study. IL-8 is known to activate the PI3K/Akt and JAK/STAT through its signalling pathways (Liu et al., 2016). The immunomodulator TNFα is raised in MRD risk patients and reduced in weight gain patients, this also indicates acute phase inflammation (Page et al., 2018). Visfatin is also increased in MRD risk compared to the weight gain cohort and is implicated in the severity of inflammation (Park et al., 2020). Increased visfatin is also a biomarker of cardiac complications in survivors of ALL (Morel et al., 2020). Unfortunately, we can only speculate that infection and the acute phase inflammatory response could be driving MRD risk status as we do not have the clinical information as part of the study to confirm this, this does warrant further investigation. The cross talk involved in both sets of adipokine profiles is complex and they do have differences, they also have similarities and each set of adipokine profiles can act as modulators or indicators of increased risk. Both can benefit from the targeting of similar pathways to impact ALL cell viability. The next section discusses the adipokines most associated with weight gain and MRD risk.

4.11 Identification of adipokine changes in patients associated with weight gain

4.11.1 Leptin

The results for leptin expression in patients show a significant increase in levels in patients who gain weight within this cohort, in contrast leptin levels are not associated with MRD risk between MRD risk and low risk patients. Linear regression analysis shows positive correlation between start BMI percentile and leptin expression at the start of induction therapy, analysis at the end of induction therapy shows a significant positive correlation between leptin expression and BMI percentile. It is well established that leptin is a known adipokine to positively correlate with body weight (Ekmen et al., 2016), high levels of leptin can be detected in the blood of obese patients (Knight et al., 2010), in studies evaluating long term body composition and increased weight confirmed that patients with a childhood diagnosis of ALL had higher body fat percentage and higher leptin levels compared to control subjects (Jahnukainen et al., 2015). Further studies in ALL have confirmed our findings that leptin is increased in ALL patients who gain weight (Shahramian et al., 2016, Skoczen et al., 2011). Leptin is also significantly increased in our patients who presented obese or overweight at diagnosis compared to patients in the normal weight range, see appendix table 7.2. A major factor in weight gain during induction therapy is the administration of steroid treatment which contributes to significant weight gain and in the following study it was found that dexamethasone was significantly more intense in contributing to increased leptin levels and insulin resistance than prednisolone (Wallace et al., 2003). The impact of steroids occurs early from commencing treatment with markers of insulin resistance increasing after just 4 days of dexamethasone treatment in the following study (Warris et al., 2016). A further study observed that leptin is increased in response to steroids in induction therapy (Tavil et al., 2012). Steroids are an important part of induction therapy but also cause significant side effects one being raised leptin which are known to contribute cancer progression and chemoresistance (Ma et al., 2019).

4.11.2 IL-8

This study has confirmed as significant decrease in IL-8 expression levels in patients who gain weight in our study, further to this there is no significant difference in IL-8 patients expression levels between MRD risk patients and low risk patients after induction therapy. Linear regression analysis displayed a non-significant negative correlation between start BMI percentile and IL-8 expression at the end of induction therapy, analysis at the end of induction therapy shows a non-significant negative correlation between day 29 BMI percentile and IL-8 expression. Studies linking ALL to IL-8 confirm that IL-8 is a proinflammatory adipokine that is significantly higher in ALL patients at diagnosis compared to a control group without disease and in the absence of infection (Pérez-Figueroa et al., 2016), similar to our findings this study did not link IL-8 to MRD risk. The reduced levels seen in weight gain patients at day 29 may be an indication of the protective factor of having some weight gain as suggested from the findings of this study, also a positive outcome of chemotherapy treatment on the disease process. In the following study early reduction in IL-8 levels from chemotherapy treatments predicted a positive outcome in melanoma and non-small-cell lung cancer patients (Sanmamed et al., 2017), the same was observed in breast cancer (Tiainen et al., 2019).

4.11.3 Endocan

Analysis of endocan expression data has confirmed that endocan is significantly increased within the cohort of the patients who gain weight. In addition to this there is a small but non-significant increase in the patients who were MRD risk at the end of induction compared to low risk patients. At the beginning of treatment, there is a slight non-significant negative correlation in endocan expression and BMI percentile, by the end of induction therapy this changes to a slight positive correlation in endocan expression and BMI percentile. Endocan expression is known to be increased in obesity at higher levels (Klisić et al., 2021) which would explain the significant increase in patients who gain weight, some weight gain has also shown to be protective during induction therapy in this study. There is also a slight increase in endocan expression levels in patients who are MRD risk compared to low risk patients, a possible explanation for this could be the known link to acute leukaemia's and higher circulating endocan levels which have been directly implicated to sepsis and poorer outcome

(Kiral et al., 2016). Endocan levels are negatively correlated with BMI percentile and are lower at the start of induction therapy which could add to risk at diagnosis with higher levels, this changes to a slight positive correlation by the end of induction therapy which may indicate a better outcome, endocan levels returning to normal levels can be explained by the impact of chemotherapy actually improving outcome and correcting levels leading to a positive outcome as at relapse levels can increase again (Xu et al., 2014b).

4.11.4 Pref-1/DLK1

This study has confirmed a significant increase in pref-1 expression in our group of patients who gain weight by the end of induction therapy and a non-significant decrease in patients that are MRD risk compared to low risk patients by the end of induction therapy. At the start of induction therapy results show a slight positive correlation with start BMI percentile, this changes to a significant positive correlation with BMI percentile at the end of induction therapy. To our knowledge this is the first study showing that pref-1 is increased at the end of induction therapy in a group a patients who gain weight. Higher levels of pref-1 are linked to inflammation, the opposite is observed in lower expression levels were obese individuals has an improved inflammatory profile (O'Connell et al., 2011). This finding could be an indicator that adipogenesis is being impacted negatively in the weight gain group and the subsequent higher circulating pref-1 could be contributing to disease progression through activation of the NOTCH receptor as shown in the following study (Wei et al., 2013). This may not be a strong factor driving disease in induction therapy but may change later in treatment. The higher levels of pref-1 could also be a direct result of chemotherapy treatment and reduced adipogenesis, results in chapter 3, figures 3.7 and 3.8 show that treatment with vincristine and daunorubicin raised levels of pref-1 in our 3T3-L1 mouse model which would be an indicator of reduced adipogenesis. We did not perform further western blot studies to confirm activation of downstream pathways for pref-1.

4.11.5 Adiponectin

Patients adiponectin expression levels during induction therapy has confirmed a significant decrease in our group of patient who gain weight compared to patients who stay within the normal weight range or lose weight, there is no change in expression between the MRD risk

and low risk group of patients. Linear regression analysis shows small positive correlation between start BMI and Adiponectin expression at the start of induction, there is no correlation between adiponectin expression and BMI percentile at the end of induction. The reduction in adiponectin levels observed in the results of this study fit in with other studies where reduced levels are associated and inversely correlated with ALL and also obesity (El-Baz et al., 2013a, Ma et al., 2016, Srivastava et al., 2015a). The targeting of the adiponectin downstream pathways have become a novel target in treating a range of disorders and obesity related conditions and cancer (Nigro et al., 2018, Yu et al., 2019b). Promising novel treatment strategies include the adiponectin agonists ADP355 and AdipoRon which have similar effects on the downstream pathway's AMPK and PPAR-α (Okada-Iwabu et al., 2013, Otvos et al., 2015). In ALL more recent research is investigating the role of the mTOR pathway by developing anti-mTOR compounds which would counteract the effects of low adiponectin (Simioni et al., 2019). More recently AICAr which is an AMPK activator has been used to raise AMPK levels in cells which adiponectin would target has led to cell cycle arrest and apoptosis in ALL cells (Du et al., 2019). The most promising candidate drug which would mimic the actions of adiponectin and has been studied for usage in ALL alongside current chemotherapy is metformin an AMPK activator and anti-diabetic drug (Biondani and Peyron, 2018). This type of repurposing of drugs with known good safety profiles would be good options for paediatric cancers by raising AMPK levels and supressing the PI3K/Akt/mTOR pathway and the PPARα and NF-kβ pathways would be promising ways of counteracting low adiponectin levels in ALL.

4.12 Identification of adipokine changes in patients associated with MRD risk

4.12.1 Resistin

The results for resistin expression in patients has confirmed a significant decrease in patients who are MRD risk at the end of induction therapy compared to low risk patients, in contrast to this there is non-significant increase in resistin expression in patients who gain weight. Resistin expression at the start of induction therapy shows a non-significant positive corelation with BMI percentile, this is a similar finding to resistin expression and BMI percentile at the end of induction therapy. Resistin has been implicated in pro inflammatory conditions and cardiometabolic disorders and positively correlated with increasing BMI, in the

following study this was confirmed in obese adolescents where circulating resistin levels and childhood obesity related conditions were associated (Makni et al., 2013). In childhood ALL a study confirmed that resistin levels in children were similar in obese and non-obese individuals (Srivastava et al., 2015a), this study has confirmed a similar finding, when comparing resistin levels at diagnosis between patients who present as overweight or obese and normal weight there is no difference in resistin expression, p= 0.9982, see appendix table 7.2. A study also confirmed our finding that resistin levels were higher at diagnosis and that resistin levels may serve as a diagnostic biomarker to detect disease relapse (El-Baz et al., 2013a). A possible explanation is that low resistin levels are associated with MRD risk and that high resistin levels are protective, a protective role for resistin was found in the following breast cancer study (Georgiou et al., 2016). This may or may not be the case with ALL, in this study comparing resistin expression levels at diagnosis in those patients who go onto to become MRD risk at day 29 show raised levels of resistin compared to low risk patients although not significant, p=0.2315, see appendix table 7.3. A different explanation is that lower resistin levels in MRD risk patients at day 29 which were higher at diagnosis may be due to the impact of chemotherapy on reducing levels, this was shown in the following study (Moschovi et al., 2010a). Reduced levels of resistin were also present in the 3T3-L1 cells treated with vincristine in this study, see figure 3.7, this could be linked to the positive impact of chemotherapy in restoring normal levels of adipokine secretions in the case of resistin.

4.12.2 SerpinA12 (Vaspin)

This study has confirmed a significant increase in serpinA12 in our patients who are MRD risk at the end of induction therapy in contrast there is a small non-significant decrease in our group of patients who gain weight. Linear regression analysis of start and end BMI and expression levels of SerpinA12 show no correlation with start BMI and expression levels at induction and a non-significant negative correlation with day 29 BMI and expression of serpinA12. This is the first time to our knowledge that serpinA12 has been associated with MRD risk in ALL. SerpinA12 data is lacking in ALL but more is known about its target receptor the heat shock protein GRP78 which is involved in the unfolded protein response (Uckun et al., 2011) activation of this receptor targets the downstream pathway PI3K/Akt (Wey et al., 2012). Serpin A12 promotes insulin sensitivity (Nicholson et al., 2019) and also positively

corelates with increasing BMI (Heiker, 2014). These findings differ in our patient results where it is decreased in expression in our weight gain patients and there is a negative correlation with end BMI. Low serum serpinA12 levels are associated with weight loss which could explain the negative correlation with BMI (Teshigawara et al., 2012).

4.12.3 Cathepsin-S

This study has confirmed that cathepsin-S is significantly associated with MRD risk and compared to low risk patients' levels are reduced after induction therapy. Cathepsin-S data is very limited in ALL research and this is the first time that this adipokine has been associated with MRD risk in ALL. The data linking cathepsin-S and our group of patients who gain weight has shown the opposite pattern where it is increased in expression compared to patients who lose weight or stay in the normal weight category. Linear regression analysis used to measure correlations between start and end BMI and cathepsin-S at the beginning of induction therapy and the end both show positive correlations with end BMI and cathepsin-S expression being significant. With limited information regarding cathepsin-s and ALL it is difficult to explain these findings in the context of ALL with no information regarding lower levels of cathepsin-S and poor outcomes in acute leukaemia's that would explain this finding. An explanation may be linked to cathepsin-S and its strong correlation with increasing BMI (Taleb and Clement, 2007), our results show increases in weight gain and as MRD risk in this study is linked to reduced weight this may explain this opposite finding, therefore lower levels in ALL are associated with increased risk at the end of induction therapy.

4.12.4 IGFBP-4

Results for IGFBP-4 a non-significant reduction in expression of IGFBP-4 in MRD risk patients compared to low risk patients, results between patients who gain weight compared to patients who lose weight or stay in the normal weight category showed a very small non-significant increase. Linear regression analysis for start and end BMI and IGFBP-4 expression displayed positive non-significant correlations with weight gain. The lower expression levels were observed in another study after induction therapy in ALL (Wex et al., 2005) although this group did not link this finding to MRD risk. IGFBP-4 has a protective role against cancer cell proliferation when expressed at higher levels, it achieves this by keeping the levels of

proliferative insulin growth factor-1 in check (Ryan et al., 2004), lower levels in MRD risk may lose this protective effect. The opposite is observed in weight gain patients and the positive correlations with start and end BMI show the reverse and have a protective effect as shown with increased weight in this study.

4.12.5 Angiopoietin-2

The results for angiopoietin-2 and MRD confirm lower levels in MRD patients compared to low risk patients, this reduction is not significant, this finding was observed in a study in childhood ALL patients but it was not associated with MRD after initial induction therapy (Karakurt et al., 2016) the opposite is observed in weight gain with a non-significant increase in weight gain compared to patients who lost weight or stayed within the normal weight range. Both start and end BMI positively correlated with increased angiopoietin-2 expression levels, end BMI was a significant correlation. Raised levels of angiopoietin-2 levels have been patients had insulin resistance and endothelial dysfunction (Gozal et al., 2017).. This study has confirmed that angiopoietin-2 is significantly higher in patients who present as obese or overweight at diagnosis compared to normal weight individuals, none of these patients went onto be MRD risk, a possible explanation for this could be the slightly raised levels may be protective at antagonising angiopoietin-1 levels, see appendix table 7.3. The next chapter involves the studies in vitro work and examines the impact of chosen adipokines on Molt4 cell viability and the cellular pathways activated.

5.0 CHAPTER 5: Adipokine in vitro work

The following chapter covers the in vitro work completed for the study. Due to the study design patient samples were collected throughout the period of the PhD placement, rather than analysing the patients data first to identify significant adipokines and then completing the invitro work. To begin to identify the adipokines of interest the patient data was analysed when patient 040 was processed which identified the following five adipokines which warranted further experimental work. This explains why the five chosen from the two patient data sets are different from the actual significant adipokines when analysing the whole data set (up to patient 057). At the time Angiopoietin-2 was significant and after the full analysis the significance had changed but it was still in the top five for MRD risk. The following adipokines were chosen out of the most significant to incubate with the Molt4 cell line using trypan blue exclusion assays, which are known to activate shared pathways.

5.1 Leptin has a protective effect on Molt4 cells and increases cell viability

In this study we wanted to examine the effects of leptin on the ALL cell line Molt4 and activation of the OB-Rb receptor and subsequently the activation of the PI3K/Akt pathway by using trypan blue exclusion assays and western blotting for pAKT (S473). Leptin exerts its effects on cancer cells by stimulating a number of pathways including STAT3 and PI3K/Akt which boosts cell growth and proliferation (Kumar et al., 2017) (Achkar et al., 2019). The pathways are activated by circulating leptin binding to its receptor OB-Rb (Tartaglia et al., 1995). Molt4 cells were cultured over a five day period in 0.3%FBS and incubated with increasing concentrations of leptin. Cells were first incubated for 24 hours in reduced 0.3% FBS concentrated media with no additions, after 24 hours cells were treated with 5ng/mL, 50ng/mL and 500ng/ml and a control with no added leptin. After a further 24 hours cells were collected and proteins extracted for western blotting, cells were then counted again on day 4 and 5. To count the cells trypan blue staining was used to count live cells and to count live and dead cells to calculate %viability over the five day period, the results are shown in figures 5.1 a-d. Figure 5.1a shows the trypan blue exclusion assay results, We can see from figure a that from days 0 to 2 the cell counts show little variability between leptin concentrations, by

day 3 and 4 the control well results can be seen decreasing and the higher concentrations of leptin are increasing. Here by day 4 there are significant differences between the control and 50ng/mL of leptin, p=0.0255 n=3, there is also differences between the control and 500ng/mL of Leptin although not significant p=0.0531. there is no significant difference between the control and 5ng/mL of leptin and the control, p=0.5054. Figure b shows the same results for % cell viability counts. These graphs display similarities for all the leptin concentrations compared to the control, by day 3 the control % viability can be seen to decrease slightly compared to the other concentrations and by day 4 there are significant differences. Figure 5.1b confirms the differences demonstrating an increase %viability in the cells treated with 50ng/mL compared to the control, p=0.0003, n=3 and also in the cells treated with 500ng/mL of leptin compared to the control, p=0.0005, n=3. The difference between the cells treated with 5ng/mL and the control were not significant, p=0.2612, n=3. Figure 5.1c and d show the western blot results for the cells extracted on day 2 of the trypan blue exclusion assay for cells incubated with increasing concentrations of leptin. Graph 5.1c shows that leptin activates the PI3K/akt pathway and this is confirmed by increased level of phosphorylation of the pAKT (S473) protein. Activation of the pathway is dose dependant on leptin concentration, cells incubated with 500ng/mL showed a significant difference compared to the control cells, p=0.0373. Cells incubated with 50ng/mL displayed an increase in phosphorylation, but this was not significant p=0.2496, cells incubated with 5ng/mL showed a smaller non-significant increase p=0.6097, n=3. Figure d has images of the western blots, similar results were observed from the three separate experiments. To calculate the relative band intensity pAKT and AKT were both normalised to their respective GAPDH internal controls for each concentration and control then these figures were divided by each other to obtain the relative band intensity and the proportion of the AKT protein which is activated or phosphorylated. Appendix figures 7.4a and b show the trypan blue exclusion assay results for live cell counts over the five day period of the experiment, cell count $x10^4$ plotted against day of incubation.

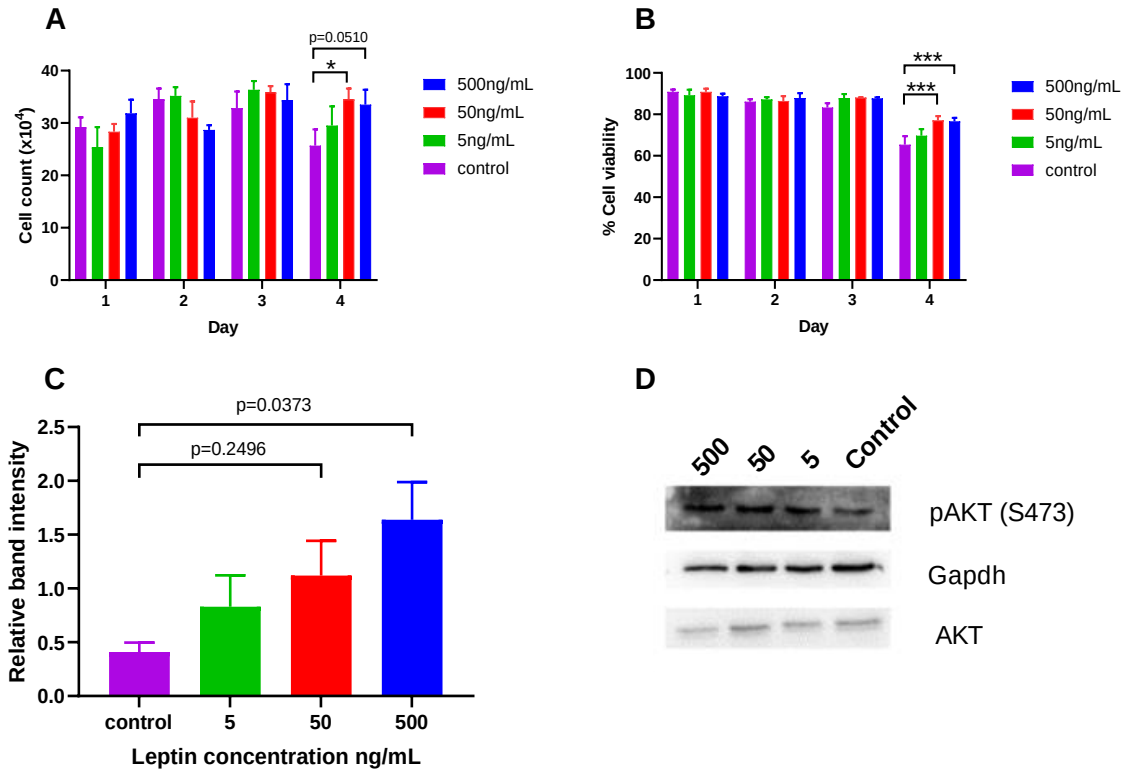

Figure 5.1: A-D, Leptin stimulates Molt4 ALL cancer cell viability by activating the PI3K/Akt pathway. Molt4 cells were incubated for 5 days in increasing concentrations of leptin. A, Trypan blue live cell counts, day 4 shows significant differences between 50ng/mL and control, p=0.0255, also 500ng/mL compared to control is increased, p=0.0531 n=3. Figure B, % viability results, day 4 showed significant results for 500ng/mL and 50ng/mL compared to control, p=0.0005 and p=0.0003 respectively, n=3. Figure C, western blot results, leptin activates the PI3K/Akt pathway compared to control in a dose dependant manner, 500ng/mL increased phosphorylation significantly compared to control, p=0.0373, n=3. D, western blot images, error bars shown as standard error.

5.2 Leptin has no impact on cell viability in the presence of Vincristine or Daunorubicin

Studies have confirmed that adipokines have the capabilities to change chemotherapy sensitivity. Leptin in other studies in cancer decreased the sensitivity of chemotherapy drugs (Gu et al., 2019a). It has been shown in this study that leptin levels are significantly increased in patients who gain weight or become overweight or obese during induction therapy. To test if leptin interferes with the chemosensitivity of ALL chemotherapy drugs we used Molt4 cells incubated with or without leptin at a concentration of 50ng/mL and observed any changes in cell viability when increasing concentrations of daunorubicin (0.781nM to 200nM) and

vincristine (0.079nM to 20nM) were added using an MTS assay, see methods section 2.10. Wells with daunorubicin alone and daunorubicin with leptin were compared, results are displayed in figure 5.2. Two way ANNOVA was used to analyse the changes in cell viability with a Sidak's multiple comparisons test to compare each concentration of drug alone to the drug incubated with leptin. There were no notable differences in cell viability between daunorubicin alone, highlighted in green and daunorubicin with leptin, highlighted in red in Molt4 cells. The same experiment was performed in the presence of vincristine, see figure 5.3. There were no changes in cell viability between cells incubated with vincristine alone, highlighted in green and vincristine and leptin, highlighted in red.

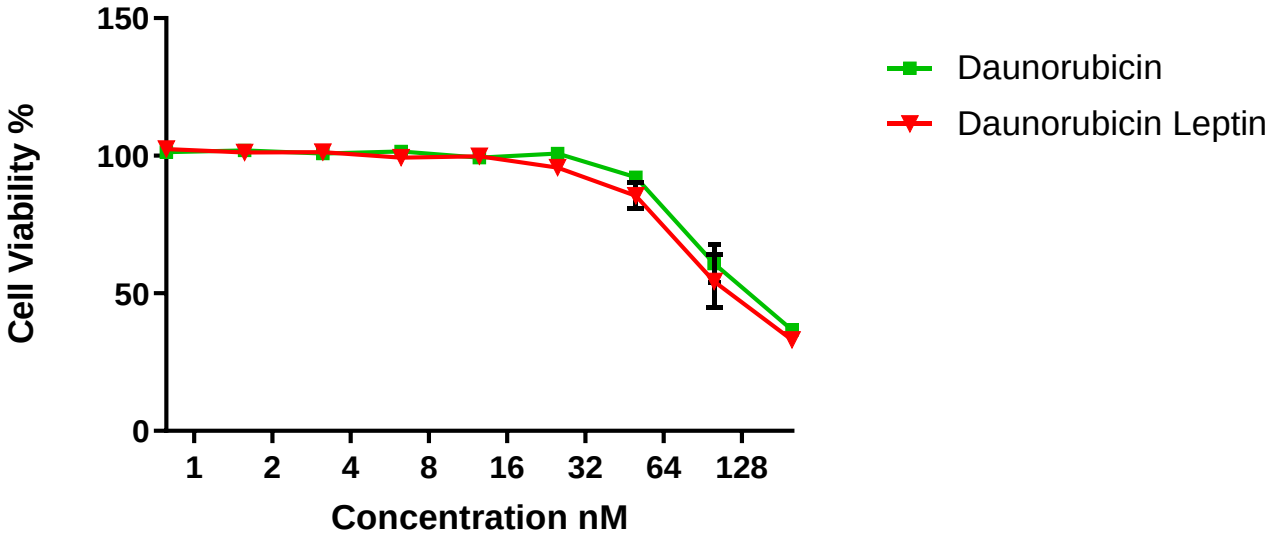

Figure 5.2: MTS chemosensitivity results for daunorubicin incubated with leptin. Molt4 cells incubated with or without leptin at a concentration of 50ng/mL and increasing concentrations of daunorubicin 0.78nM to 200nM. 2-ANNOVA was used to analyse the changes in cell viability, there were no significant changes in chemosensivity at any of the daunorubicin concentrations, n=3

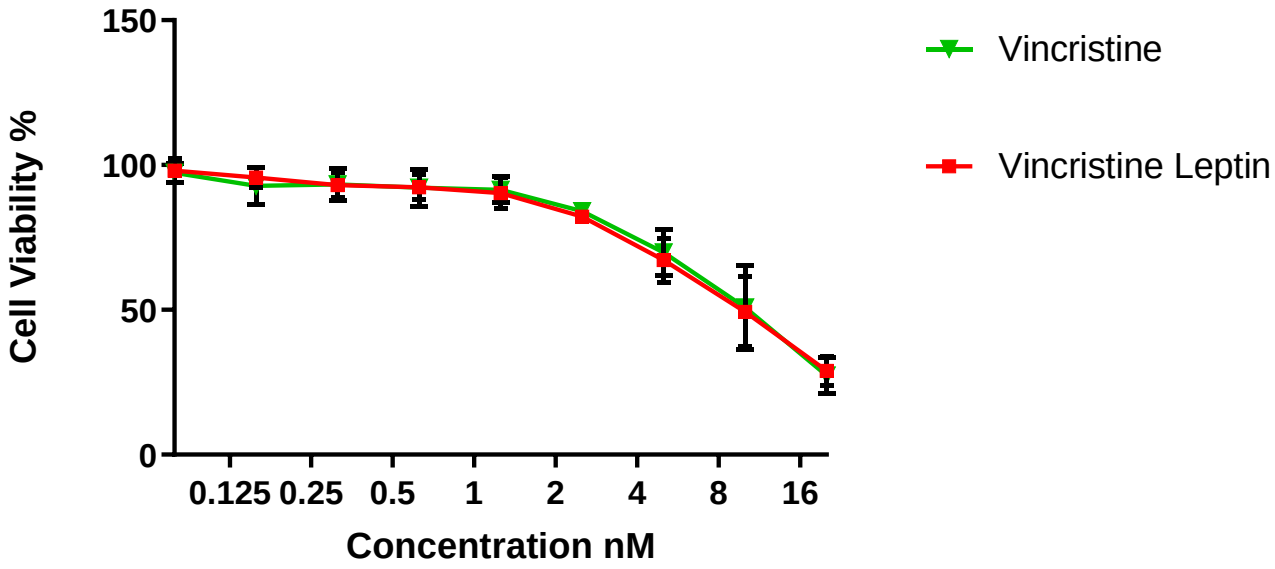

Figure 5.3: MTS chemosensitivity results for vincristine incubated with leptin. Molt4 cells incubated with or without leptin at a concentration of 50ng/mL and increasing concentrations of vincristine 0.078nM to 20nM. 2-ANNOVA was used to analyse the changes in cell viability, there were no significant changes in chemosensivity at any of the vincristine concentrations, n=3

5.3 IL-8 has a protective effect on Molt4 cells and increases cell viability

Molt4 cells were cultured over a five day period in 0.3% FBS and incubated with increasing concentrations of IL-8. It has been shown that IL-8 acts through the receptors CXCR1/2 to activate a number of downstream pathways including PI3K/Akt and JAK/STAT3 in cancer (Liu et al., 2016). Cells were first incubated for 24 hours in reduced 0.3% FBS concentrated media with no additions, after 24 hours cells were treated with 5ng/mL, 50ng/mL and 500ng/ml and a control with no added IL-8. After a further 24 hours, cells were collected and proteins extracted for western blotting on the basis that further testing could confirm pathways that are activated in the Molt4 cells incubated in IL-8, cells were then counted again on day 4 and 5. To count the cells trypan blue staining was used to count live cells and also to count live and dead cells to calculate %viability over the five day period, the results are shown in figures 5.4 a and b . Appendix figures 7.41a and b show the trypan blue exclusion assay results for live cell counts over the five day period of the experiment, cell count $x10^4$ plotted against day of incubation. We can see from figure 5.4a that there is variability between IL-8

concentrations, with no significant differences by day 4 in actual cell counts between the control and the treated cells. Figure b shows the results for % cell viability counts. These results do show significant differences in % viability on day 3 and 4 of the treatment. On day 3 the control % viability can be seen to drop slightly compared to the other concentrations and by day 4 there are significant differences. The changes can be seen as increased %viability in the cells treated with 5ng/mL and the control, p=0.0069, 50ng/mL of IL-8 compared to the control, p=0.0004 and also in the cells treated with 500ng/mL of IL-8 compared to the control, p=<0.0001, n=3. These results differ to leptin concentrations as even at the smaller concentration of 5ng/mL of IL-8 significant differences are displayed compared to the control.

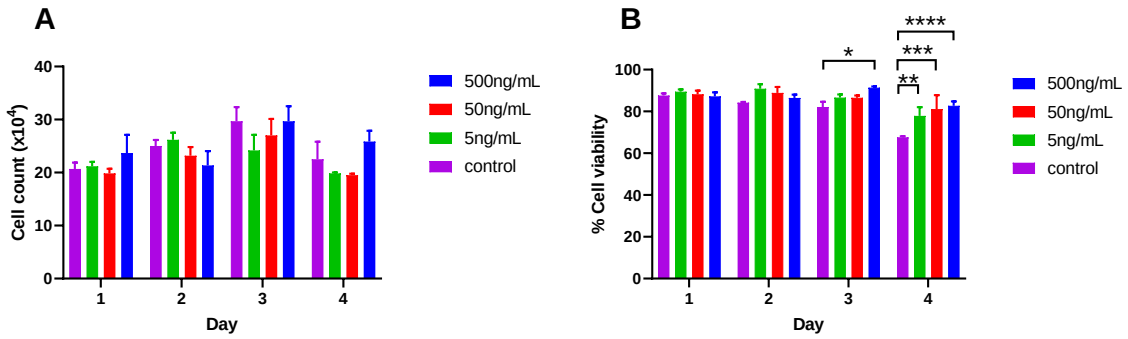

Figure 5.4: IL-8 stimulates Molt4 ALL cancer cell viability. Molt4 cells were incubated for 5 days in increasing concentrations of IL-8. A, Trypan blue live cell counts, there are no significant differences between the cells treated with IL-8 and control cells on any day, n=3. Figure B, % viability results for day 3 showed significant results for 500ng/mL and the control, p=0.0162, day 4 showed significant results for 500ng/mL, 50ng/mL, 5ng/mL and the control, p=<0.0001, p=0.0004 and p=0.0069 respectively, n=3, error bars shown as standard error.

5.4 Resistin has no impact on Molt4 cell growth and viability

In this study we wanted to examine the effects of resistin on the ALL cell line Molt4 by using trypan blue exclusion assays to count live cell numbers and also calculate percentage viability by counting live and non-viable cells. In other studies high not low levels of resistin are associated with increased risk in ALL patients (El-Baz et al., 2013b). To determine if any concentrations of resistin impacted cell viability Molt4 cells were cultured over a five day period in 0.3% FBS and incubated with increasing concentrations of resistin. Cells were first incubated for 24 hours in reduced 0.3% FBS concentrated media with no additions, after 24 hours cells were treated with 5ng/mL, 50ng/mL and 500ng/ml of resistin and a control. After

a further 24 hours cells were collected and proteins extracted for western blotting, cells were then counted again on day 4 and 5. To count the cells trypan blue staining was used to count live cells and also to count live and dead cells to calculate %viability over the five day period, the results are shown in figures 5.5a and b. Appendix figures 7.42a and b show the trypan blue exclusion assay results for live cell counts over the five day period of the experiment, cell count x10^4 plotted against day of incubation. We can see from figure 5.5a that from days 0 to 2 the cell counts show little variability between resistin concentrations, by day 3 and 4 there is more variability with no differences between the treated and the control wells. By day 4 there are no significant differences between any of the resistin concentrations and the control well. Figure b shows the same results for % cell viability counts. The bar chart in figure 5.5b confirms there are no significant differences between the wells treated with resistin and the control well, there is a non-significant increase in the 500ng/mL of resistin compared to the control, p=0.1512, n=3.

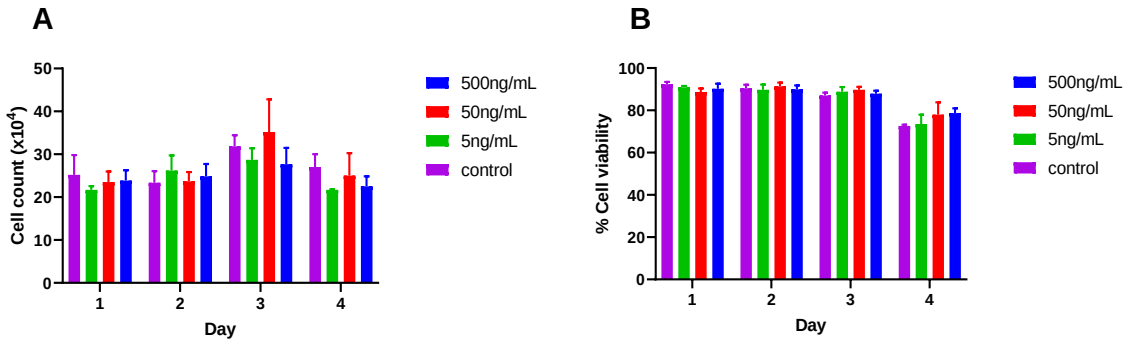

Figure 5.5: Resistin has minimal impact on Molt4 cell growth and viability. Molt4 cells were incubated for 5 days in increasing concentrations of resistin. A, Trypan blue live cell counts, there are no significant differences between the cells treated with resistin and control cells on any day, n=3. Figure B, % viability results, there are no significant differences between the cells treated with resistin and control cells on any day although there was a small non-significant increase in the results for days 4: 500ng/mL and the control, p=0.1512, n=3, error bars shown as standard error.

5.5 Serpin A12 has a protective effect on Molt4 cells and increases cell viability

Results indicate serpin A12 is significantly associated with MRD risk in this study. Information on serpin12 is lacking in ALL although information on its target receptor GPR78 has been explored. (Uckun et al., 2011) highlighted that GRP78 which is an important component of

the unfolded protein response (UPR) signalling network and was abundantly expressed in relapsed B ALL. Studies in liver cancer confirmed the protective activity of serpinA12 at concentrations of 5ng/mL on Hep-3B cells alongside this there was a reduction of reactive oxygen species and NO (Skonieczna et al., 2019). Further to this knockdown of the GRP78 receptor in AML cells lines suppressed the proliferative effects of the PI3K/Akt pathway (Wey et al., 2012). Molt4 cells were cultured over a five day period in 0.3%FBS and incubated with increasing concentrations of serpin A12. Cells were first incubated for 24 hours in reduced 0.3% FBS concentrated media with no additions, after 24 hours cells were treated with 5ng/mL, 50ng/mL and 500ng/ml and a control with no added serpin A12, the results are shown in figures 5.6a and b. Appendix figures 7.43a and b show the trypan blue exclusion assay results for live cell counts over the five day period of the experiment, cell count $x10^4$ plotted against day of incubation. We can see from figure 5.6a that from days 1 to 4 the cell counts show little variability between serpin A12 concentrations, by day 3 and 4 the control well results can be seen decreasing and the higher concentrations of serpin A12 are increasing but this is not significant. Figures 5.5b are results for % cell viability counts, these do have significant differences in % cell viability at day 3 and 4. There is increased %viability in the cells treated with 500 ng/mL, 50ng/mL and 5ng/mL at day 3 compared to the control, p=0.0145, p=0.0111 and p=0.0277 respectively. Day 4 showed significant results for 500ng/mL and the control, p=0.0001, 50ng/mL displayed an increase but not significant p=0.0550 and 5ng/mL to control showed a significant increase p= 0.0009, n=3, error bars shown as standard error.

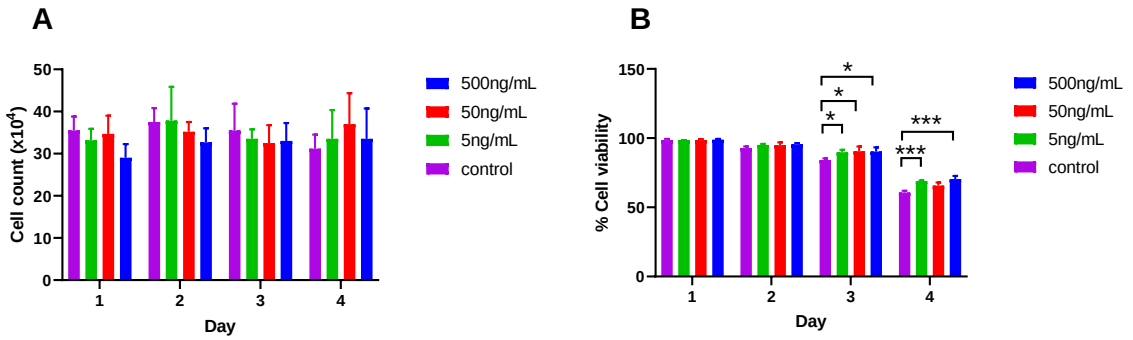

Figure 5.6: Serpin A12 stimulates Molt4 cell viability. Molt4 cells were incubated for 5 days in increasing concentrations of serpin A12. A, Trypan blue live cell counts, there are no significant differences between the cells treated with serpin A12, n=3. Figure B, % viability results for day 3 showed significant results for 500ng/mL, 50ng/mL, 5ng/mL treated with serpin A12 and the control, p=0.0145, p=0.0111 and p=0.0277 respectively, day 4 showed significant results for 500ng/mL and the control, p=0.0001, 50ng/mL displayed an increase but not significant p=0.0550 and 5ng/mL to control showed a significant increase p= 0.0009, n=3, error bars shown as standard error.

5.6 Angiopoietin-2 has a protective effect on Molt4 cells at high expression levels and increases cell viability

Angiopoietin-2 acts as a partial agonist and antagonist for angiopoitin-1 in the Tie2 signalling pathways which activates the downstream PI3K/Akt and has been shown to be lowered in this study. To examine the effects of angiopoietin-2 on the ALL cell line Molt4 by using trypan blue exclusion assays to count live cells and also percentage viability. Molt4 cells were cultured over a five day period in 0.3%FBS and incubated with increasing concentrations of angiopoietin-2. Cells were first incubated for 24 hours in reduced 0.3% FBS concentrated media with no additions, after 24 hours cells were treated with 5ng/mL, 50ng/mL and 500ng/ml of Angiopoietin-2 and a control with no added angiopoietin-2. After a further 24 hours cells were collected and proteins extracted for western blotting, cells were then counted again on day 4 and 5. To count the cells trypan blue staining was used to count live cells and also to count live and dead cells to calculate %viability over the five day period, the results are shown in figures 5.7a and b. Appendix figures 7.44a and b show the trypan blue exclusion assay results for live cell counts over the five day period of the experiment, cell count x10^4 plotted against day of incubation. Over the course of the five day experiment there are no significant differences in live cell numbers in the treated cells compared to the control,

see figure 5.7ba. Figure b shows the same results for % cell viability counts, there are no differences in percentage viability up until day 4, by day 4 there is a significant difference in % viability between the cells in the control well and the cells treated with 500ng/mL of angiopoietin-2, p=0.0044, n=3.

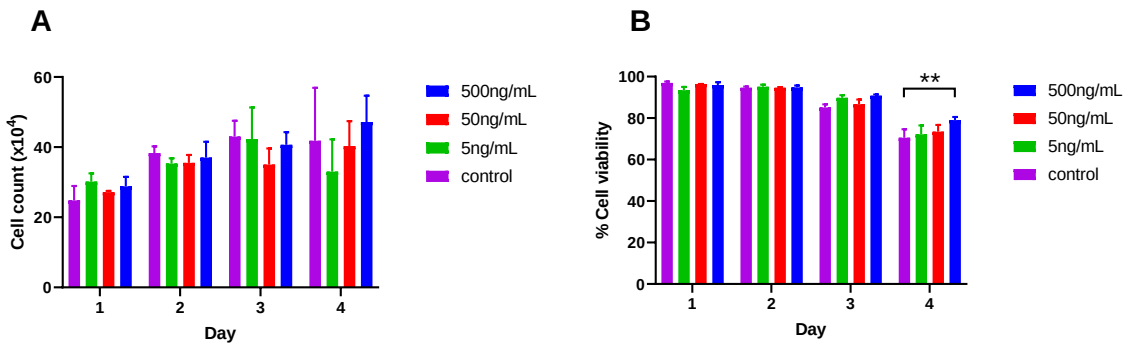

Figure 5.7: Angiopoietin-2 increases Molt4 cell viability. Molt4 cells were incubated for 5 days in increasing concentrations of angiopoietin-2. A, Trypan blue live cell counts, there are no significant differences between the cells treated with angiopoietin-2 and control cells on any day, Figure D, % viability results, day 4 showed significant results for 500ng/mL and the control, p=0.0044, n=3, error bars shown as standard error.

5.7 CHAPTER 5 DISCUSSION

5.7.1 Identification of adipokine associated with changes in cell viability

The analyses of the adipokine expression data from patients during induction therapy has identified a group of significant adipokines that are associated MRD risk with weight gain at the end of induction therapy compared to patients who stay within the normal weight range or gain less than 5% of their starting body weight or lose weight by the end of induction treatment. To our knowledge this is the first study that has identified a large group of adipokines that are associated with increased risk using these parameters at this early stage in treatment. There were significant changes in the following adipokines in relation to weight gain at day 29; leptin, IL-8, endocan, pref-1 and adiponectin, none of these adipokines were associated with MRD risk. Adipokines linked to MRD risk were resistin, serpinA12, cathepsin-S and Angiopoietin-2. The following sections discuss each adipokine tested using the trypan

blue assay, these are leptin ad IL-8 for weight gain and resistin, serpinA12 and Angiopoietin-2 linked to MRD risk.

5.7.2 Leptin

The results for the trypan blue exclusion assays have shown that leptin is having an impact on cell viability by increasing this at day 4 of the experiments. In this case it seems that leptin has a protective effect on Molt4 cells towards the end of the experiments and is possibly boosting survival in the presence of decreased nutrients in the cell media, or starvation conditions. In another study leptin was able to boost survival of ovarian cancer cells in serum starved conditions by activation of the JAK/STAT, PI3K/Akt and MEK/ERK1/2 pathways, this stimulated the expression of Mcl-1 and cyclin D1 exerting antiapoptotic effects (Chen et al., 2013a). A study on the Molt3 cell line it was confirmed that leptin increased cell viability by upregulation of the GLUT1 receptor which enhanced glucose signalling (Han et al., 2018). In this set of experiments, we confirm that leptin is able to activate the PI3K/Akt pathway and this may be contributing to the increased cell viability in our trypan blue exclusion assay experiments. It is likely that there are other mechanisms involved in this finding such as activation of other pathways shown to be involved in cell proliferation as described in the above ovarian cancer study and other cancers (Liu et al., 2011). The final set of experiments looked at leptins ability to change chemosensitivity of daunorubicin and vincristine using an MTS assay. Leptin is known to change chemosensitivity in other cancers but here there was no change when Molt4 cells were treated with increasing levels of daunorubicin or vincristine in the presence of absence of 50ng/mL of leptin. A possible explanation for this may be that there wasn't enough leptin present in the set up to have an impact on cell viability in the presence of the chemotherapy drugs. Another explanation could be that leptin does not work on its own to change chemosensitivity in ALL and it is a number of adipokines that work together to do this. Leptin is well studied in cancer and in ALL, in recent studies fasting has been used to control leptin physiology which upregulated leptin receptors throughout the body which in turn reduced leptin levels in circulation and had a positive impact on reducing disease burden in a mouse model (Lu et al., 2017). In addition, fasting may be beneficial to a positive outcome due to lower available nutrients in supply to fuel ALL cells and not just due to reduced leptin levels, in summary leptin does have an important role to play in the

development of ALL in particular patients who gain weight and are MRD risk but it is unlikely to be acting alone in its contribution to the disease process.

5.7.3 IL-8

The results from the IL-8 trypan blue exclusion assay confirm that IL-8 has the ability to protect Molt4 cells and increase their viability in starvation conditions at day 3 and 4 of the experiment. IL-8 is protective of Molt4 cells at low and high doses so having low concentrations at day 29 may still be protective of ALL cells in a patient regardless of their weight status. IL-8 may be an adipokine that can directly drive the disease at low and high concentrations in circulation further into treatment. A study by (AA) confirms that in healthy children IL-8 would not be in the circulation so it being at low or high concentrations is a marker of risk and inflammation in paediatric disease. Increased IL-8 levels are linked to poorer outcomes in other cancers by activation of downstream pathways PI3K/Akt and JAK/STAT3 (Liu et al., 2016). IL-8 may be an adipokine that in the circulation increases risk in weight gain and MRD patients, unfortunately we were unable to confirm potential activated pathways in this study.

5.7.4 Resistin

At present there is very little research regarding resistin and its receptors in ALL cells, in the trypan blue exclusion assays there were no significant differences in Molt4 cells incubated with increasing levels of resistin although a non-significant increase was seen in cell viability at day 4 of incubation in the well treated with 500ng/ml of resistin. Explanations for this may be that the receptors are simply not present in the membrane of Molt4 cells, there are no other studies to confirm this at present or that it could mean there is a receptor there but it needs very high levels to activate it which could not be confirmed in this set of experiments. No western blots were carried for Molt4 cells incubated with resistin so we were unable to know if the PI3K/Akt or the JAK/STAT pathways were activated as this has been confirmed in other cancers (Gong et al., 2018a).

5.7.5 Serpin A12 (Vaspin)

The trypan blue assay results for serpinA12 confirms significant increases in %cell viability on day 3 and 4 of the experiment, to our knowledge this is the first time serpinA12 has been shown to have a protective effect on ALL cells. By day 3 and 4 of the experiment with nutrient depletion serpinA12 may be contributing to increased cell viability by boosting survival in the presence of less nutrients. The trypan blue assay experiments also show this effect at the three concentrations tested which may imply that serpinA12 is able to have an impact on cell viability at low and high levels of expression, or in weight gain and weight loss. Although we have not confirmed by western blot which downstream pathway was activated in this set of experiments, in a study in liver cancer 5ng/mL of serpinA12 was enough to supress apoptosis by reduction of ROS and NO (Skonieczna et al., 2019). In a study using prostate and breast cancer cells GRP78 was able to relocate to the cell surface and form a complex with PI3K which activated the PI3K/Akt pathway and promoted chemoresistance (Zhang et al., 2013). The possibility of targeting other pathways involved in the UPR in ALL could also be considered alongside antagonising the GRP78 receptor (Masouleh et al., 2015)

5.7.6 Angiopoietin-2

The angiopoietin-2 trypan blue assays confirm no significant differences in numbers between cells incubated with increasing levels of angiopoietin-2 compared to the control, although the results for cell viability showed a significant difference between the control and the cells treated with 500ng/mL of angiopopietin-2, this finding confirms and fits in with previous research that at lower concentrations the growth factor acts as an antagonist against the Tie-2 receptor and at higher concentrations it can activate the receptor and downstream pathways (Kim et al., 2000). Lower levels of angiopoietin-2 in MRD risk patients also fit in with other studies as angiopoietin-2 acts as an antagonist at lower expression levels for angiopoeitin-1 and inhibits the activation of the downstream pathway PI3K/Akt/mTOR, lower levels in MRD risk would indicate that it is failing to block the action of angiopoeitin-1 and activating the PI3K/Akt pathway (Maisonpierre et al., 1997). We were unable to confirm activated downstream pathways by western blot for Angiopoietin-2.

The identification of adipokines associated with MRD risk has begun to identify risk profiles and potential adipokines that could be driving the disease process this is more likely to be occurring with serpinA12/vaspin in the five adipokines discussed, angiopoietin-2 seems to be having an indirect role expressed at low levels as it is unable to antagonise the action of angiopoietin-1. The results for resistin are not significant but there is a trend for increased cell viability in high concentration of 500ng/mL compared to the control. These three adipokines are all known to activate the PI3K/Akt pathway even though we have been unable to confirm this with western blotting. Resistin and SerpinA12 are also known to be involved the UPR and upregulation of ABC transporter pumps even though we have not confirmed these findings in this study. Resistin levels are lower in MRD risk patients at day 29, is the opposite protective in ALL? or are reduced levels of resistin due to a positive response to chemotherapy by the end of induction treatment, could we begin to track adipokine expression to predict effective response to treatment. Interestingly we have shown that a lower weight at the end of induction therapy is associated with MRD risk and that adipokine expression changes fit in with findings in the literature, the opposite is seen in weight gain patients, for example serpinA12 is raised in MRD risk in this study, but in weight loss group of patients levels reduce which matches the findings that MRD risk and associated adipokines are linked with weight loss.

6.0 CONCLUSION AND FUTURE WORK

This study has identified circulating adipokine expression changes during induction treatment that are significantly associated with patient sub-groups including those who gain weight and those who are MRD risk at the end of induction therapy. With this information we can build on this knowledge so that we can improve outcomes for obese and overweight patients with ALL, and perhaps also help to monitor ongoing risk throughout and after successful treatment for ALL. Current drug treatments are effective in curing most patients with ALL but leave many children with long term health complications. There is also little input given in current treatment protocols that considers the impact of weight gain during and after treatment, or the more aggressive management of weight loss which is linked to MRD risk early on in treatment.

We have shown that increased weight gain in this study is not linked to early stage MRD risk after induction therapy. It is clear though from other studies (Butturini et al., 2007, Eissa et al., 2017b, Gelelete et al., 2011, Orgel et al., 2014b) that excess weight poses an increased risk to patients throughout treatment and later in life and reduces overall survival and event free survival, it also puts the patient at risk of metabolic disorders which can reduce normal expected lifespan. What this work has highlighted is just how complex the subject of obesity and ALL is. If we break down the different aspects of risk in ALL patients without adding the further complexity of obesity, adipose tissue acting as an ALL cell niche and a source of fuel, dose capping of chemotherapeutics, adipokines or chemoresistance, the range of presentations of the disease is vast. Just taking into account the clinical data and the current standard risk strategies there are large differences in risk between a patient who is below 10 years of age, has a low white cell count at diagnosis, is female, who receives a regimen A, has good B ALL cytogenics and is low risk compared to an older male who has a high white count at diagnosis, receives regimen B, has T cell cytogenics and who is MRD risk at the end of induction therapy, often the only difference is the addition of daunorubicin to the treatment plan which is known to have many toxic side effects, possible changes in steroid treatment timing of doses or the addition of drugs if the disease has spread to the central nervous system.

It is important to identify patient subgroups who could benefit from new treatments and possibly omit more toxic drugs like daunorubicin which can further add to the burden of complications later in life or reduce or omit steroid treatment if possible, which contributes to weight gain and metabolic disorders. There are of course treatments in trials in particular small molecule inhibitors but is it time to investigate broader options that could be available to treat ALL. When we add in the increased risk of weight gain throughout treatment and also obese and overweight patients with dysregulated adipokines, could they be considered an at-risk group similar to patients with unfavourable genetics such as patients with T cell disease or a higher white cell count at diagnosis? Patients who present with increased weight during and after induction could benefit from their own treatment arm in clinical protocols throughout consolidation, maintenance therapy and any later follow up therapy especially if they present as MRD risk and obese or overweight. We have discussed in the chapter 4 adipokine summary that presenting as MRD alone without weight gain subjects ALL cells to

raised adipokines, also cross talk that can activate the PI3K/Akt and JAK/STAT pathways from serpinaA12 and IL-6. Drugs that target these pathways would benefit both at risk groups.

In the case of adipokines and targeting specific receptors such as the leptin receptor through drugs or fasting may not be the best approach, it is clear from this study and other studies that situation is much more complex, rather than target one or even two of the receptors directly it would be a better approach to target the shared downstream pathways. To combat dysregulated adipokine profiles contributing to increased risk drugs that target the various points in the PI3K/Akt/mTOR pathway and or the JAK/STAT pathway could be considered when it is found safe to treat children with them. For adipokines profiles that change chemosensitivity and it is found that this impacts the Bcl-2 family of proteins and consequently shifts levels of apoptosis to a pro survival phenotype in ALL cells Bcl-2 inhibitors could be included in treatment, there is increased interest in the usage of these drugs. There has been more recent interest in the drug metformin in the treatment of acute lymphoblastic leukaemia, recent research is adding to the all-round benefits of this drug which sensitises the AMPK pathway which in turn supresses the PI3K/Akt/mTOR and JAK/STAT pathways. Metformin also induces cell cycle arrest and apoptosis in drug resistant leukaemia cells. There are numerous new drug approaches that could be considered when treating patients where chemoresistance is apparent and the child is at risk of relapse further into treatment such as targeting the dysregulated unfolded protein response, autophagy or ABC transporter upregulation, adipokines have all been shown to have an impact in these pathways. More research is needed into the mechanisms of chemoresistance in ALL as this is not a straightforward approach. In the case of autophagy and the UPR both are finely balanced networks in the cell and both can act in an acute phase and chronically activated way. Early in treatment they may contribute a positive effect to outcome by driving apoptosis but later on in treatment when cells are exposed to chronic levels of dysregulated adipokine profiles particular overweight and obese profiles, this may switch the pathways to a pro survival phenotype to protect ALL cells, this could be an underlying contributor to poorer outcomes in obese and overweight children or MRD risk patients as they move through the treatment phases. Not all adipokines are direct drivers of disease by activating downstream pathways in the cell, for example leptin or IL-8. Some indirectly influence ALL by interacting with other adipokines or acting as antagonist such as IGFBP4 or angiopoietin-2, some are protective, an

example would be adiponectin. Adipokines such as pentraxin-3 can give an indication of infection or sepsis in a patient which can add to risk. Adipokines such as resistin could be used as potential biomarkers and used to track response to chemotherapy and relapse throughout treatment, we have shown in the 3T3-L1 work that adipose tissue does respond to chemotherapy treatments, further research is needed to understand the impact chemotherapy has on long term function of adipose tissue and if this contributes to late effects. Further research and studies are needed to identify adipokine patterns in MRD risk patients and patients who gain weight to be confident in knowing that they present with a particle adipokine profile combined with their clinical risk and then researching their main drivers of disease or chemoresistance and choosing the most suitable kinder drug combination treatments for them, this would involve larger studies to collect more patient data and further research into the most suitable treatment options.

Levels of obesity are on the rise and there is a call for newer targeted treatments for cancer patients who have the added risk of weight gain. There is no clear evidence that weight gain is a cause of developing ALL, but it is clear that through various mechanisms discussed in this study that it adds risk to the patient. This work has begun to identify just one part of the complex reasons for increased risk associated with being obese or overweight and will add to the field of knowledge to other paediatric cancers that are made worse by weight gain and also adult cancers

This study has highlighted the need for future research in this area, this study had more work planned but unfortunately patient recruitment and the work planned in chapter 5 has been severely impacted by the Covid-19 pandemic. This led to reduced time in the laboratory to complete the planned experiments, there are also limitations that need to be considered of the study.

For this study the number of patients in the cohort needed to be larger, this would allow for more overweight or obese patients to be recruited, also MRD risk patients. Higher patient numbers would have the advantage of increasing the statistical power of the study overall, this could provide a more definitive answer to know if presenting or becoming obese or overweight with ALL significantly contributes to MRD risk during induction therapy. Future patient work could include extending the study in the consolidation and maintenance phases of treatment and taking plasma samples again with weight and later MRD risk status, this

could provide more answers into how adipokine profiles change throughout the whole of the treatment. Patients could even be followed up after successful treatment to monitor adipokine expression and see if it correlates with patients who have stayed at a higher BMI percentile and if they have markers of metabolic syndrome and other late effects. One option would be to obtain patients cells from bone marrow biopsies that are taken as part of the treatment protocols to measure MRD risk and use these in experiments with adipokines. The use of ALL cell lines are useful in experiments to test hypothesis but they have their own limitations and lack the genetic variability of a patient cohort.

A limitation of the study is that we are only looking at adipokines in the circulation (plasma) as there may be different expression levels in different tissues, some adipokines may not be present in the circulation if they are acting locally within the bone marrow tissue for example. This could explain why certain adipokines were not detectable on the array or there was no change in expression from diagnosis to the end of induction therapy.

Included in the appendix are tables 7.2 and 7.3 which confirm that adipokines could be used as biomarkers to predict MRD risk at diagnosis and which adipokines are increased in patients who presented as obese or overweight at diagnosis compared to normal weight patients. Future work could include researching these adipokines further particularly the ones which may be responding to treatment, these could be used to monitor disease progression and relapse.

We have not yet proven cause and effect between adipokine expression and MRD risk, so far, we have only confirmed correlations and identified adipokines associated with MRD risk and weight gain. After identifying potential adipokines from our patient data we have begun to show that adipokines do have a protective effect on Molt4 cells in our trypan blue exclusion assay experiments, this work is incomplete though and we planned to build on these findings. From the adipokines tested further research suggested that they shared common downstream pathways which included PI3K/Akt and JAK/STAT, the planned work was to western blot to check which of the pathways were activated in the adipokines we had chosen to complete the trypan blue exclusions assay from and collect the incubated cells. Then perform an MTS chemosensitivity assay for each individual adipokine selected. The full set of data can be seen for leptin. Due to time missed in the laboratory the work for the other adipokines was incomplete. Once we knew the results for each individual adipokine we

planned to use cocktails of adipokines in the MTS sensitivity assay. Planned cocktails were adipokines which reflected an MRD risk profile, then adipokines which were involved in weight gain and finally a cocktail of adipokines that would represent an MRD risk patient who also gained weight. If any of the adipokine cocktails changed chemosensivity the plan was to then attempt a new set of trypan blue exclusion assay experiments which would involve using inhibitors of the PI3K/Akt pathways and/or JAK/STAT pathways to rescue the protective effect of the adipokines and collect incubated cells and confirm these findings with further western blots to check if the inhibitors worked.

There were limitations to the trypan blue cell viability experiments in that by day 4 cell viability was also reduced in the control well. This suggests that the cells may have been running out of nutrients, this was still useful data as some of the adipokines were still having a protective effect on cell viability which is interesting in itself and may involve other mechanisms that haven't been discussed in this study or that the activated pathways by the selected adipokines protect the cells in starvation conditions.

One area that would need more future research is the impact of steroids on weight gain and disease progression and also in the future if they could ever be omitted from paediatric treatments, research indicates that steroids do impact adipose tissue and cause weight gain, future work could include the use of steroid in the adipokine cocktail experiments. There are also major issues with glucocorticoid resistance in ALL treatment, using dexamethasone or prednisolone in these experiments could look at how resistance develops in ALL treatment and which mechanisms are activated and how that can be overcome. It would also be important to include an ALL cell line from B cell origin in future work, B cell lines are limited in paediatric leukaemia and the most common used is infected with the herpes simplex virus so this was not chosen to study.

One surprising finding in the study was identifying adipokines associated with MRD risk which wasn't expected as much of the literature regarding adipokines is in relation to weight gain and obesity. Serpin A12 or vaspin was probably the best example and likely to be a good future target for further investigation due to its interaction with the GRP78 receptor and the UPR. It would be useful to investigate if the GRP78 is relocated to the outer cell membrane and is activating the PI3K/Akt pathway in our experiments. There is a lot of recent research looking into the UPR in acute leukaemia's and how it contributes to chemoresistance, also

the potential drug targets which could work alongside it to improve efficacy of current chemotherapy protocols.

Other new treatment strategies from this work indicate that inhibitors of the PI3K/Akt/mTOR pathways the JAK/STAT pathways could be helpful in treatment and kinder for long term effects, also drugs that mimic the action of adiponectin, these include AMPK activators or metformin which switch off the PI3K/Akt/mTOR and JAK/STAT pathways. Metformin has also been shown to stabilise insulin levels which in turn improves glucose uptake in fat tissues and skeletal muscle which can act to starve ALL cells of glucose. Further experiments in cells treated with dysregulated adipokine cocktails could look for changes in apoptosis and the Bcl-2 family. This could be observed using flow cytometry then western blots to confirm changes in Bcl-2 expression, Bcl-2 inhibitors could then be considered as a treatment. Another option would be to investigate if there are changes in autophagy in the cells treated with adipokine cocktails, autophagy can contribute to chemoresistance and could be checked by western blotting for changes in the LC3-I/LC3-II ratio, if this is changed drugs that target autophagy could be useful in treatments.

The one finding in this study is that the situation is complex, there are a lot of other research articles in other cancers and ALL that allude to the targeting of one adipokine or its receptor, there is much more to learn about the complex crosstalk between cells in ALL, the findings from this study would suggest that targeting one adipokine or its downstream pathway may not be the best course of treatment stratification, adipokine profiles are complex it would be useful to target common pathways as suggested above and in very up to date research. The most recent review for ALL and obesity which covers some of the suggestions above is here (Dushnicky et al., 2020). It is clear from this study and others that more research is needed in these areas in the hope to improve outcomes for obese, overweight and MRD risk patients and to develop kinder drug treatments for paediatric ALL patients.

7.0 APPENDIX

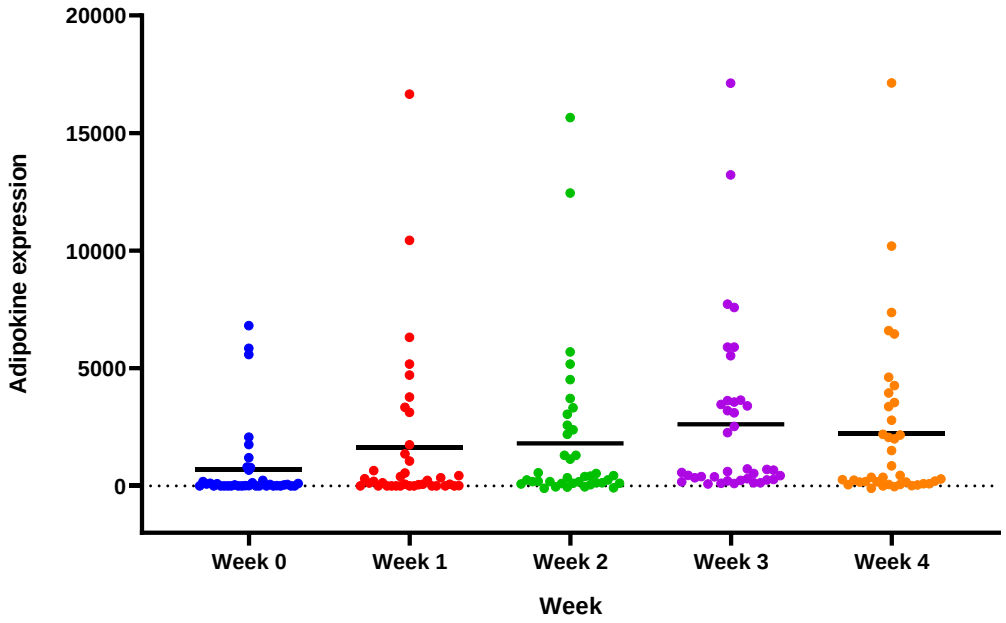

Figure 7.1: Mouse Adipokine array results and pixel intensity values from 3T3-L1 differentiated and undifferentiated cells over the four-week differentiation period. Adipokine expression levels from each week 0, 1, 2, 3 & 4 are displayed grouped to show the mean increases over the whole experiment, n=1.

Table 7.1: Mouse Adipokine array results and pixel intensity values from 3T3-L1 differentiated and undifferentiated cells over a four-week differentiation period. The summary table indicates increases (green) and decreases (red) of array pixel intensity for each corresponding adipokine. Results for differentiated cells (fat) and undifferentiated (control) wells for weeks 1, 2, 3 & 4 compared to pre-adipocytes at 24 hours after seeding on a 24 well plate. White boxes indicate no data available.

ADIPOKINE	Week 1 Control	Week 1 Fat	Week 2 Control	Week 2 Fat	Week 3 Control	Week 3 Fat	Week 4 Control	Week 4 Fat
Adiponectin	2108.93	3128.51	4967.36	5692	3823.75	5898.66	3952.16	3540.62
AgRP	139.51	61.92	248.59	188.87	299.07	526.59	76.69	90.61
ANGPT-L3	88.32		173.22	108.58	158.37	298.79	116.4	-4.18
CRP	90.46		105.28	-88.47	121.83	123.54	67.69	46.41
DPPIV	97.7		100.33	-52.88	115.86	109.69	190.92	158.23
Endocan	104.12	113.02	215.54	341.48	313.02	439.33	83.99	363.13
Fetuin A	50.46		226.52	108.64	359.38	384.87	-1.57	52.25
FGF acidic			31.82	180.13	84.02	265.73	-24.49	-105.08
FGF-21	488.77	192.12	573.92	429.92	647.02	665.64	694.63	845.07
HGF	167.11	50.57	116.77	129.29	274.88	563.82	147.29	258.62
ICAM-1	117.88	63.93	134.89	126.73	214.52	246	332.12	237.19
IGF-I	2906.13	295.51	1991.56	253.82	2842.71	3098.87	5133.88	4264.85
IGF-II	997.91	435.37	1922.74	1129.88	2345.95	3403.37	3157.86	3945.7
IGFBP-1	118.18	67.32	159.95	183.2	202.18	433.44	80.59	164.17
IGFBP-2	129.1	4.75	217.38	75.89	149.95	206	323.83	157.25
IGFBP-3	4022.64	647.95	5704.43	1295.26	4132.9	3617.16	5151.75	1997
IGFBP-5	1164.85	540.99	1854.09	1300.68	1893.2	2261.04	2160.94	2058.26
IGFBP-6	4817.78	4712.06	4376.53	3041.91	4728.91	3557.56	5757.32	4620.61
IL-6	32.78		37.68	-110.05	167.84	95.23	-139.09	52.65
IL-10	23.34		49.59	100.04	148.09	170.23	-138.48	-33.48
IL-11	112.59		138.64	248.96	206.08	339.71	-20.05	40.44
Leptin	1288.49	342.6	1792.76	556.35	1614.21	2526.98	1472.87	2185.95
LIF	172.45	123.32	216.41	173.25	298.99	391.69	237.57	287.94
Lipocalin-2	4393.02	6313.74	4385.31	5178.34	4779.82	7726.38	5831.93	7372.76
MCP-1	5474.24	3335.99	6384.57	3710.8	5313.74	7581.2	7792.67	6593.2
M-CSF	1124.47	1361.64	2559.08	2189.68	2097.33	3643.28	2008.71	2157
Oncostatin M	109.07		130.82	44.17	129.81	129.11	222.86	181.62
Pentraxin 2	153.33		194.88	-49.45	252.84	233.63	141.27	83.83
Pentraxin 3	2439.01	1747.76	1400.28	2378.21	2513.43	3460.46	2789.82	2786.56
Pref-1	1348.37	1047.36	2274.56	2572.46	2915.47	3198.47	1571.15	1501.37
RAGE	59.76		36.56	-42.21	120.03	80.36	-140.79	1.25
RANTES	107.37	229.05	97.02	416.5	182.39	604.34	-128.48	439.35
RBP4	71.61		253.55	521.56	519.8	697.05	338.47	197.59
Resistin	15445.17	16657.52	14712.28	15656.87	14808.22	17116.25	18301.23	17131.8
Serpin E1	4825.3	3769.57	5094.75	4516.47	4880.25	5526.56	3391.33	3371.48
TIMP-1	5886.77	5173.68	4087.79	3320.69	4449.47	5897.74	6551.29	6455.87
TNF-alpha	384.12	392.47	459.39	392.29	356.75	718.98	673.27	365.36
VEGF	9316.23	10437.37	11067.84	12450.35	8938.93	13217.86	11625.11	10198.07

7.1 Individual patient data

7.1.1 Patient 009

The individual adipokine expression and clinical data for patient 009 is shown in figure 7.2. This patient is a 13.6 year old male, due to the patient being over 10 years of age they were

assigned induction regimen B. Their BMI percentile was calculated at 10 at the start of induction and increased to 25 by day 29 at the end of induction, with a body weight increase of 6.96% during the induction period from their starting weight. The patient's cytogenic risk was good and their MRD risk low. This patient did put on weight and plasma adipokine profiling between day 0 and day 29 shows increases in leptin and resistin, with highest increases in visfatin. The largest decreases for this patient were IL-10 and growth hormone.

7.1.2 Patient 010

The individual results for patient 010 are displayed in figure 7.3. Patient 010 is a 6.4 year old female who received induction regimen A as their treatment option. Their BMI percentile at day 0 was 86 which is in the overweight category and by the end of induction this had decreased to 73 which put them in the normal percentile range, this resulted in a 3.2% reduction in body weight during the induction period. The patients cytogenic risk was good, and their MRD risk status was recorded low. This patients highest recorded adipokine increase was resistin, angiopoietin-1 and TNF-alpha, here leptin expression was decreased and the patient's largest decreases in expression were growth hormone and MCP-1.

7.1.3 Patient 011

The clinical data and adipokine expression profile for patient 011 is displayed in figure 7.4. Patient 011 is a 6.9 year old male who received induction regimen A. Their BMI percentile at day 0 was 64 which is in the normal weight range and rose to 93 at day 29 which placed the patient in the overweight percentile category. During the induction period patient 011 put on 13.23% of their starting body weight. The patients cytogenic risk was intermediate and their MRD risk was recorded low. The highest increases in adipokine expression were IL-11 and TNF-alpha and the largest decreases from day 0 to day 29 were BAFF and BMP-4.

7.1.4 Patient 012

The adipokine expression profile and clinical data for patient 012 is displayed in figure 7.5. Patient 012 is a 1.8 year old male and received induction regimen A. Their BMI percentile at

day 0 was 75 which is in the normal range and by the end of induction therapy it has dropped slightly to 70, still in the normal range. The percentage weight change for this patient decreased by 2.62%. the patients cytogenic risk was good, and the MRD risk status was low. This patients' largest increases in adipokine expression were IL-8, LAP(TGF-beta1) and TIMP-3 the largest decreases were growth hormone and IL-11.

7.1.5 Patient 013

The individual results for patient 013 are displayed in figure 7.6. Patient 013 is the youngest patient in our cohort and is male and 1.1 years of age. The patient received induction regimen A. Their BMI percentile was in the normal range at the beginning of induction at 72 and increased to 88 by day 29, this figure put them in the overweight percentile range with a 5.34% body weight increase from day 0. The patients cytogenic risk was good and their MRD risk was recorded low. The largest increase in adipokine expression from day 0 was leptin, resistin and VEGF. The largest decreases for patient 013 was growth hormone and BMP-4.

7.1.6 Patient 014

Results for patient 014 are displayed in figure 7.7. Patient 014 is a 16 year old female and due to them being over the age of 10 they have been placed on induction regimen B. Their BMI percentile at day 0 was 21 which is at the lower end of the normal range percentile category and had risen to 38 by the end of induction, their body weight increased by 3.13%. The patients cytogenic risk was intermediate and their MRD risk low. The largest fold change increases for this patient were angiopoietin-1 and CRP. The largest decreases were adiponectin and growth hormone.

7.1.7 Patient 015

Patient 015 was the first patient in the cohort recorded as MRD risk and their results are displayed in figure 7.8. The patient is a 3.7 year old male who received induction regimen A. Their BMI percentile at the start of induction was recorded at 74 which is in the normal weight percentile category and by day 29 was greater than 95 which is the obese percentile category, this patient also gained 22.22% of their starting body weight during induction. Patient 015 cytogenic risk was good and MRD risk was recorded at day 29. The largest fold change

increases in adipokines were leptin, myeloperoxidase and serpin A12 (vaspin). The largest decreases were growth hormone and MCP-1.

7.1.8 Patient 017

The individual adipokine expression results and clinical data for patient 017 are shown in figure 7.9. Patient 017 is a 12 year old female and due to being over 10 years of age have been given induction regimen B. Their BMI percentile was recorded at 40 at the beginning of induction on day 0 which is normal and dropped to 11 by day 29, the patient lost 11.93% of their body weight by the end of induction treatment. The patients cytogenic risk was intermediate, and they were MRD risk. Patient 017's overall adipokine expression is low compared to other patients with most of their adipokines decreasing by day 29. The largest fold change increases were IL-8, pentraxin-3 and serpinA12. leptin and resistin were decreased and growth hormone and angiopoietin-1 were decreased further.

7.1.9 Patient 018

The adipokine expression profile and clinical data for patient 018 is displayed in figure 7.10. The patient is an 11.8 year old female and was placed on induction regimen B. the patients BMI is in the normal range at day 0 although at the low end and is recorded at 18, by day 29 this had dropped further to 13 with a percentage decrease in weight of 2.63% during the induction period. The patients cytogenic risk was intermediate, and they were also MRD risk. The largest fold change increases in adipokines were CRP and BMP-4 and the largest decreases were HGF, TIMP-3.

7.1.10 Patient 020

The results for patient 020 are displayed in figure 7.11. Patient 020 is a 6.2 year old female who received regimen A during induction. Their BMI percentile at day 0 was in the normal range and recorded at 66 and by day 29 this has risen slightly to 69, the patient gained 0.66% of their starting body weight during induction. Their cytogenic risk was good and their MRD risk was low. The largest increases in adipokine expression were leptin and MCP-1 and the largest decreases in expression were HGF and BAFF.

7.1.11 Patient 021

The adipokine profile and clinical data for patient 021 is shown in figure 7.12. Patient 021 is a 5.5 year old female who has received induction therapy B. As the patient is under 10 years of age, we must assume that the patients initial white cell count was >10,000 cells as that would be the only indication for regimen B, this information has not been recorded as part of this particular study. The patients BMI percentile at day 0 was 42 which is in the normal range, but it dropped to 5 by day 29 which is the underweight percentile category, the patient lost 11.56% of their body weight during induction. Their cytogenic risk was intermediate and their MRD risk low. The highest adipokine fold change increases were CRP and IGFBP-2, the largest decreases in adipokine expression were growth hormone and BAFF.

7.1.12 Patient 023

Results for patient 023 are shown in figure 7.13. Patient 023 is a 6.7 year old male who received induction therapy regimen A. Their BMI at day 0 was recorded in the normal weight range at 16, by the end of induction therapy at day 29 it had risen to 79, the patient had a large increase in weight during induction gaining 17.27% of their initial starting weight. The patients cytogenic risk was intermediate and there were no MRD markers detected for this patient. The largest fold change increases in adipokines were ICAM-1/CD54 and myeloperoxidase, the largest decreases BMP-4 and IL-11.

7.1.13 Patient 024

The adipokine expression profile and clinical data for patient 024 is displayed in figure 7.14. Patient 024 is a 3.3 year old male who received induction therapy A. Their BMI percentile at day 0 was 75 which is in the normal percentile category and it rose to 86 by day 29 which puts the patient just into the overweight category, the patient gained 3.33% of their starting weight during induction treatment. The patients cytogenic risk was low and their MRD risk was recorded low at day 29. The largest fold change increases in adipokines were angiopoietin-1 and leptin and the largest decreases were growth hormone and BAFF.

7.1.14 Patient 025

The results for patient 025 are shown in figure 7.15. Patient 025 is a 6 year old female who received induction therapy A. Their BMI percentile was calculated at 70 on day 0 which is in the normal range before treatment, after treatment on by day 29 it has dropped to 45, still in the normal range, they recorded a 6.1% weight reduction from their initial starting weight during induction treatment. The patients cytogenic risk was good, but they were MRD risk when tested at day 29. The highest adipokine fold change increases were Il-11 and visfatin, the largest decrease from day 0 were growth hormone and BAFF.

7.1.15 Patient 026

The individual adipokine expression profile and clinical data for patient 026 is shown in figure 7.16. Patient 026 is a 4.2 year old male who was given induction regimen A for their treatment option. Their BMI percentile at day 0 was recorded at <5 which is the underweight category, by day 29 the patient had put on weight and it had risen to 34 which was a body weight percentage gain of 16.26. The patients cytogenic risk was intermediate, and they were MRD risk at the end of induction. The largest increases in adipokine expression were leptin and BMP-4 and the largest decreases were growth hormone and IL-8.

7.1.16 Patient 027

The results for patient 027 are displayed in figure 7.17. The clinical data confirms that patient 027 is a 2.6 year old male who received regimen A for their induction treatment. The patients BMI percentile at day 0 was calculated at 56 which is in the normal range and by day 29 this was unchanged and recorded at 56 as there was zero weight change during induction. The patients cytogenic risk was good, and their MRD risk status was low. The largest increases in fold change adipokine expression was for leptin and VEGF, the largest decreases in adipokine expression from baseline day 0 levels were BMP-4 and angiopoietin-Like 2.

7.1.17 Patient 028

The individual adipokine expression profile and clinical data for patient 028 is shown in figure 7.18. Patient 028 is a 7.2 year old male who received induction regimen B, as they are younger than 10 years old this would indicate a high initial white cell count which as discussed previously this data has not been collected as part of this study. The patients BMI percentile at day 0 was calculated at >95 which puts the patient in the obese category and by the end of induction they remained in the obese category with their BMO percentile at >95, this patient gained 1.7% of their initial starting weight during induction therapy. The patients cytogenic risk was good, and their MRD risk status was low. The largest increases for adipokine expression were CRP and pentraxin-3, the largest decreases in adipokine expression were BAFF and growth hormone.

7.1.18 Patient 030

The adipokine profile and clinical data set for patient 030 is shown in figure 7.19. Patient 030 is a 9.4 year old female who received regimen A as part of their induction treatment. Their BMI percentile at day 0 was in the overweight category at 88 and by day 29 this had risen to 91 which is still in the overweight category, during the induction period the patient gained 4.05% of their initial body weight. The patient cytogenic risk was good, and their MRD risk status was low. The patients adipokine profile showed the largest increases in CRP and angiopoietin-1 and the largest decreases in growth hormone and BAFF.

7.1.19 Patient 031

The results for patient 031 are displayed in figure 7.20. Patient 031 is a 12.7 year old male who received induction regimen B as part of their treatment. The patients BMI percentile at induction was calculated at 25 which is in the normal category and by the end of induction has increased to 30 which is still within the normal range. During the induction period the patient gained 1.66% of their initial starting body weight. The patients cytogenic risk indicating T-cell disease which is the highest risk category for cytogenetics in ALL, the patient also has an MRD risk status at day 29. The patients largest adipokine expression increases

were leptin and serpinA12 and the largest decreases were growth hormone and angiopoietin-2.

7.1.20 Patient 032

The individual adipokine expression profile and clinical data for patient 032 is shown in figure 7.21. Patient 032 is a 11.5 year old male who was placed on induction therapy B for their treatment. The patients BMI percentile was calculated at 29 at the beginning of induction and increased to 43 by the end of induction, both measurements falling into the normal category. The patients weight increased by 4.5% during the induction period. their cytogenic risk was recorded good and the MRD risk status low. The largest increases in fold change adipokine expression were complement factor D and the largest decreases in adipokine expression were growth hormone and TNF-alpha.

7.1.21 Patient 034

The adipokine profile and clinical data set for patient 034 are shown in figure 7.22. Patient 034 is male patient who is 1.8 years of age and received induction regimen A for their treatment option. The patients BMI percentile at day 0 was calculated at 31 which is in the normal range, by day 29 it had risen to 45 which is still in the normal range, this resulted in a weight change increase of 2.67% during the induction period. The patients cytogenic risk was low, and their MRD risk status was also low. The largest individual increases in adipokine expression were for pentraxin-3 and angiopoietin-1, the largest decreases were CCL2/MCP-1 and growth hormone.

7.1.22 Patient 035

The results for patient 035 are displayed in figure 7.23. Patient 035 is a 12.1 year old female who received induction regimen B. The patients BMI percentile at day 0 was in the normal range and calculated at 9 which is the lower end of the range, this increased to 16 by the end of induction therapy, still in the normal range, their body weight increased by 3.68% during the induction period. The patients cytogenic risk was intermediate and their MRD risk status high. The highest fold change increase in adipokine expression was for pappalysin-1 and IL-10, the lowest BAFF and angiopoietin-2.

7.1.23 Patient 036

The individual adipokine expression profile and clinical data for patient 036 are displayed in figure 7.24. Patient 036 is a 2.7 year old male who was treated with induction regimen A. The patients BMI percentile at induction was calculated at 31 which is in the normal category, this increased by a large amount to >95 which is classed as treatment acquired obesity, their body also weight increased by 27.36% by day 29 compared to day 0. The patients cytogenic risk is intermediate and their MRD risk low. The largest increases for adipokine expression where leptin and angiopoietin-1, the largest decreases were growth hormone and EN-RAGE.

7.1.24 Patient 037

Results for patient 037 are shown in figure 7.25. Patient 037 is a 4.6 year old female who received induction regimen A as part of their treatment for ALL. The patients BMI was calculated at 25 at the beginning of induction, which is in the normal weight range, this increased to 86 by day 29, this is classed at treatment acquired overweight category, the patient also gained 16.77% of their starting body weight. The patients cytogenic risk was intermediate and the MRD risk high. The largest increase in fold change adipokine expression was leptin and 195ngiopoietin-1 and the largest decreases adiponectin and BAFF.

7.1.25 Patient 038

The adipokine expression profile and clinical data for patient 038 is displayed in figure 7.26. Patient 038 is a 3.2 year old male who received induction therapy B as their treatment option this could indicate a higher initial white count. The patient's day 0 BMI percentile was calculated at 29 and increased slightly to 31 by the end of induction treatment, this resulted in a 0.42% body weight increase by day 29. The patients cytogenic risk was low and their MRD risk was also low. The patients largest adipokine expression increases were CRP and TIMP-3 and the largest decreases were growth hormone and angiopoietin-Like 2.

7.1.26 Patient 040

The results for patient 040 are displayed in figure 7.27. Patient 040 is a 2.9 year old female who received regimen A. The patients starting BMI percentile was calculated at 90 which is in

the overweight category and by the end of induction it had decreased to 86 which is still in the overweight percentile category, this decreased their body weight by 2.01% by the end of treatment. The patients cytogenic risk was intermediate and their MRD risk was low. The highest fold change increase in adipokine expression was for angiopoietin-1 and resistin, the lowest BAFF and IL-8.

7.1.27 Patient 041

The adipokine profile and clinical data set for patient 041 is shown in figure 7.28. Patient 041 is a 2.8 year old male who received induction A as a treatment option. Their BMI percentile at day 0 was in the normal range and recorded at 49 and by day 29 this has risen to 67, the patient gained 3.72% of their starting body weight during induction. The patients cytogenic risk was good and their MRD risk low. The patients largest adipokine expression increases were CRP and angiopoietin-1 and the largest decreases were BAFF and angiopoietin-Like 2.

7.1.28 Patient 044

The results for patient 044 are displayed in figure 7.29. Patient 044 is a 10.3 year old female who received induction regimen B. The patients BMI was calculated at 90 at the beginning of induction, which is in the overweight category, this decreased slightly to 88 by day 29, both measurements are in the overweight category, the patient also lost 2.06% of their starting body weight. The patients cytogenic risk was intermediate and their MRD risk low. The highest fold change increase in adipokine expression was for EN-RAGE and resistin, the lowest growth hormone and oncostatin M.

7.1.29 Patient 045

The individual adipokine expression profile and clinical data for patient 045 is shown in 7.30. Patient 045 is a 4.1 year old male who received regimen A during induction. Their BMI percentile at day 0 was in the normal range and recorded at 79 and by day 29 this had increased to >95, this is classed at treatment acquired obesity and the patient gained 24.43% of their starting body weight during induction. The patients cytogenic risk is good and their MRS risk low. The patients largest adipokine expression increases were leptin and BMP-4 and the largest decreases were growth hormone and BAFF.

7.1.30 Patient 046

Results for patient 046 are shown in figure 7.31. Patient 046 is a 6.2 year old male who was administered induction therapy A. The patients BMI was calculated at 47 at the beginning of induction, which is in the normal weight range, this increased to 73 by day 29, also in the normal range, the patient also gained 6.47% of their starting body weight. The patients cytogenic risk is intermediate and the MRD risk high. The highest fold change increase in adipokine expression was for VEGF and leptin, the lowest BAFF and IL-8.

7.1.31 Patient 047

The adipokine profile and clinical data set for patient 047 is shown in figure 7.32. Patient 047 is a 4.7 year old male who received induction A as their treatment option. Their BMI percentile at day 0 was in the normal range and recorded at 22 and by day 29 this has risen to 80, the patient gained 12.93% of their starting body weight during induction. The patients cytogenic risk was intermediate, and they were classed as MRD risk by day 29. The patients largest adipokine expression increases were CRP and BMP-4 and the largest decreases were BAFF and EN-RAGE.

7.1.32 Patient 048

Results for patient 048 are shown in figure 7.33. Patient 048 is a 9 year old male who received induction regimen B as part of their treatment for ALL. The patients BMI percentile was calculated at 45 at the beginning of induction, which is in the normal weight range, this decreased to 18 by day 29, the patient also lost 7.14% of their starting body weight. The patients cytogenic risk was Tcell disease which is the highest risk category and MRD risk. The largest increase in fold change adipokine expression was DPPIV/CD26 and angiopoieitn-1 and the largest decreases BAFF and RAGE.

7.1.33 Patient 049

The individual adipokine expression profile and clinical data for patient 049 is shown in figure 7.34. Patient 049 is a 4.8 year old female who was placed on induction therapy A for their treatment. The patients BMI percentile was calculated at 77 at the beginning of induction and

decreased to 59 by the end of induction, both measurements falling into the normal category. The patients weight decreased by 4.73% during the induction period. Their cytogenic risk was recorded good and they were classed as MRD by day 29. The largest increases in fold change adipokine expression were IGFBP-2 and TIMP-1 and the largest decreases in adipokine expression were growth hormone and resistin.

7.1.34 Patient 050

The results for patient 050 are displayed in figure 7.35. Patient 050 is a 6.6 year old male who received induction regimen A. The patients BMI was calculated at 29 at the beginning of induction, which is in the normal category, this increased to >95 by day 29 which is classed as treatment acquired obesity, the patient also gained a large amount of weight increasing by 37.24% of their starting body weight. The patients cytogenic risk was intermediate, and they were MRD risk at day 29. The highest fold change increase in adipokine expression was for leptin and resistin, the lowest growth hormone and BAFF.

7.1.35 Patient 051

The adipokine profile and clinical data set for patient 051 are shown in figure 7.36. Patient 051 is male patient who is 5.9 years of age and received induction regimen A for their treatment option. The patients BMI percentile at day 0 was calculated at 66 which is in the normal range, by day 29 it had risen to >95 which is in the obese category, this resulted in a weight change increase of 27.25% during the induction period and the patient having treatment acquired obesity. The patients cytogenic risk was good, and they were low risk by day 29. The largest individual increases in adipokine expression were pentraxin-3 and CRP, the largest decreases were adiponectin and growth hormone.

7.1.36 Patient 053

Results for patient 053 are shown in figure 7.37. Patient 053 is a 2.8 year old female who was administered induction therapy B. The patients BMI was calculated at 81 at the beginning of induction, which is at the high end of the normal weight range, this increased to >95 by day 29, which is in the obese range, the patient also gained 14.84% of their starting body weight. The patients cytogenic risk is intermediate and the MRD risk was low. The highest fold change

increase in adipokine expression was for angiopoietin-1 and leptin, the lowest adiponectin and growth hormone.

7.1.37 Patient 055

The individual adipokine expression profile and clinical data for patient 055 is shown in 7.38. Patient 055 is a 2.9 year old male who received regimen B during induction. Their BMI percentile at day 0 was in the normal range and recorded at 40 and by day 29 this had increased to 84, still in the normal range but at the high end, the patient gained 10.07% of their starting body weight during induction. The patients cytogenic risk is good, and they were MRD risk by day 29. The patients largest adipokine expression increases were LAP(TGF-beta1) and angiopoietin-1 and the largest decreases were growth hormone and IL-8.

7.1.38 Patient 057

The results for patient 057 are displayed in figure 7.39. Patient 057 is a 11.2 year old male who received induction regimen B. The patients BMI was calculated at 50 at the beginning of induction, this increased slightly to 54 by day 29, both measurements are in the normal category, the patient also gained 1.43% of their starting body weight. The patients cytogenic risk was high, and they were classed as MRD risk by day 29. The highest fold change increase in adipokine expression was for angiopoietin-1 and CRP, the lowest growth hormone and BAFF.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
009	13.6	M	B	10	25	Good	Low	+6.96

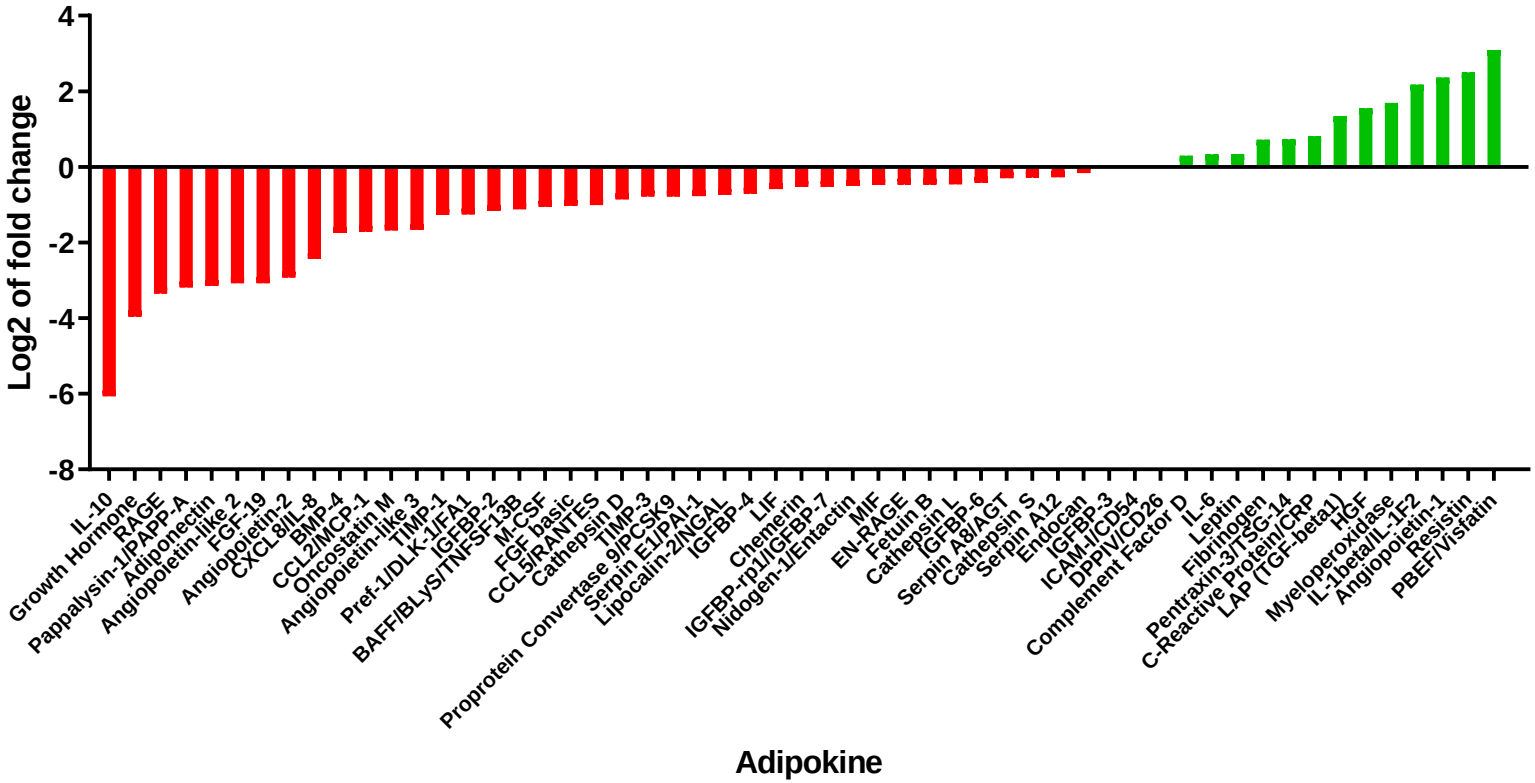

Figure 7.2: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 009 . Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in exp ression, red indicates a decrease and green an increase in expression. Patient age 13.6 years, male, induction regimen B, BMI percentile day 0=10, BMI percentile day 29=25, Cytogenetic risk good, MRD risk low, % weight change +6.96, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
010	6.4	F	A	86	73	Good	Low	-3.2

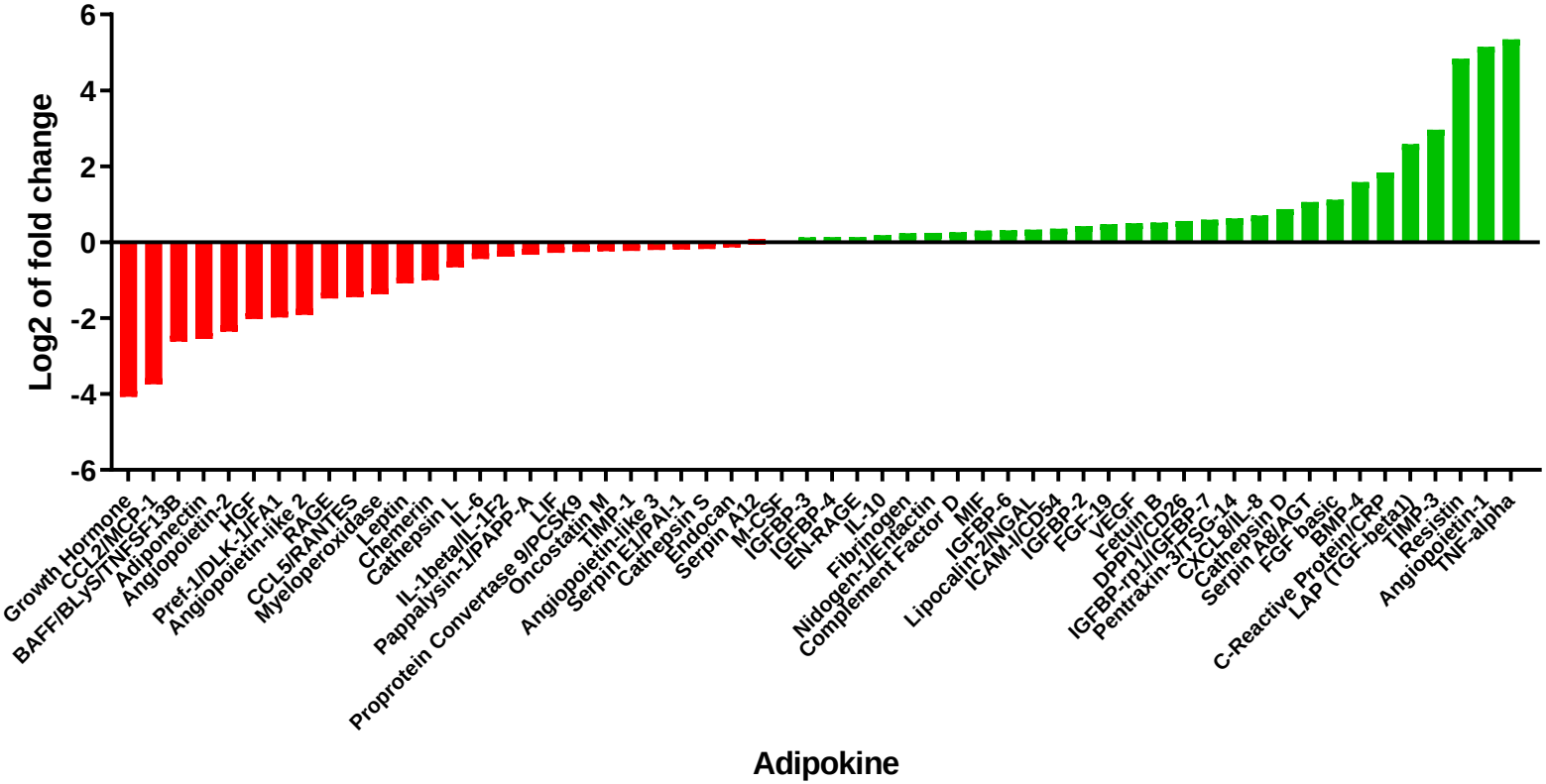

Figure 7.3: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 010. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in exp ression, red indicates a decrease and green an increase in expression. Patient age 6.4 years, female, induction regimen A, BMI percentile day 0=86, BM I percentile day 29=73, Cytogenetic risk good, MRD risk low, % weight change -3.2, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
011	6.9	M	A	64	93	Intermediate	Low	+13.23

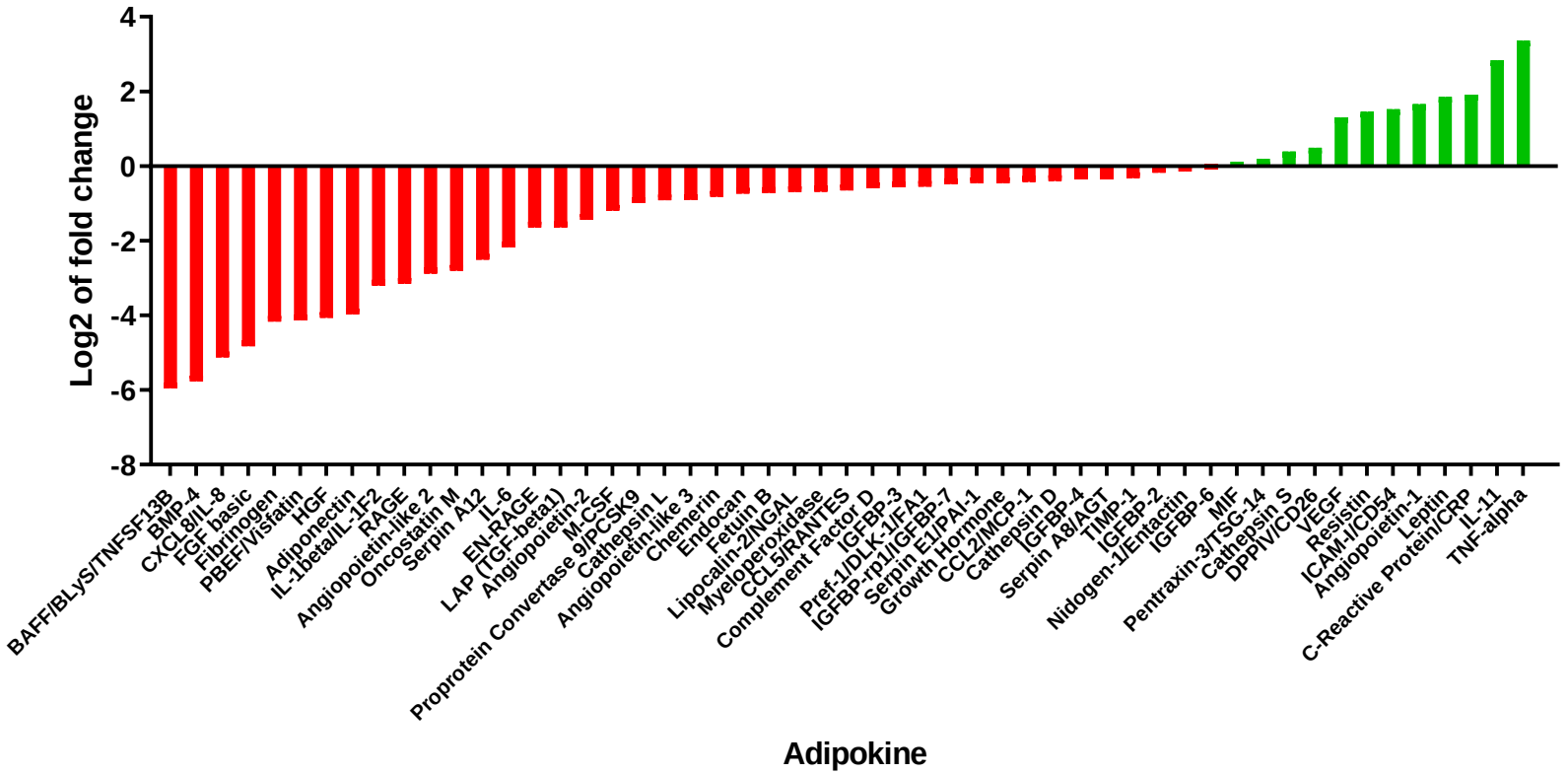

Figure 7.4: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 011. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 6.9 years, male, induction regimen A, BMI percentile day 0=64, BMI percentile day 29=93, Cytogenetic risk intermediate, MRD risk low, % weight change +13.23, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
012	1.8	M	A	75	70	Good	Low	-2.62

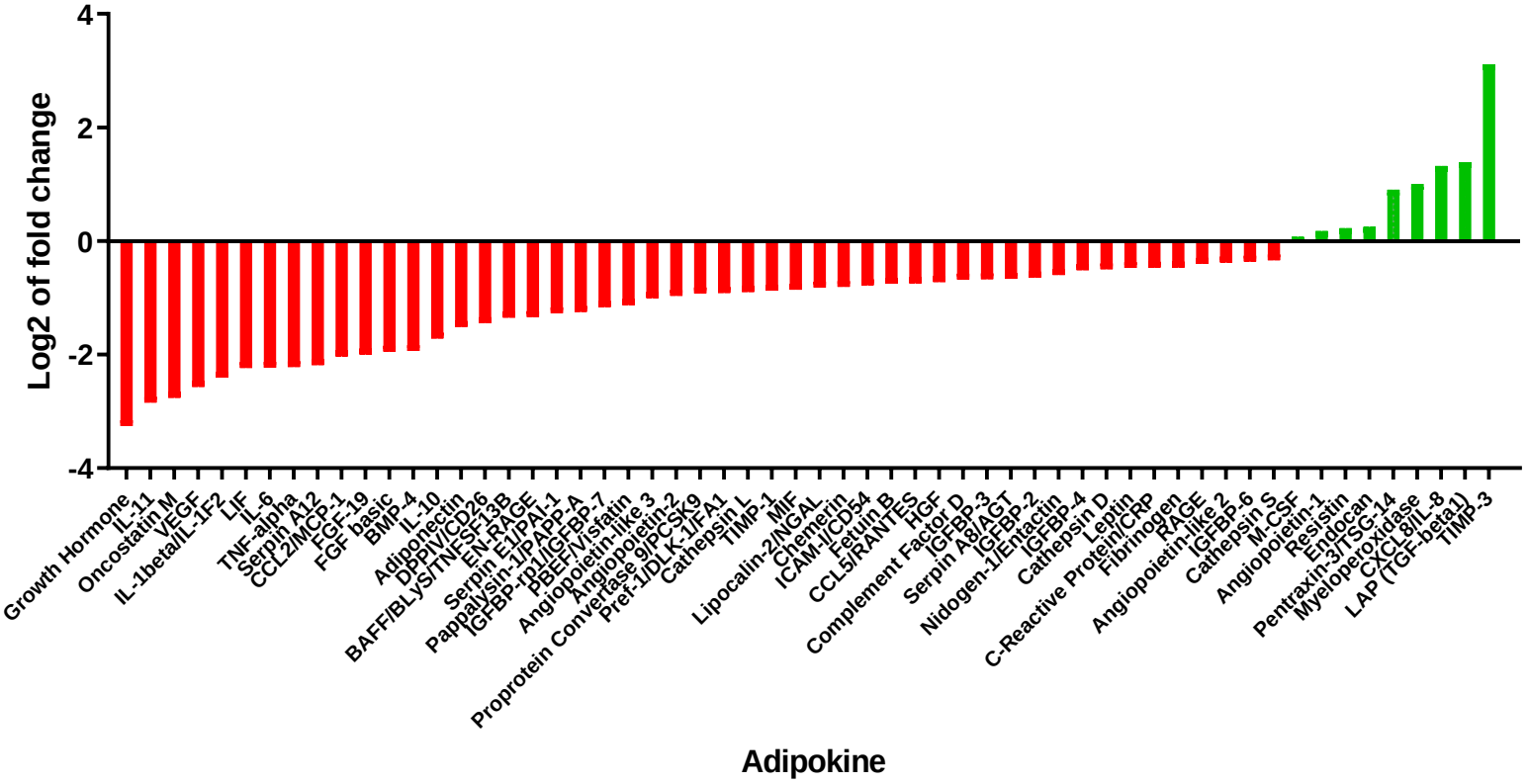

Figure 7.5: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 012. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 1.8 years, male, induction regimen A, BMI percentile day 0=75, BMI percentile day 29=70, Cytogenetic risk good, MRD risk low, % weight change -2.62, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
013	1.1	M	A	72	88	Good	Low	+5.34

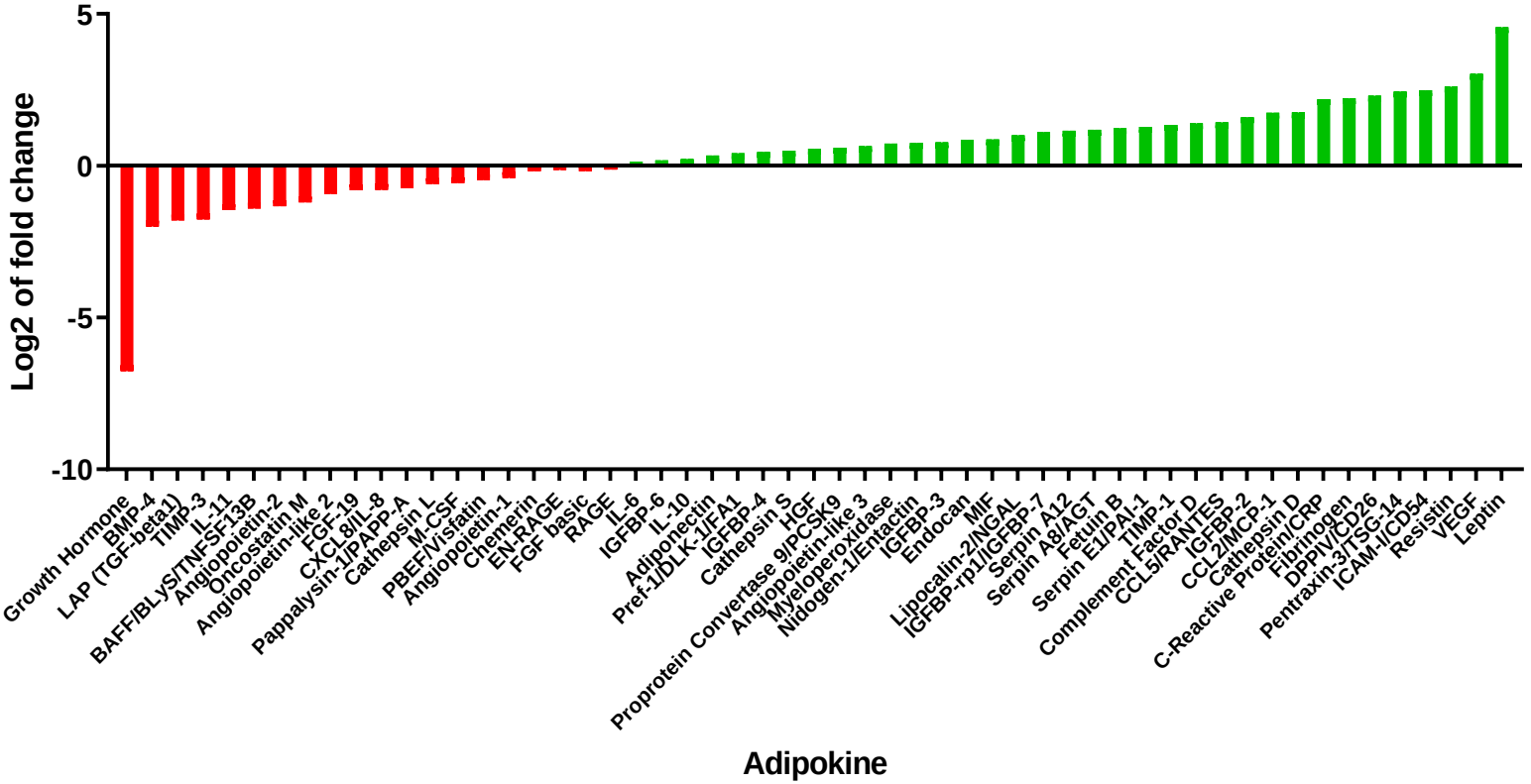

Figure 7.6: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 013 . Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 1.1 years, male, induction regimen A, BMI percentile day 0=72, BMI percentile day 29=88, Cytogenetic risk good, MRD risk low, % weight change +5.34, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
014	16	F	B	21	38	Intermediate	Low	-3.13

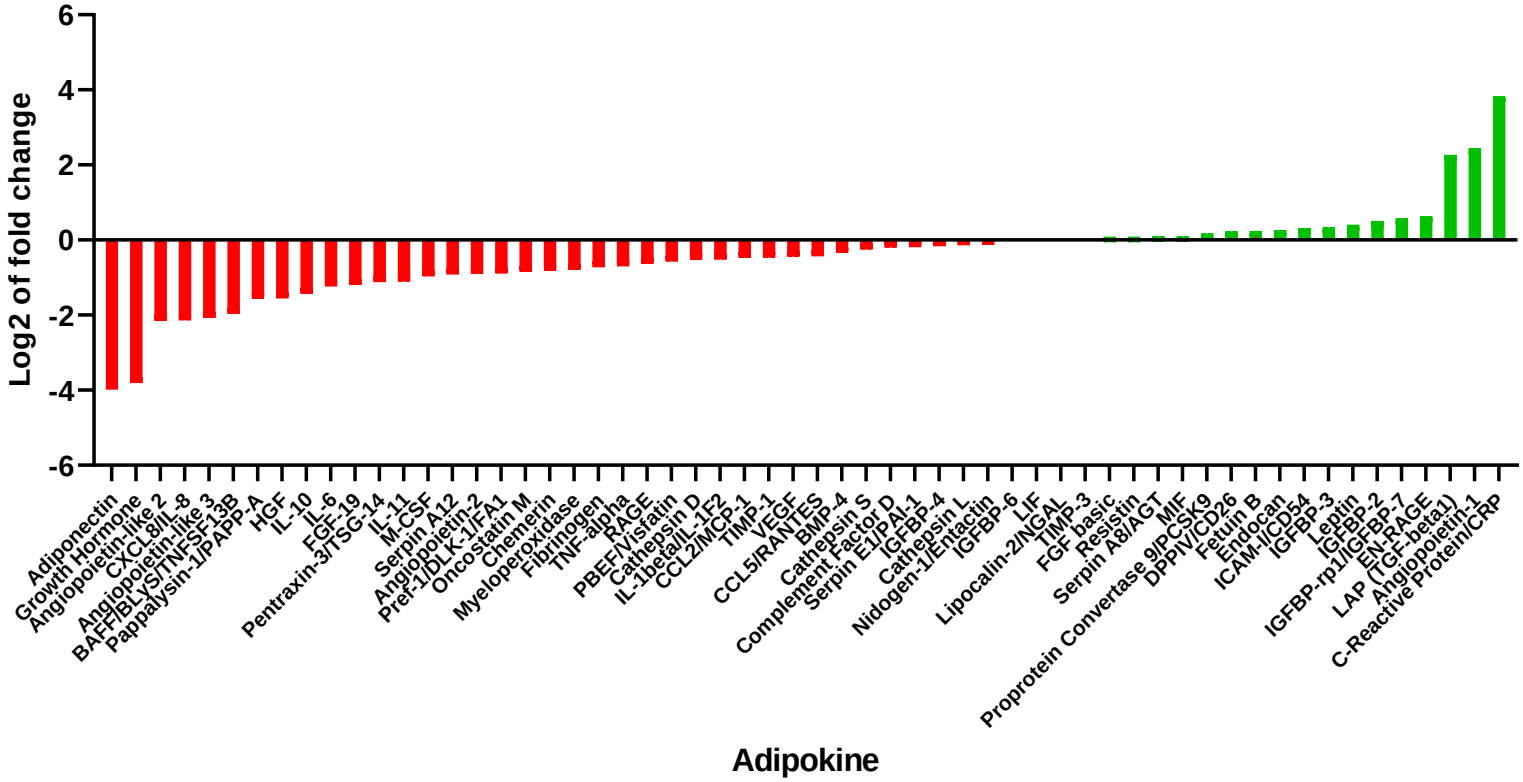

Figure 7.7: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 014 . Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in exp ression, red indicates a decrease and green an increase in expression. Patient age 16 years, female, induction regimen B, BMI percentile day 0=21, BM I percentile day 29=38, Cytogenetic risk intermediate, MRD risk low, % weight change -3.13, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
015	3.7	M	A	74	>95	Good	MRD	+22.22

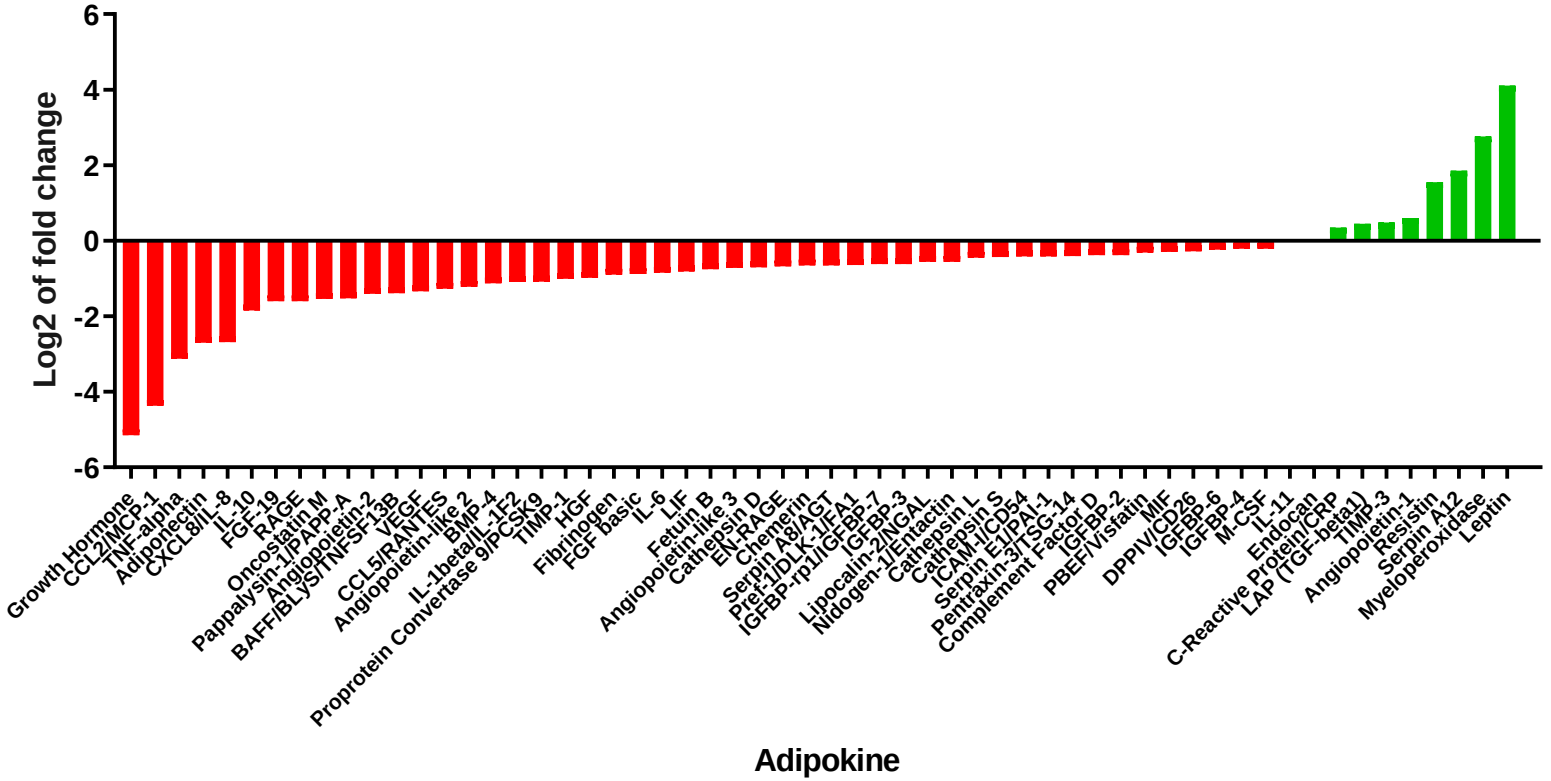

Figure 7.8: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 015 . Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 3.7 years, male, induction regimen A, BMI percentile day 0=74, BMI percentile day 29=>95, Cytogenetic risk good, MRD risk, % weight change +22.22, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
017	12	F	B	40	11	Intermediate	MRD	-11.93

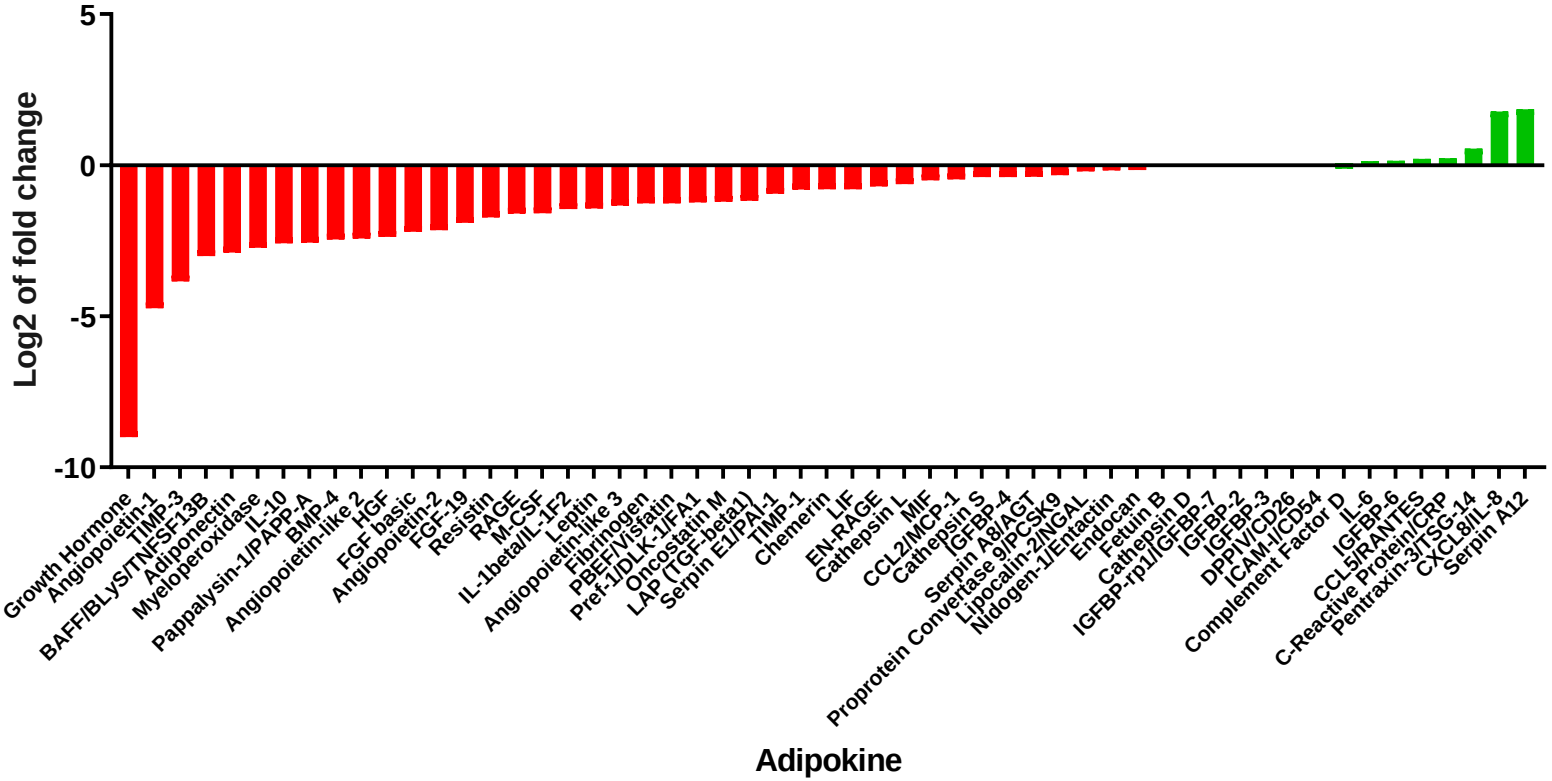

Figure 7.9: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 017 . Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in exp ression, red indicates a decrease and green an increase in expression. Patient age 12 years, female, induction regimen B, BMI percentile day 0=40, BMI percentile day 29=11, Cytogenetic risk intermediate, MRD risk, % weight change -11.93, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
018	11.8	F	B	18	13	Intermediate	MRD	-2.63

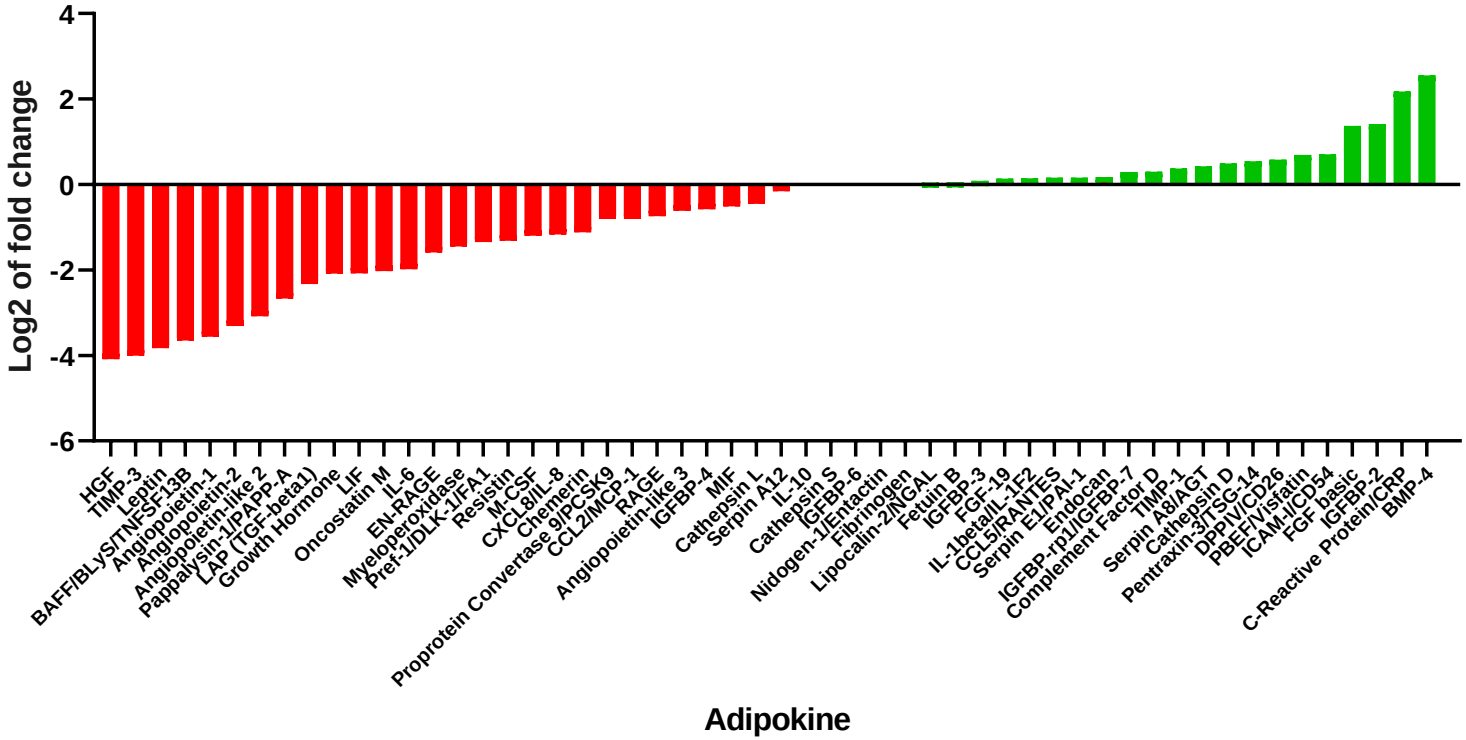

Figure 7.10: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 018. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 11.8 years, female, induction regimen B, BMI percentile day 0=18, BMI percentile day 29=13, Cytogenetic risk intermediate, MRD risk, % weight change -2.63, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
020	6.2	F	A	66	69	Good	Low	+0.66

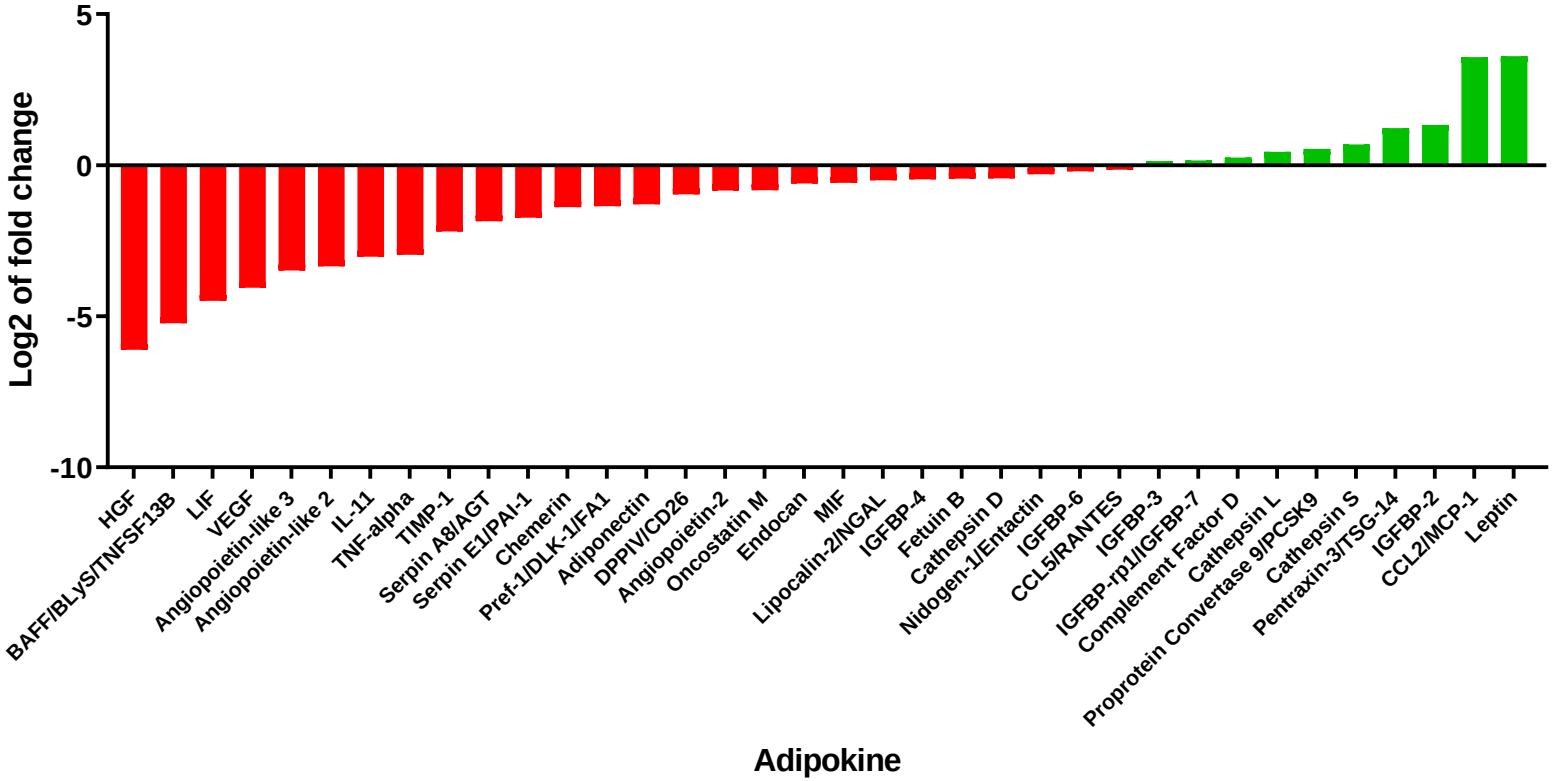

Figure 7.11: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 020. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 6.2 years, female, induction regimen A, BMI percentile day 0=66, BMI percentile day 29=69, Cytogenetic risk good, MRD risk low, % weight change +0.66, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
021	5.5	F	B	42	<5	Intermediate	Low	-11.56

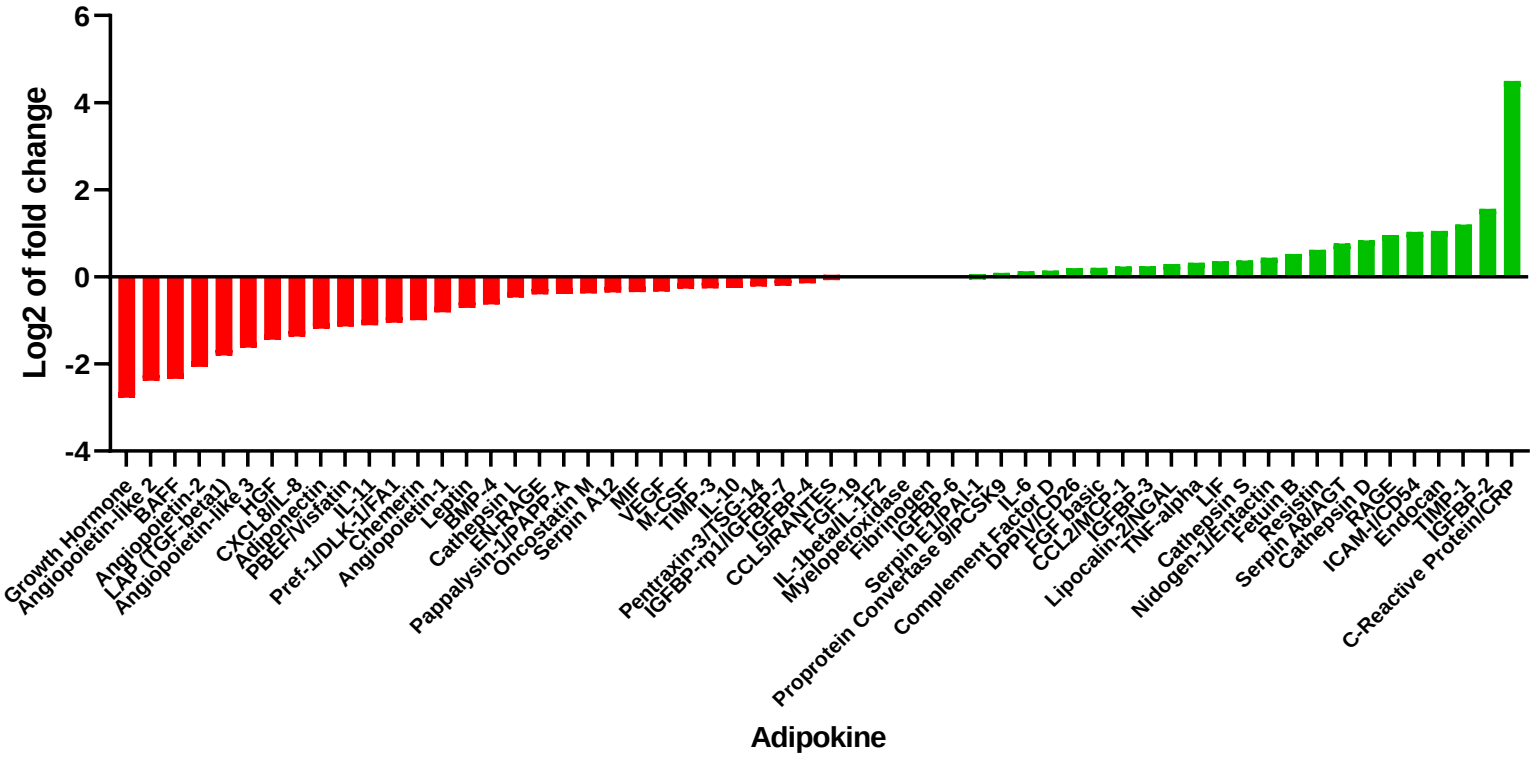

Figure 7.12: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 021. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 5.5 years, female, induction regimen B, BMI percentile day 0=42, BMI percentile day 29=<5, Cytogenetic risk intermediate, MRD risk low, % weight change +11.56, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
023	6.7	M	A	16	79	Intermediate	Mo markers	+17.27

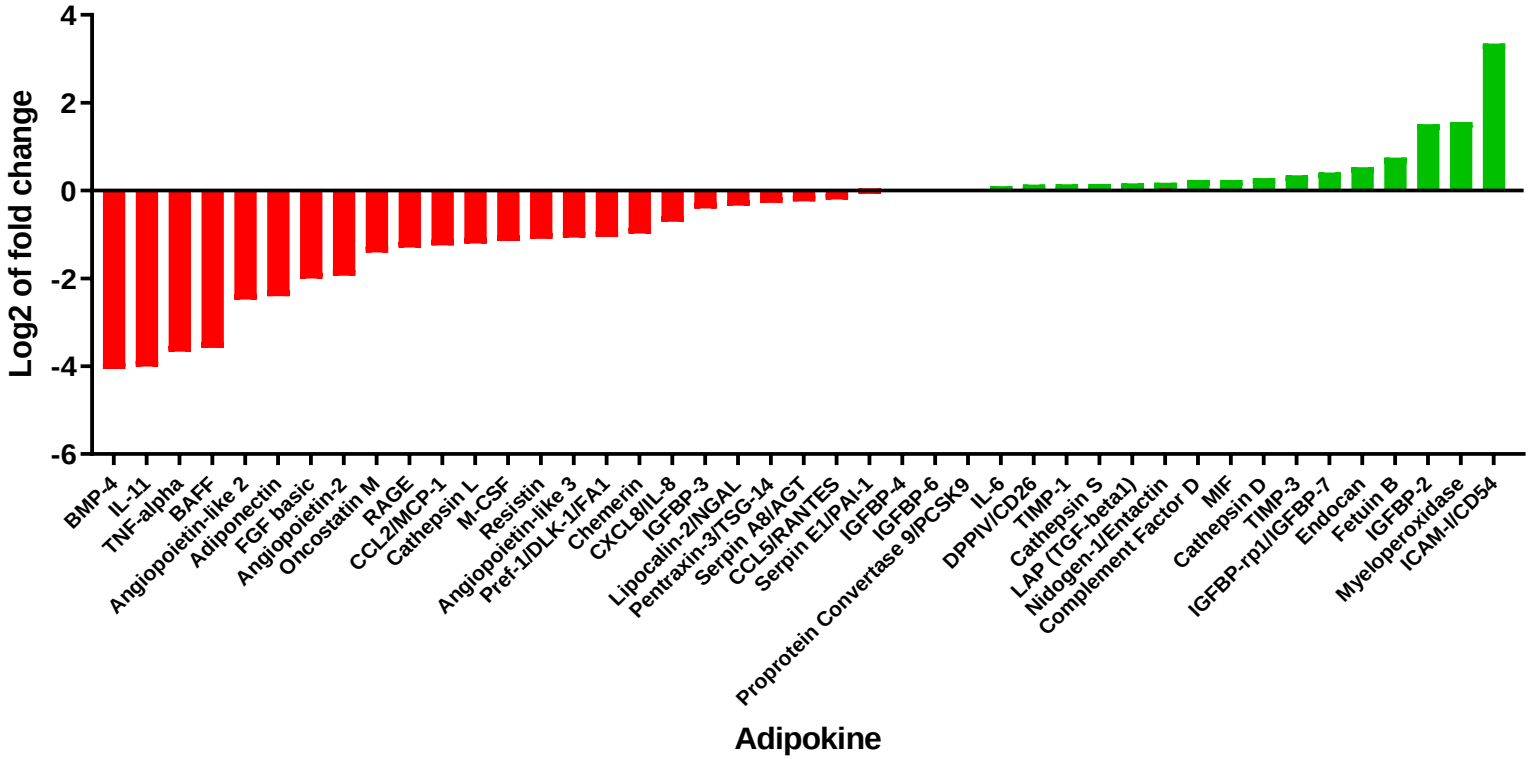

Figure 7.13: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 023. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 6.7 years, male, induction regimen A, BMI percentile day 0=16, BMI percentile day 29=79, Cytogenetic risk intermediate, no MRD markers, % weight change +17.27, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
024	3.3	M	A	75	86	Good	Low	+3.33

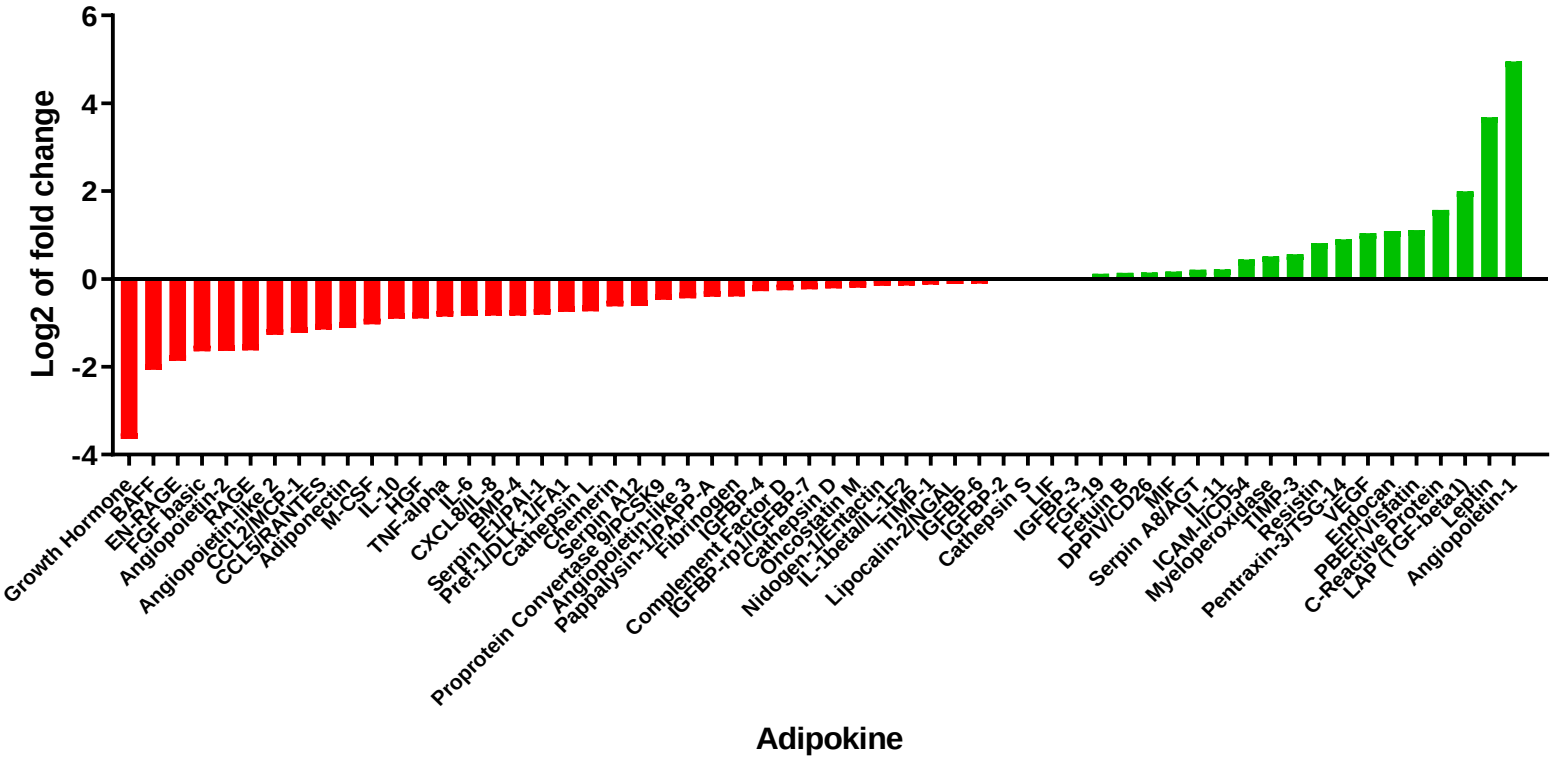

Figure 7.14: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 024. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in exp ression, red indicates a decrease and green an increase in expression. Patient age 3.3 years, male, induction regimen A, BMI percentile day 0=75, BMI percentile day 29=86, Cytogenetic risk good, MRD risk low, % weight change +3.33, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
025	6	F	A	70	45	Good	MRD	-6.1

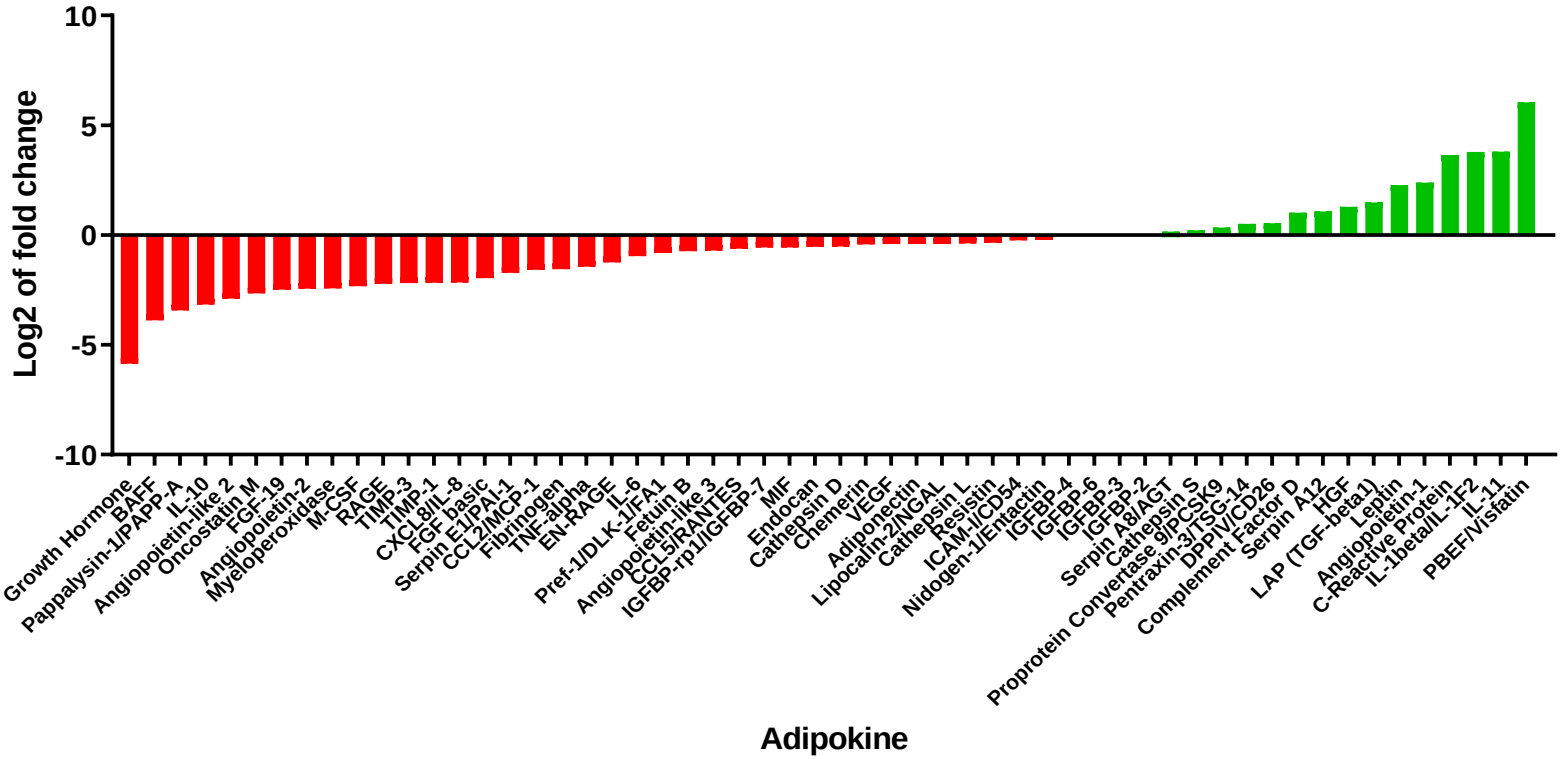

Figure 7.15: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 025. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 6 years, female, induction regimen A, BMI percentile day 0=70, BMI percentile day 29=45, Cytogenetic risk good, MRD risk, % weight change -6.1, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
026	4.2	M	A	<5	34	Intermediate	MRD	+16.26

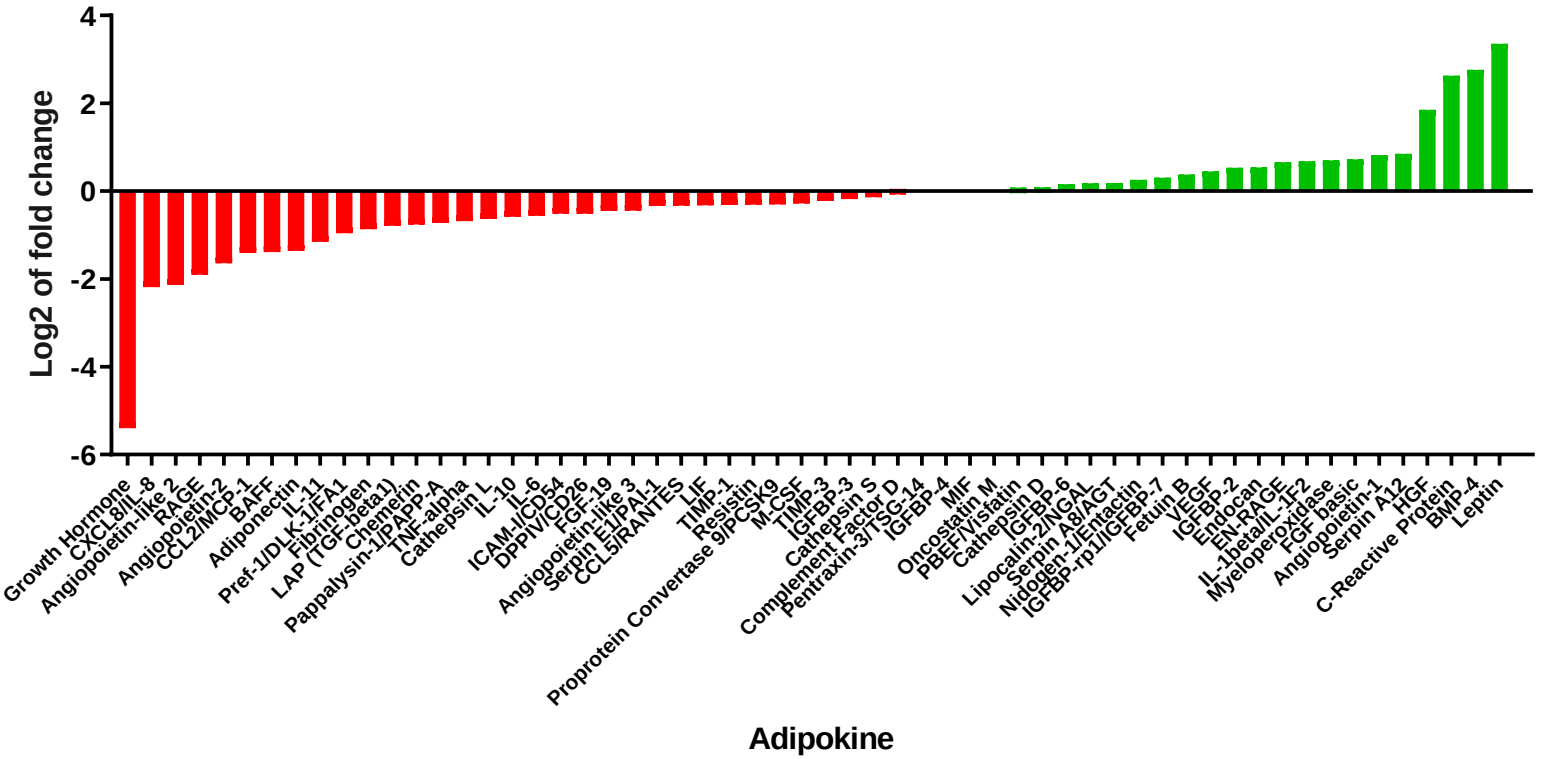

Figure 7.16: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 026. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in exp ression, red indicates a decrease and green an increase in expression. Patient age 4.2 years, male, induction regimen A, BMI percentile day 0=<5, BMI percentile day 29=34, Cytogenetic risk intermediate, MRD risk, % weight change +16.96, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
027	2.6	M	A	56	56	Good	Low	0

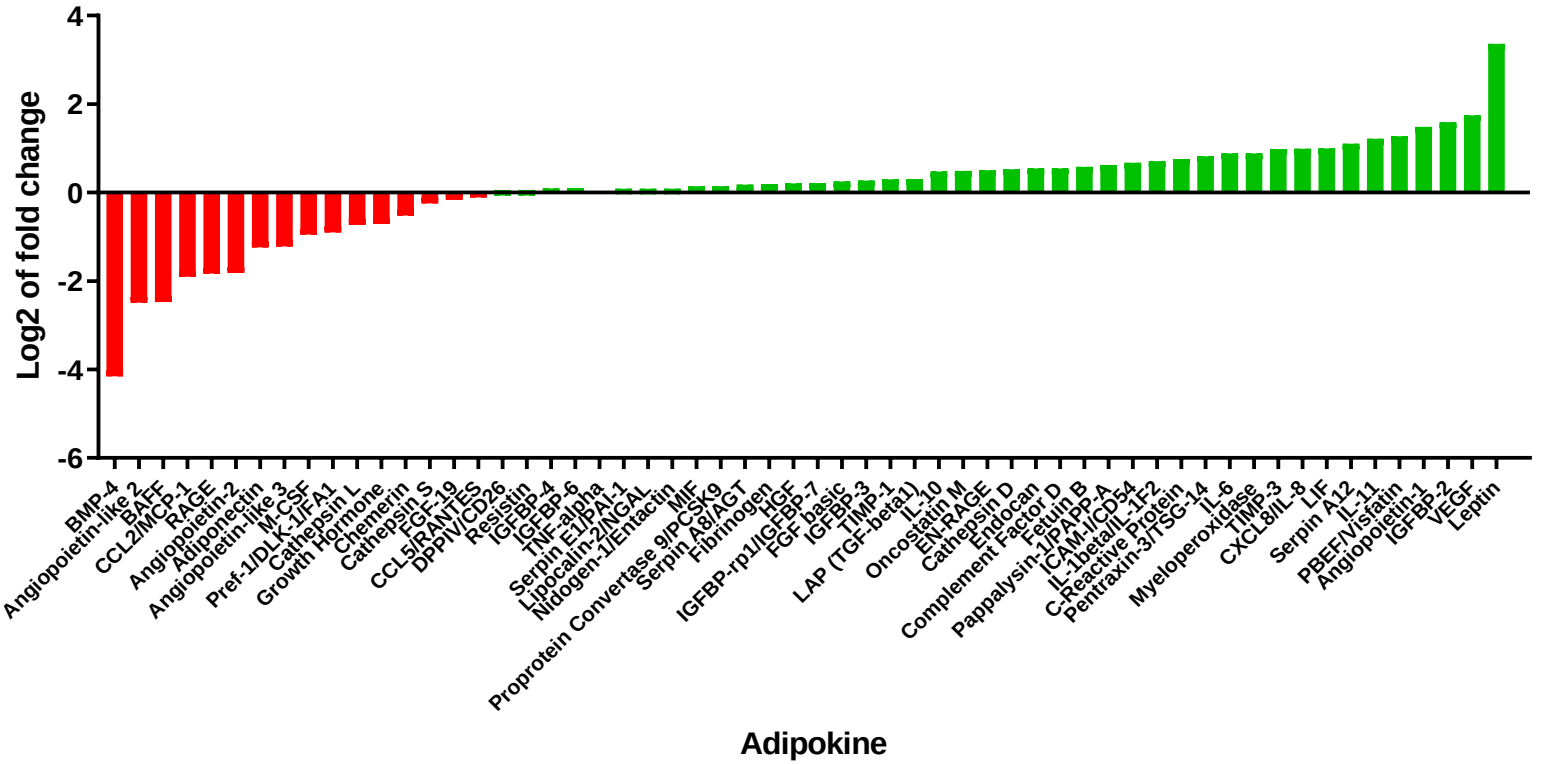

Figure 7.17: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 027. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 2.6 years, male, induction regimen A, BMI percentile day 0=56, BMI percentile day 29=56, Cytogenetic risk good, MRD risk low, % weight change 0, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
028	7.2	M	B	>95	>95	Good	Low	+1.7

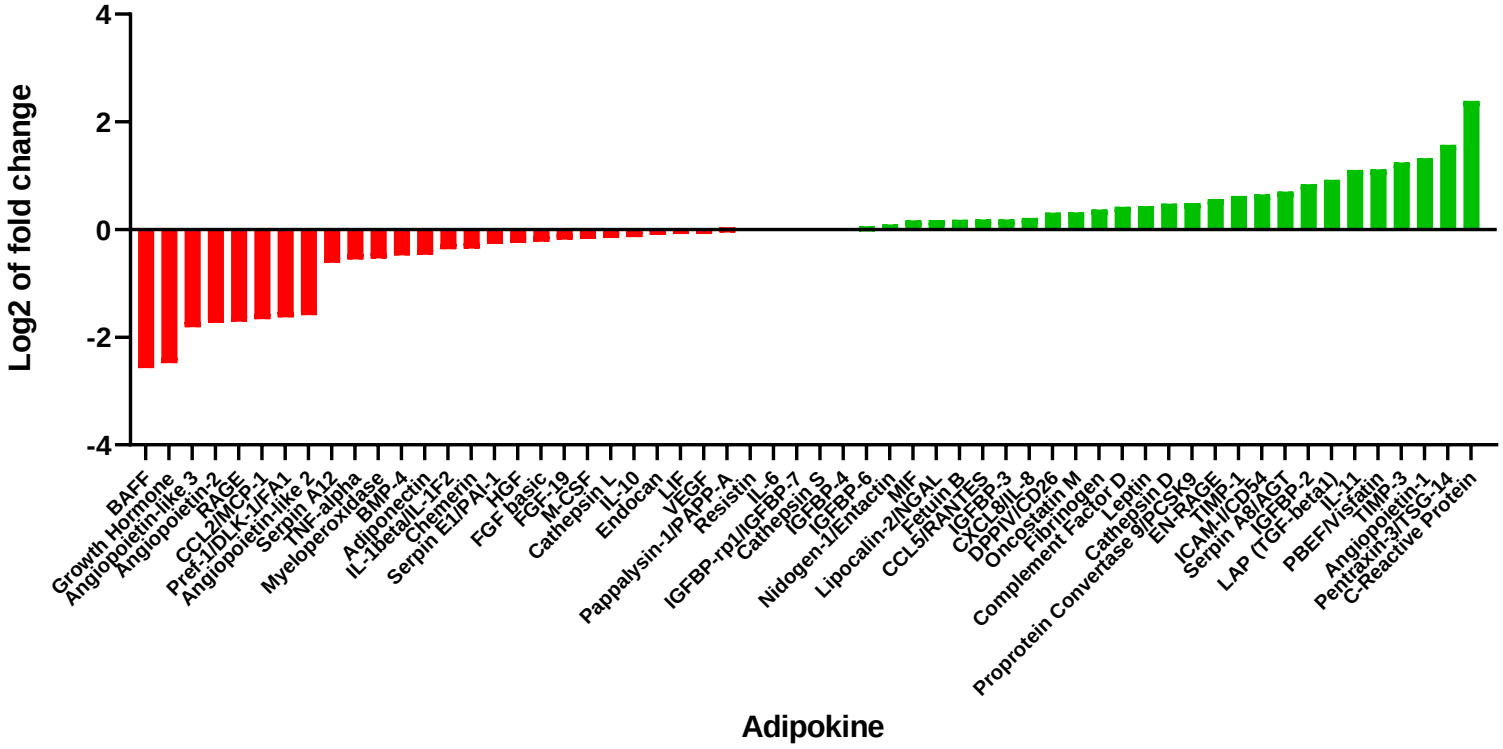

Figure 7.18: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 028. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 7.2 years, male, induction regimen B, BMI percentile day 0=>95, BMI percentile day 29=>95, Cytogenetic risk good, MRD risk low, % weight change +1.7, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
030	9.4	F	A	88	91	Intermediate	Low	+4.05

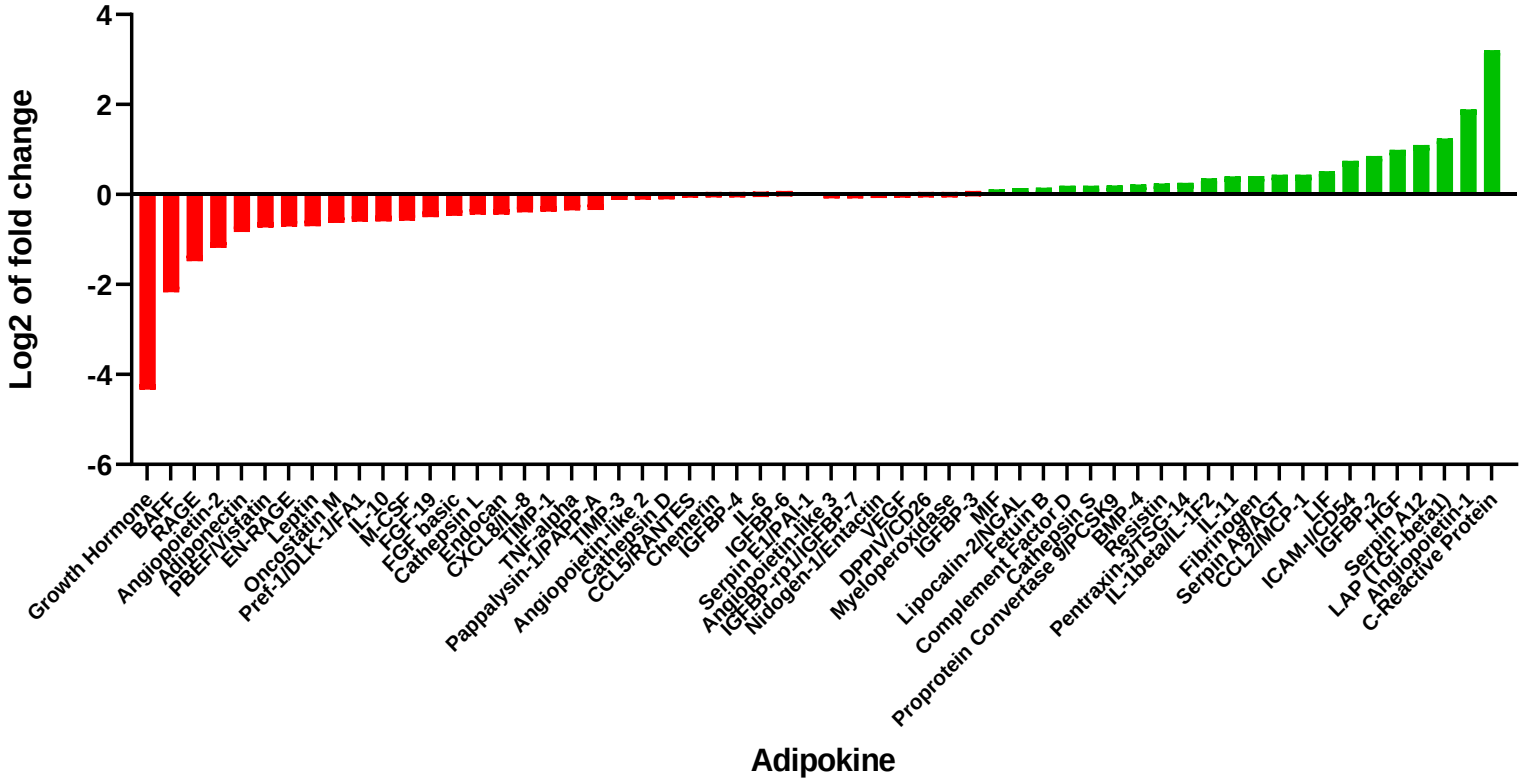

Figure 7.19: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 030. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 9.4 years, female, induction regimen A, BMI percentile day 0=88, BMI percentile day 29=91, Cytogenetic risk intermediate, MRD risk low, % weight change +4.05, n=1

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
031	12.7	M	B	25	30	T-cell	MRD	+1.66

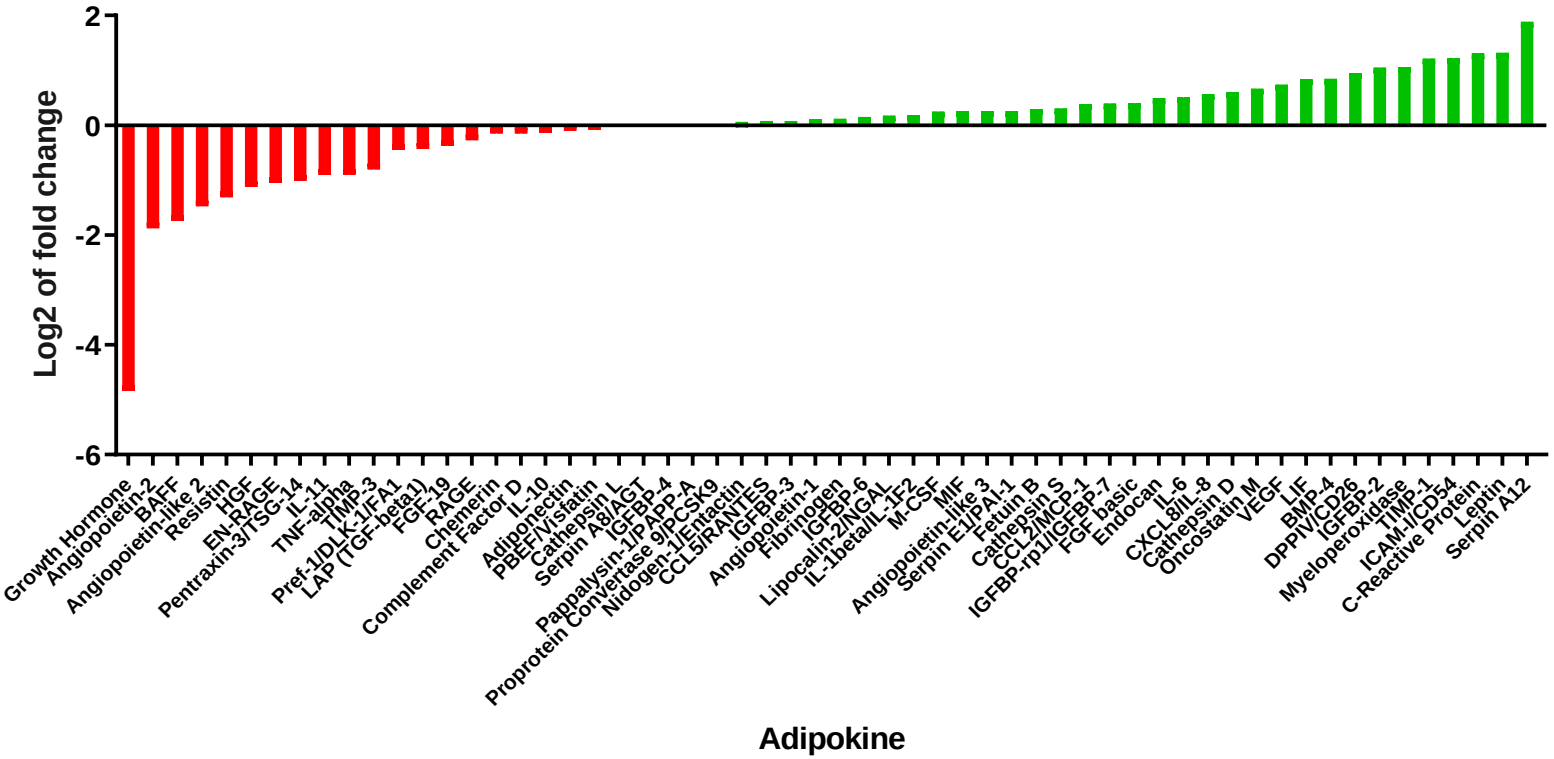

Figure 7.20: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 031. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 12.7 years, male, induction regimen B, BMI percentile day 0=25, BMI percentile day 29=30, Cytogenetic risk T-cell disease, MRD risk, % weight change +1.66, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
032	11.5	M	B	29	43	Good	Low	+4.5

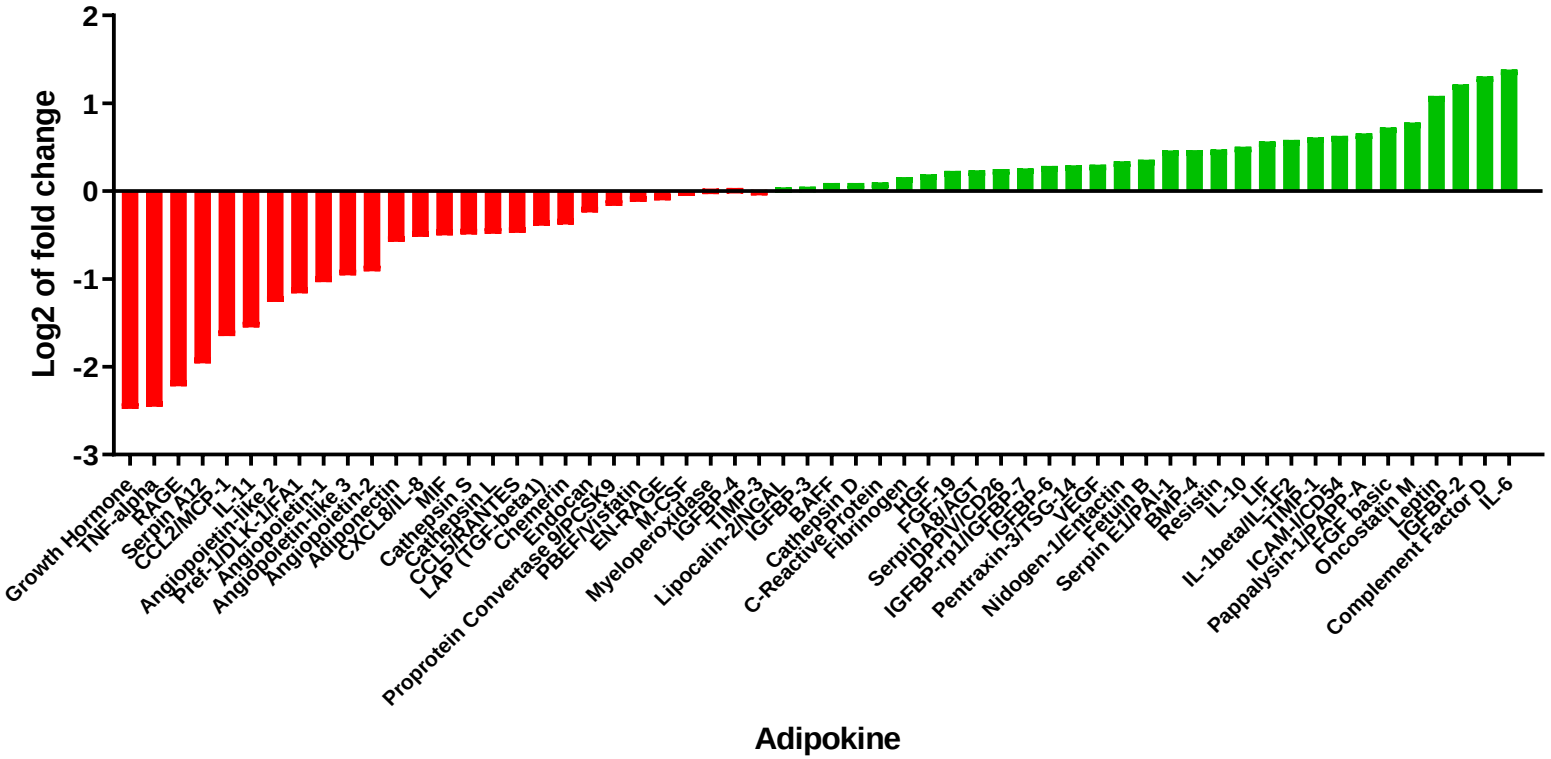

Figure 7.21: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 032. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in exp ression, red indicates a decrease and green an increase in expression. Patient age 11.5 years, male, induction regimen B, BMI percentile day 0=29, BMI percentile day 29=43, Cytogenetic risk good, MRD risk low, % weight change +4.5, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
034	1.8	M	A	31	45	Good	Low	+2.67

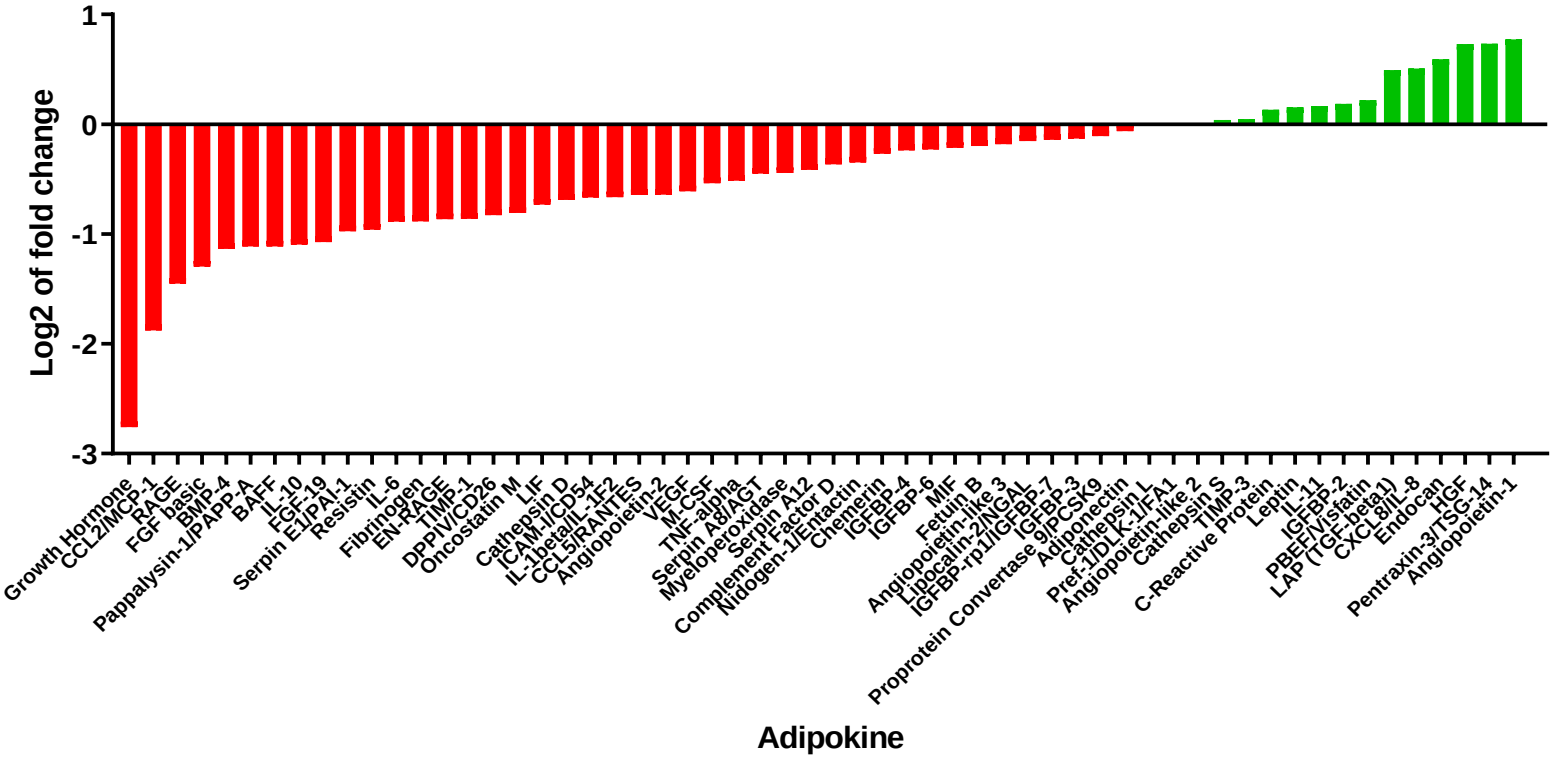

Figure 7.22: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 034. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 1.8 years, male, induction regimen A, BMI percentile day 0=31, BMI percentile day 29=45, Cytogenetic risk good, MRD risk low, % weight change +2.67, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
035	12.1	F	B	9	16	Intermediate	MRD	+3.68

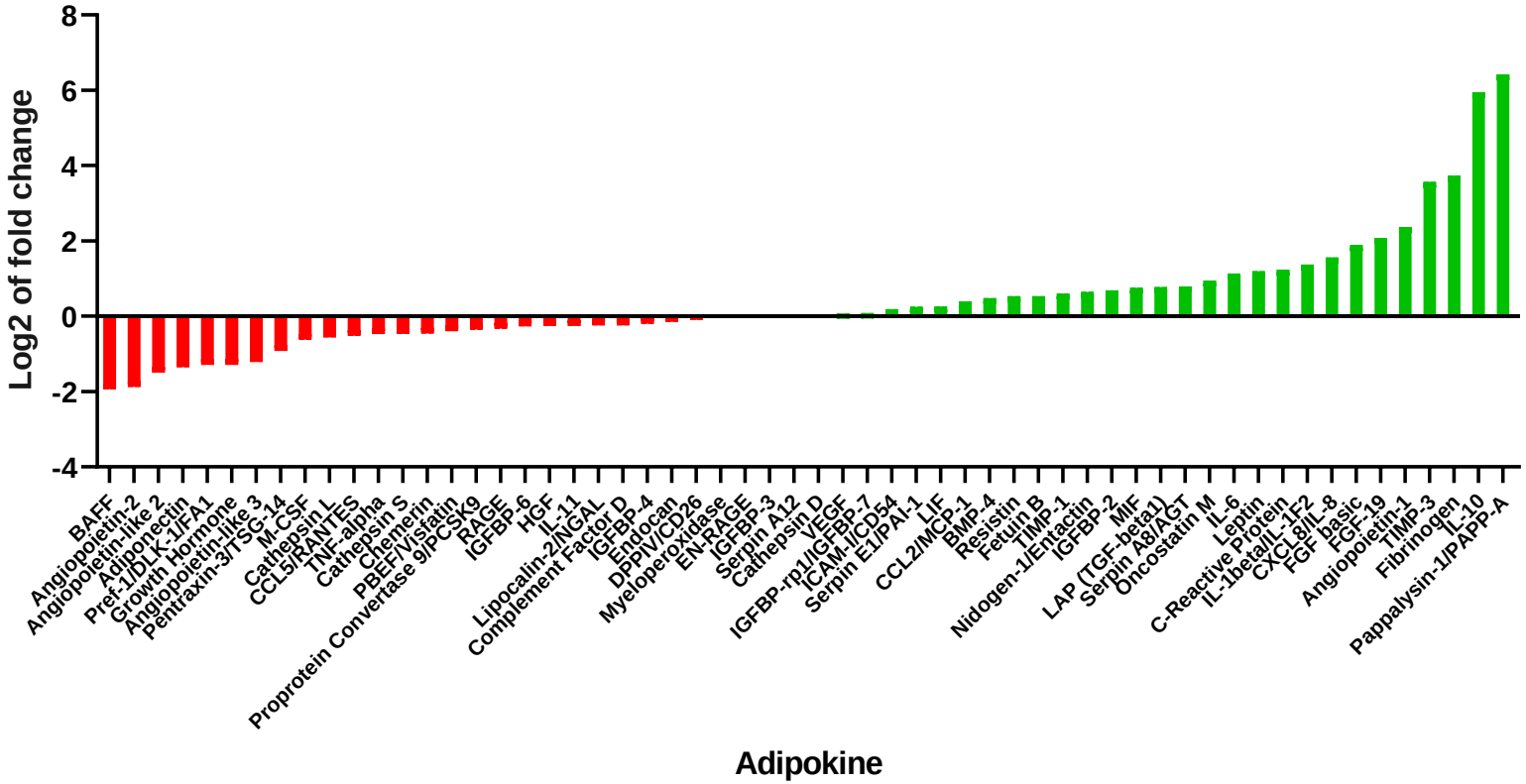

Figure 7.23: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 035 . Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in exp ression, red indicates a decrease and green an increase in expression. Patient age 12.1 years, female, induct ion regimen B, BMI percentile day 0=9, BMI percentile day 29=16, Cytogenetic risk intermediate, MRD risk, % weight change +3.68, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
036	2.7	M	A	31	>95	Intermediate	Low	+27.36

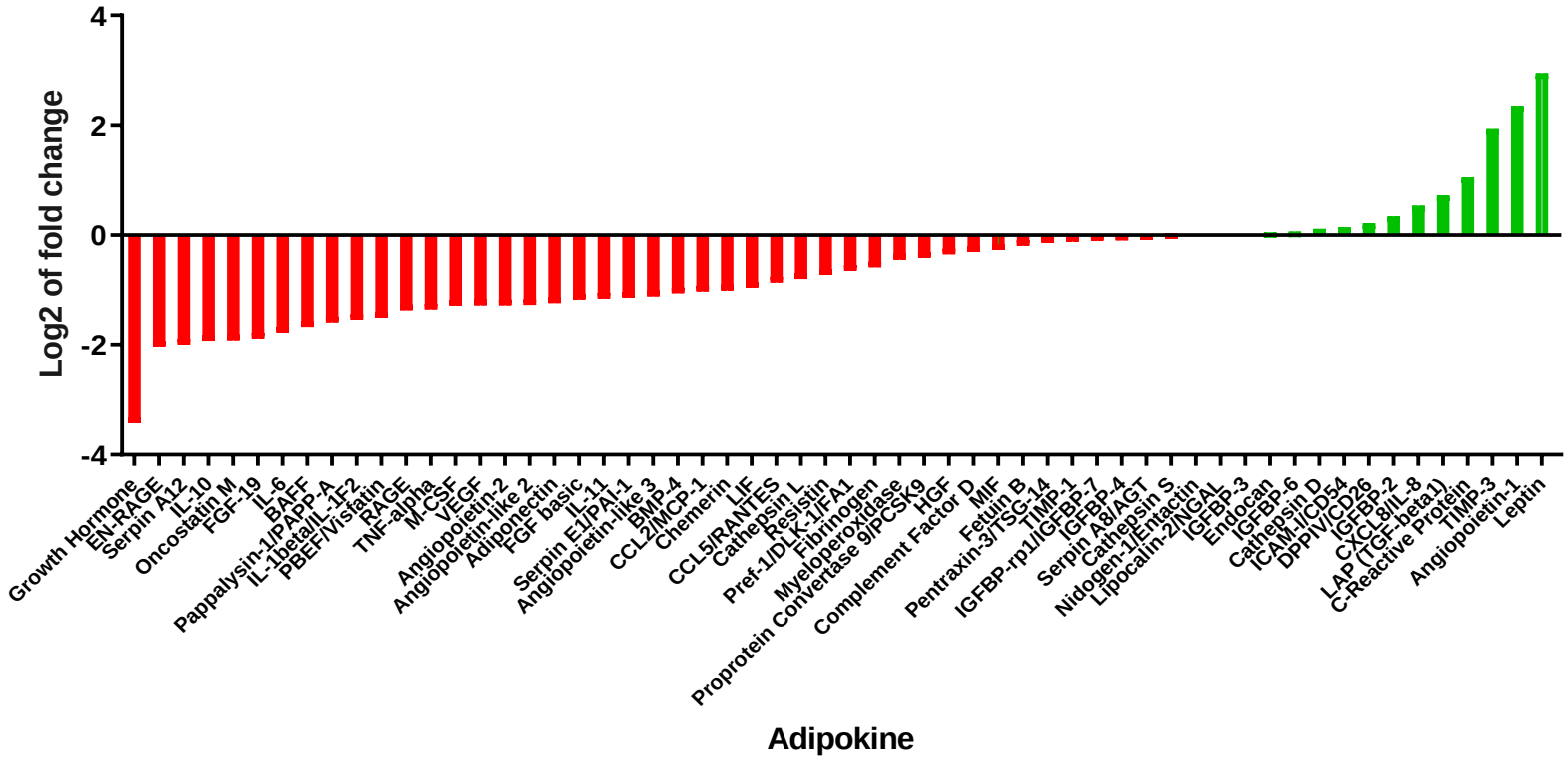

Figure 7.24: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 036. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in exp ression, red indicates a decrease and green an increase in expression. Patient age 2.7 years, male, induction regimen A, BMI percentile day 0=31, BMI percentile day 29=>95, Cytogenetic risk intermediate, MRD risk low, % weight change +27.36, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
037	4.6	F	A	25	86	Intermediate	MRD	+16.77

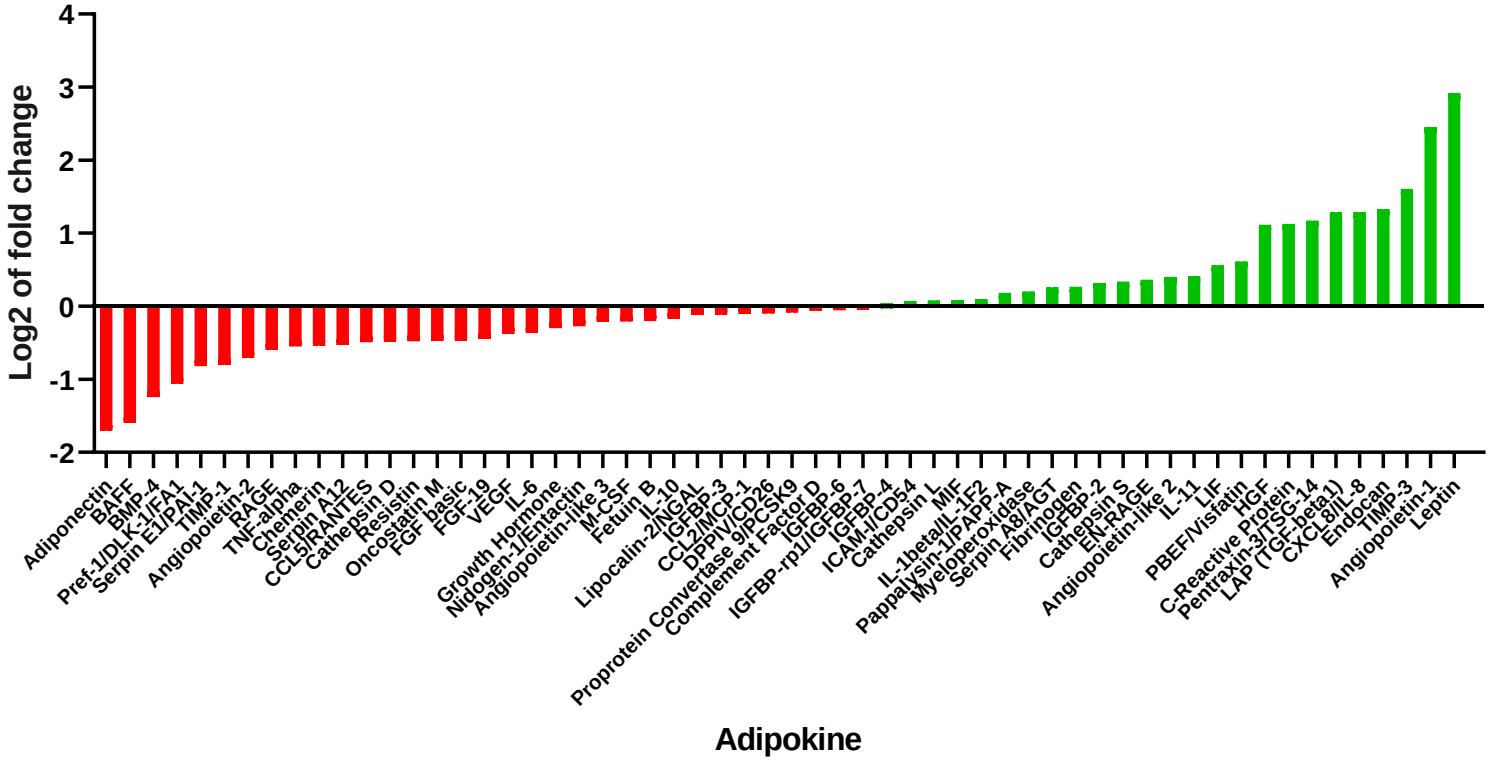

Figure 7.25: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 037. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 4.6 years, female, induction regimen A, BMI percentile day 0=25, BMI percentile day 29=86, Cytogenetic risk intermediate, MRD risk, % weight change +16.77, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
038	3.2	M	B	29	31	Good	Low	+0.42

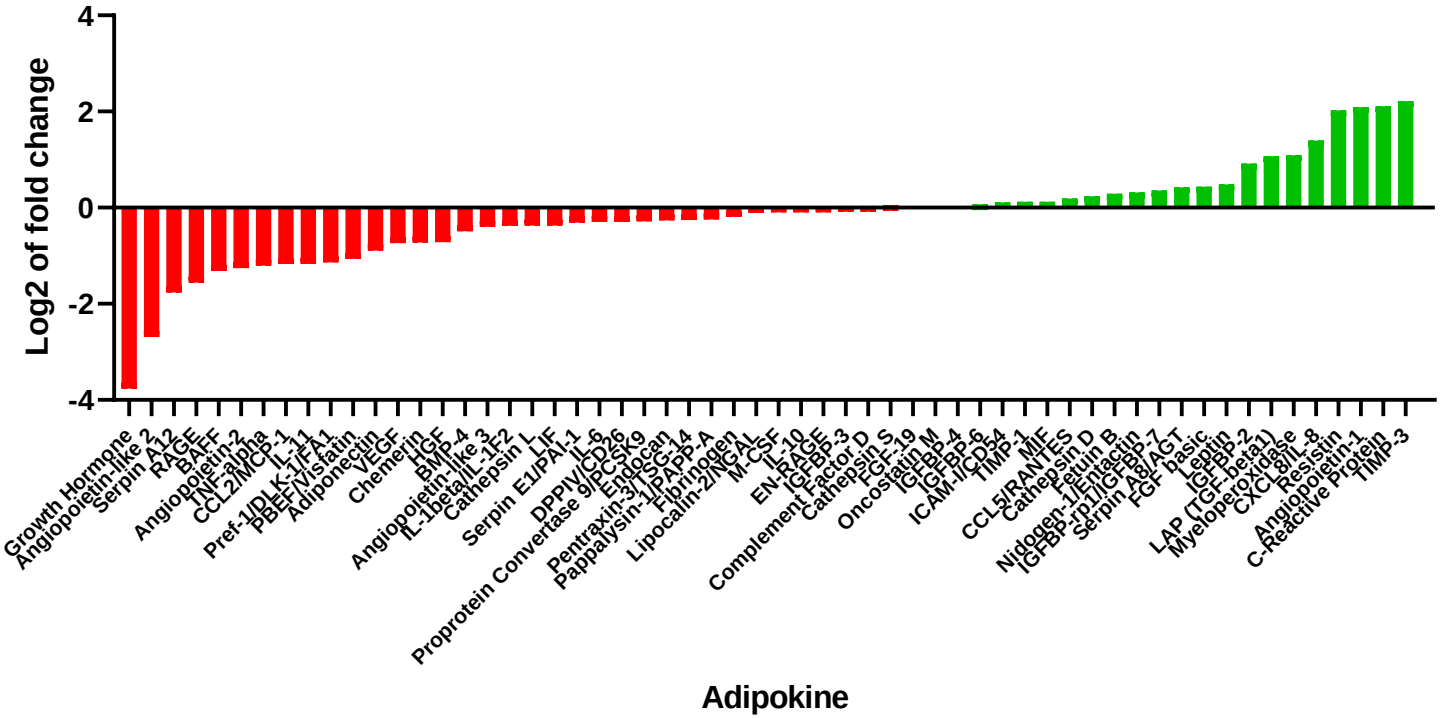

Figure 7.26: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 038. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 3.2 years, male, induction regimen B, BMI percentile day 0=29, BMI percentile day 29=31, Cytogenetic risk good, MRD risk low, % weight change +0.42, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
040	2.9	F	A	90	86	Intermediate	Low	-2.01

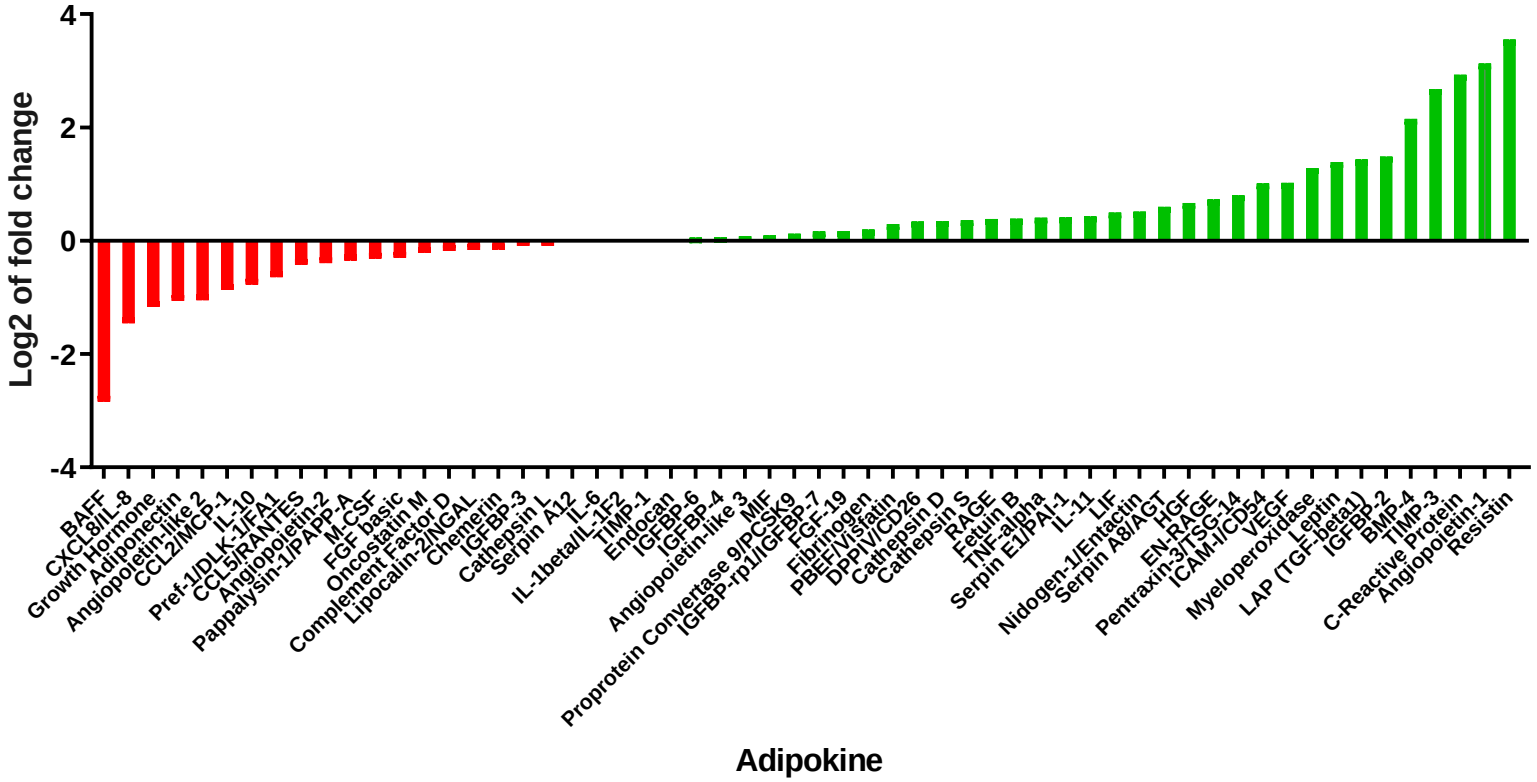

Figure 7.27: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 040. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 2.9 years, female, induction regimen A, BMI percentile day 0=90, BMI percentile day 29=86, Cytogenetic risk intermediate, MRD risk low, % weight change -2.01, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
041	2.8	M	A	49	67	Good	Low	+3.72

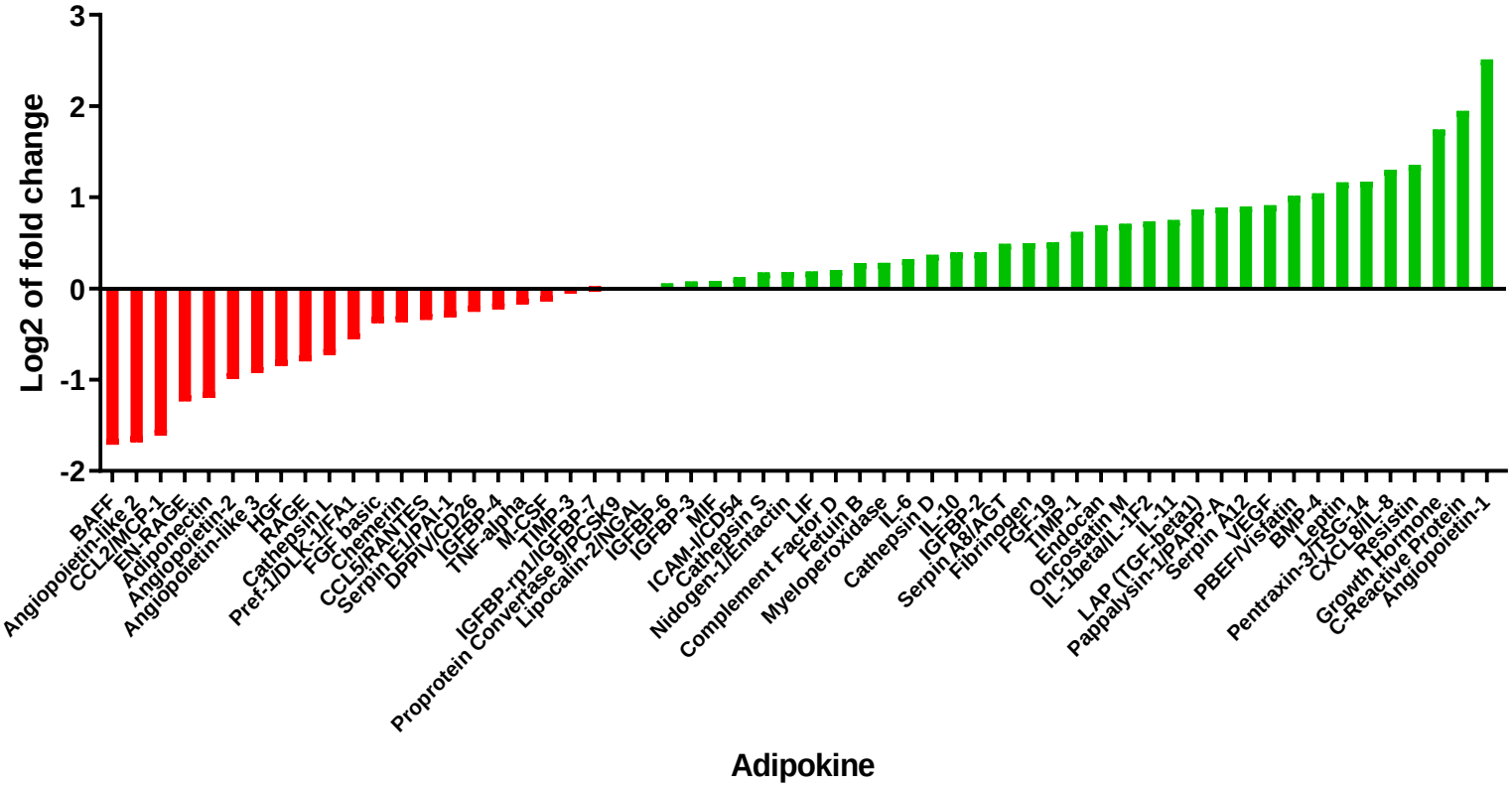

Figure 7.28: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 041. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 2.8 years, male, induction regimen A, BMI percentile day 0=49, BMI percentile day 29=67, Cytogenetic risk good, MRD risk low, % weight change +3.72, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
044	10.3	F	B	90	88	Intermediate	Low	-2.06

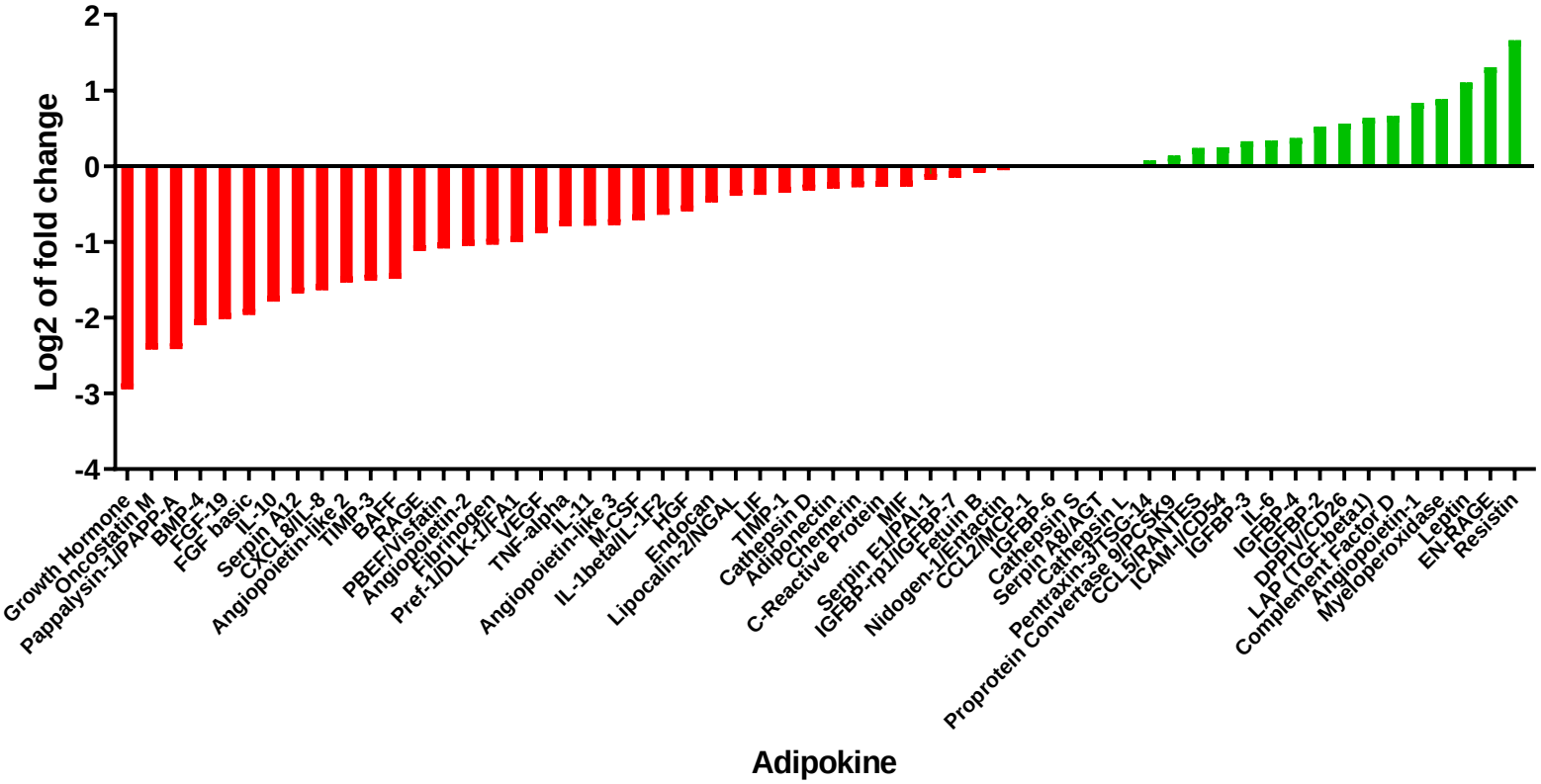

Figure 7.29: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 044. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 10.3 years, female, induction regimen B, BMI percentile day 0=90, BMI percentile day 29=88, Cytogenetic risk intermediate, MRD risk low, % weight change -2.06, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
045	4.1	M	A	79	>95	Good	Low	+24.43

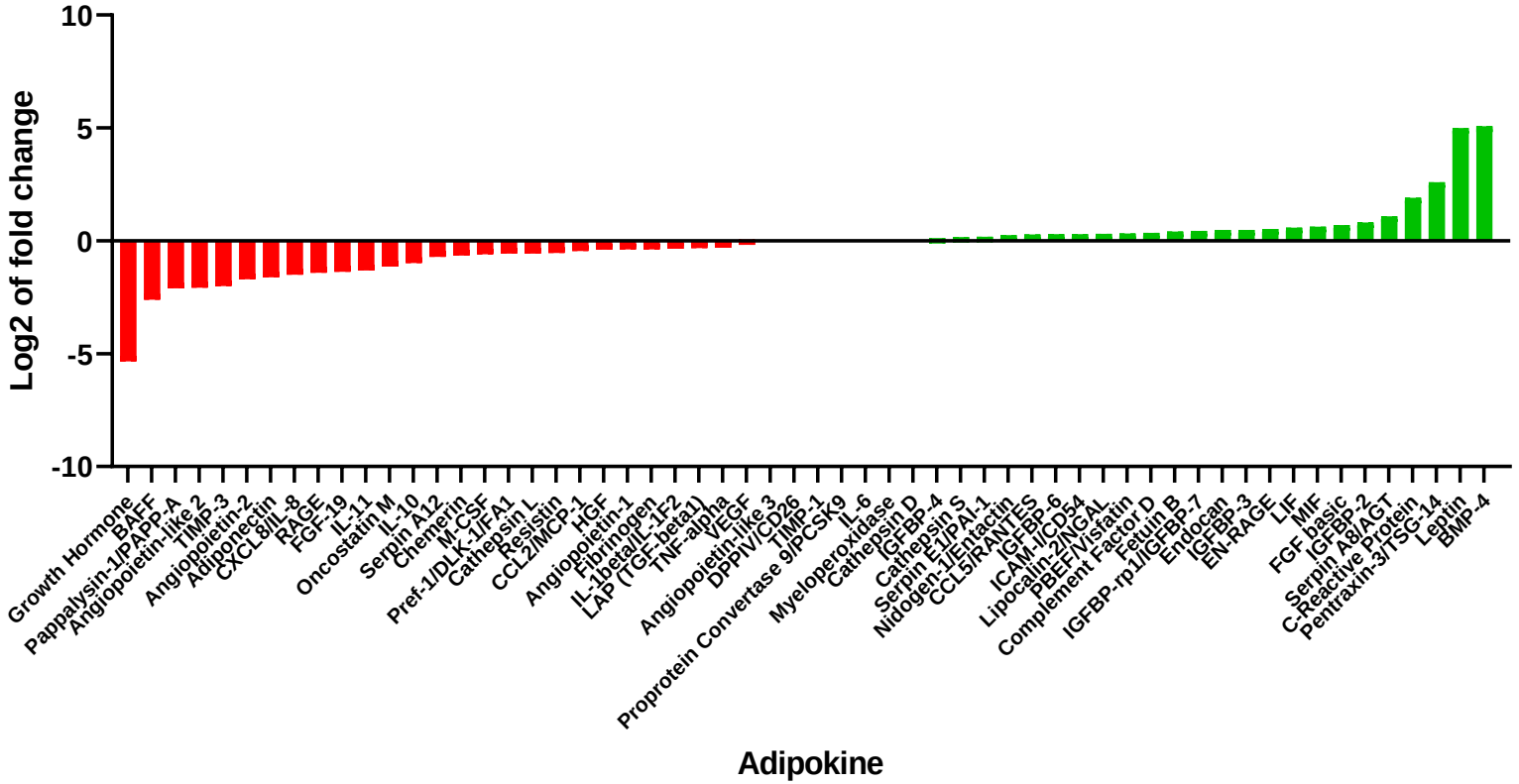

Figure 7.30: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 045 . Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 4.1 years, male, induction regimen A, BMI percentile day 0=79, BMI percentile day 29=>95, Cytogenetic risk good, MRD risk low, % weight change +24.43, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
046	6.2	M	A	47	73	Intermediate	MRD	+6.47

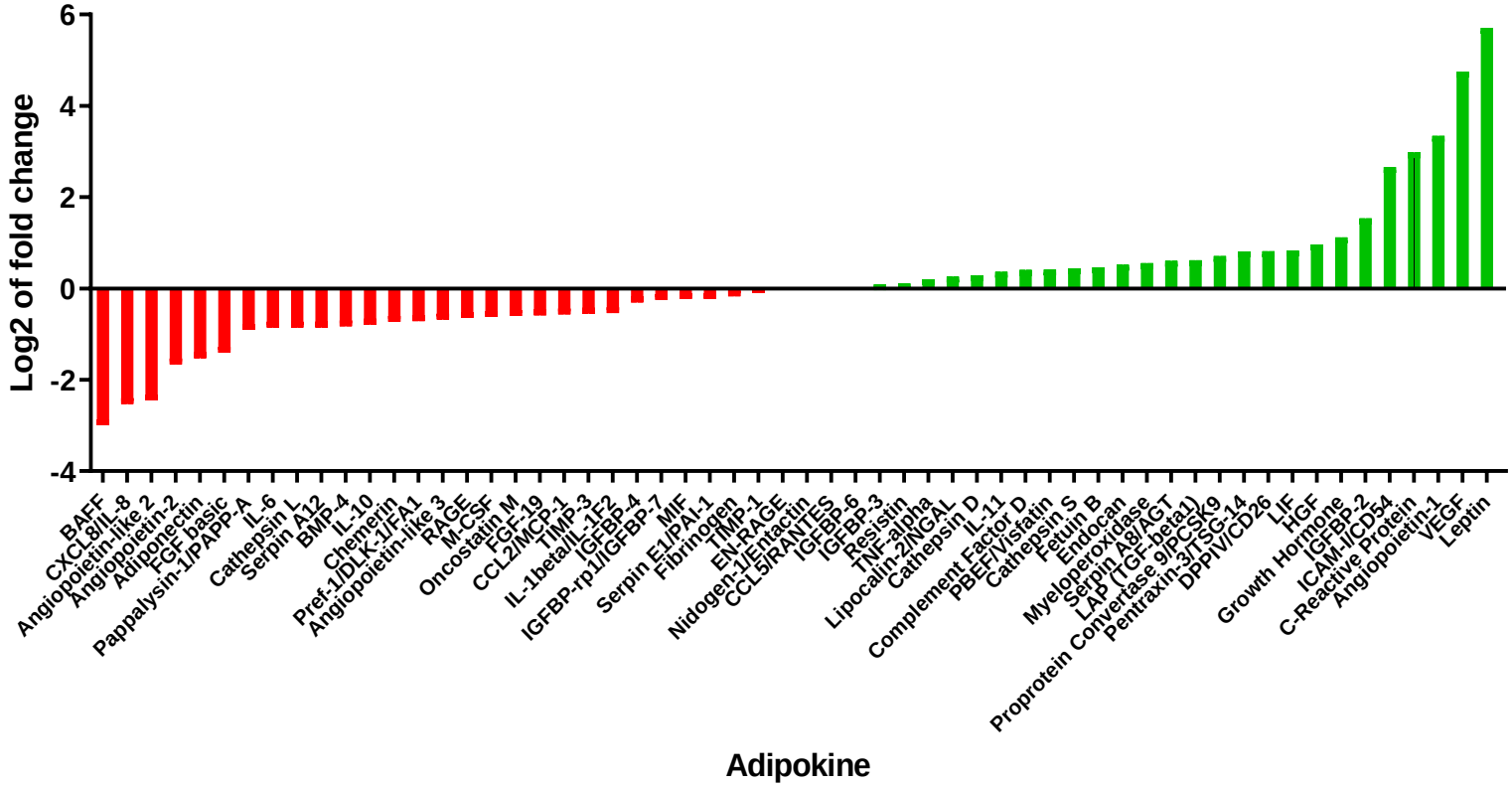

Figure 7.31: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 046 . Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in exp ression, red indicates a decrease and green an increase in expression. Patient age 6.2 years, male, induction regimen A, BMI percentile day 0=47, BMI percentile day 29=73, Cytogenetic risk intermediate, MRD risk, % weight change +6.47, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
047	4.7	M	A	22	80	Intermediate	MRD	+12.93

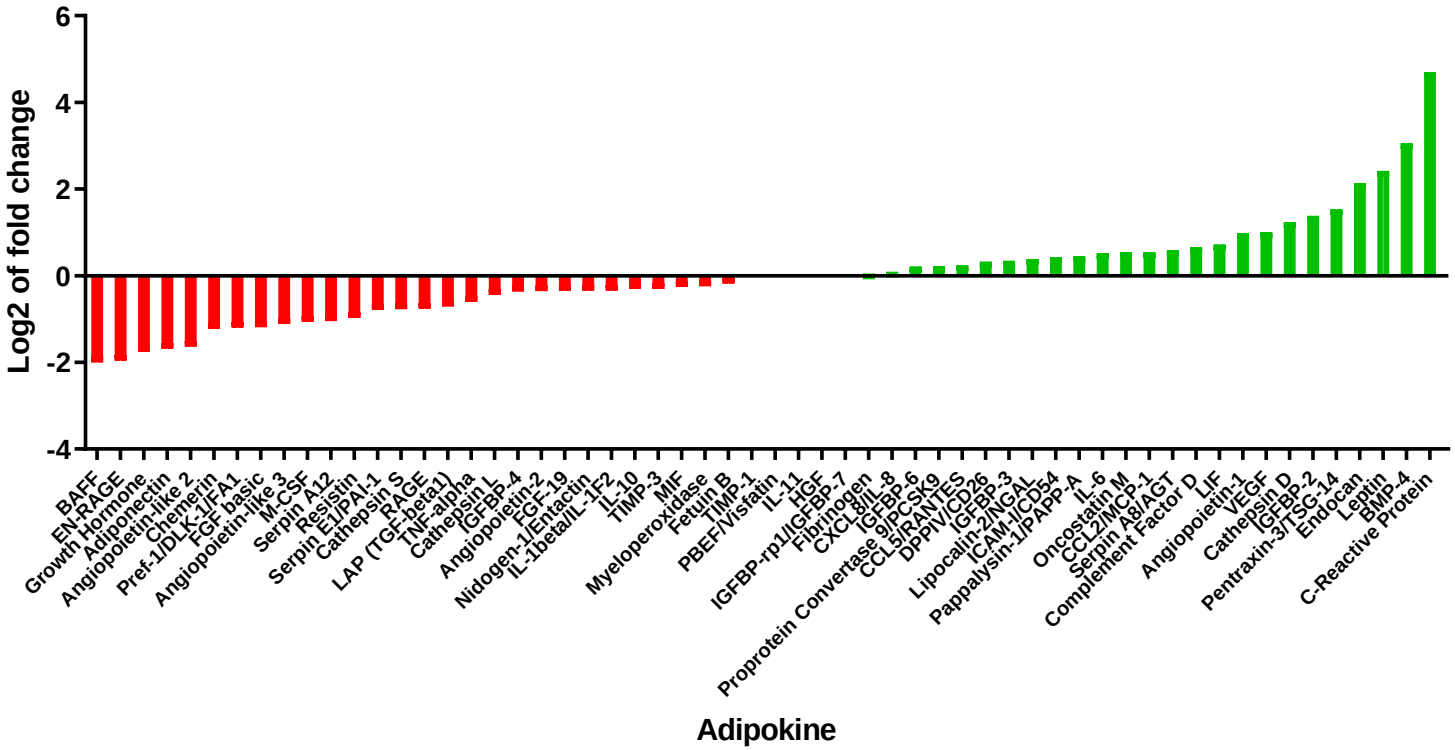

Figure 7.32: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 047. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in exp ression, red indicates a decrease and green an increase in expression. Patient age 4.7 years, male, induction regimen A, BMI percentile day 0=22, BMI percentile day 29=80, Cytogenetic risk intermediate, MRD risk, % weight change +12.93, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
048	9	M	B	45	18	Tcell disease	MRD	-7.14

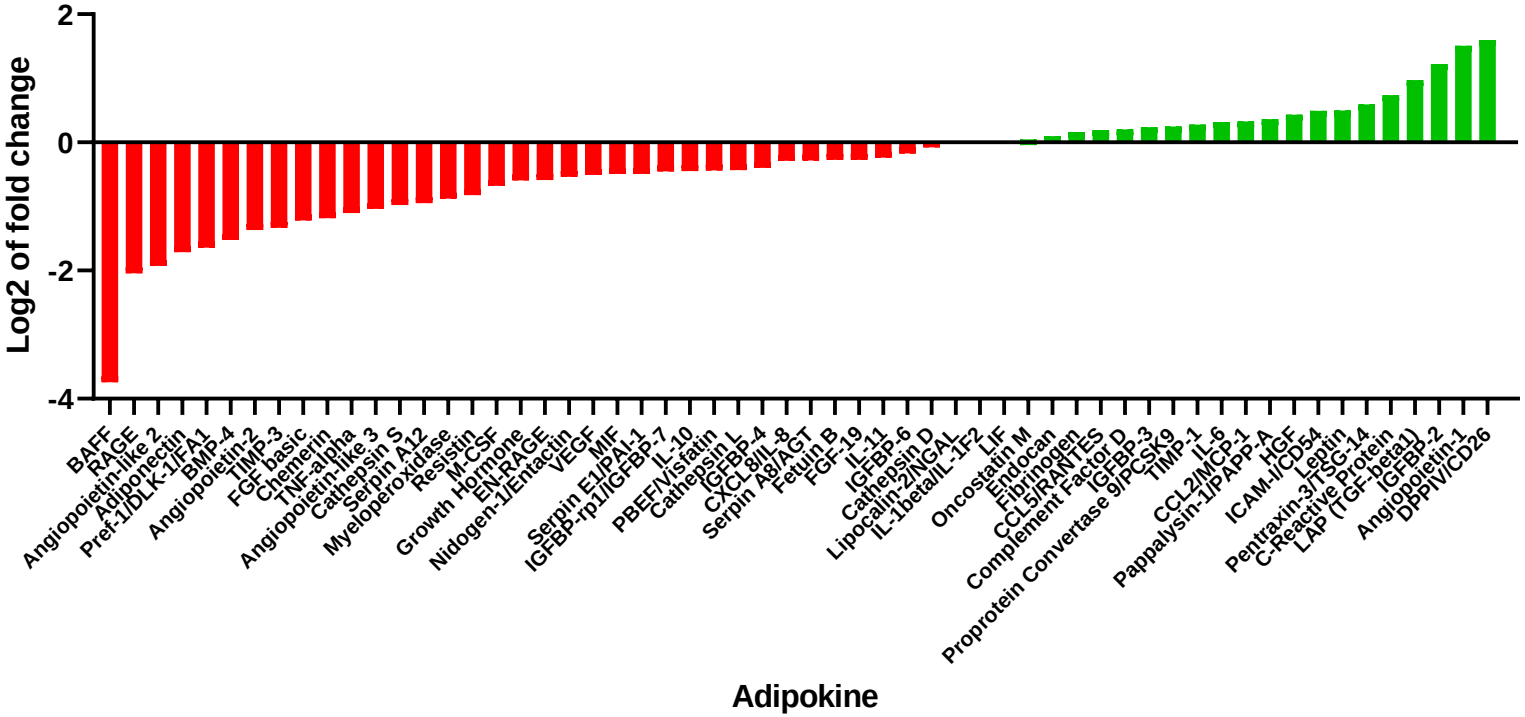

Figure 7.33: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 048 . Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 9 years, male, induction regimen B, BMI percentile day 0=45, BMI percentile day 29=18, Cytogenetic- risk T Cell disease, MRD risk, % weight change -7.14, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
049	4.8	F	A	77	59	Good	MRD	-4.73

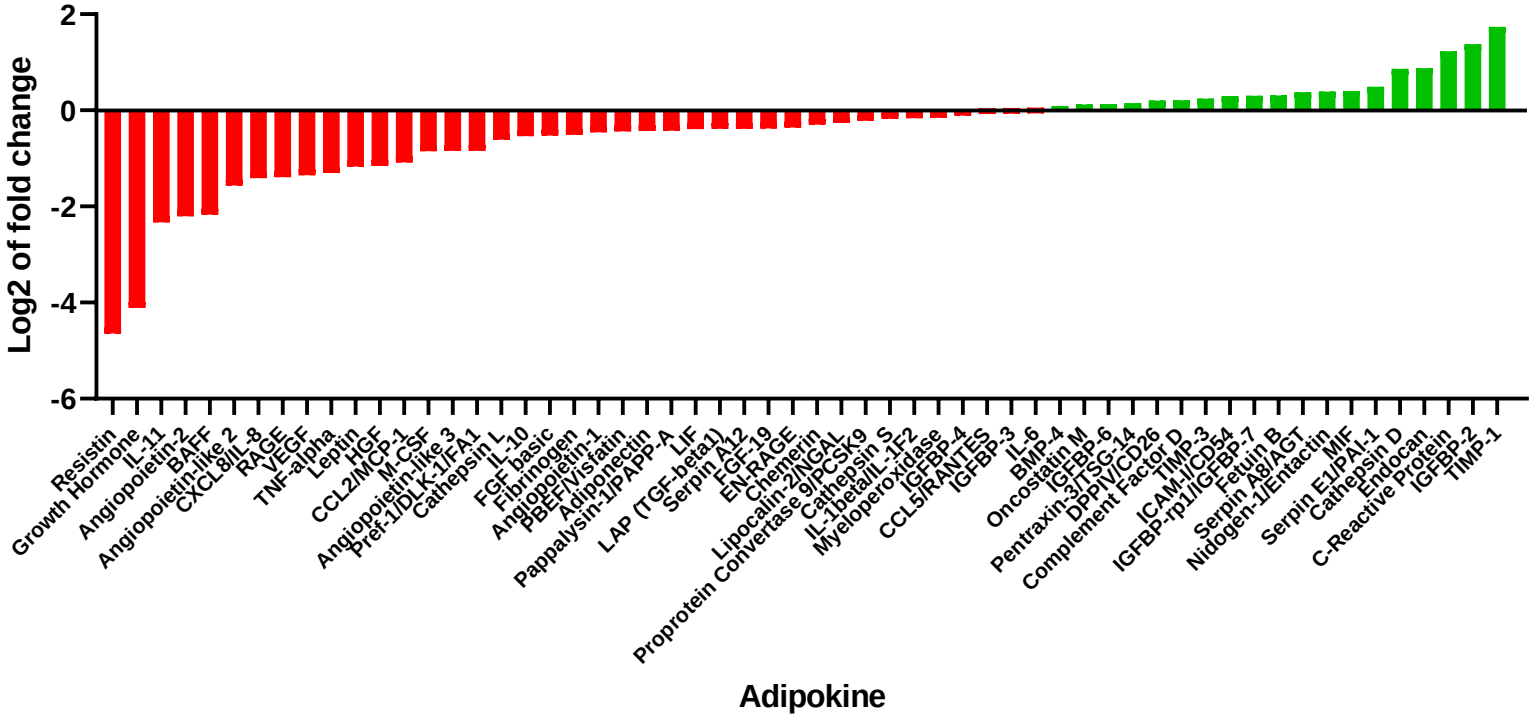

Figure 7.34: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 049. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 4.8 years, female, induction regimen A, BMI percentile day 0=77, BMI percentile day 29=59, Cytogenetic risk good, MRD risk, % weight change -4.73, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
050	6.6	M	A	29	>95	Intermediate	MRD	+37.24

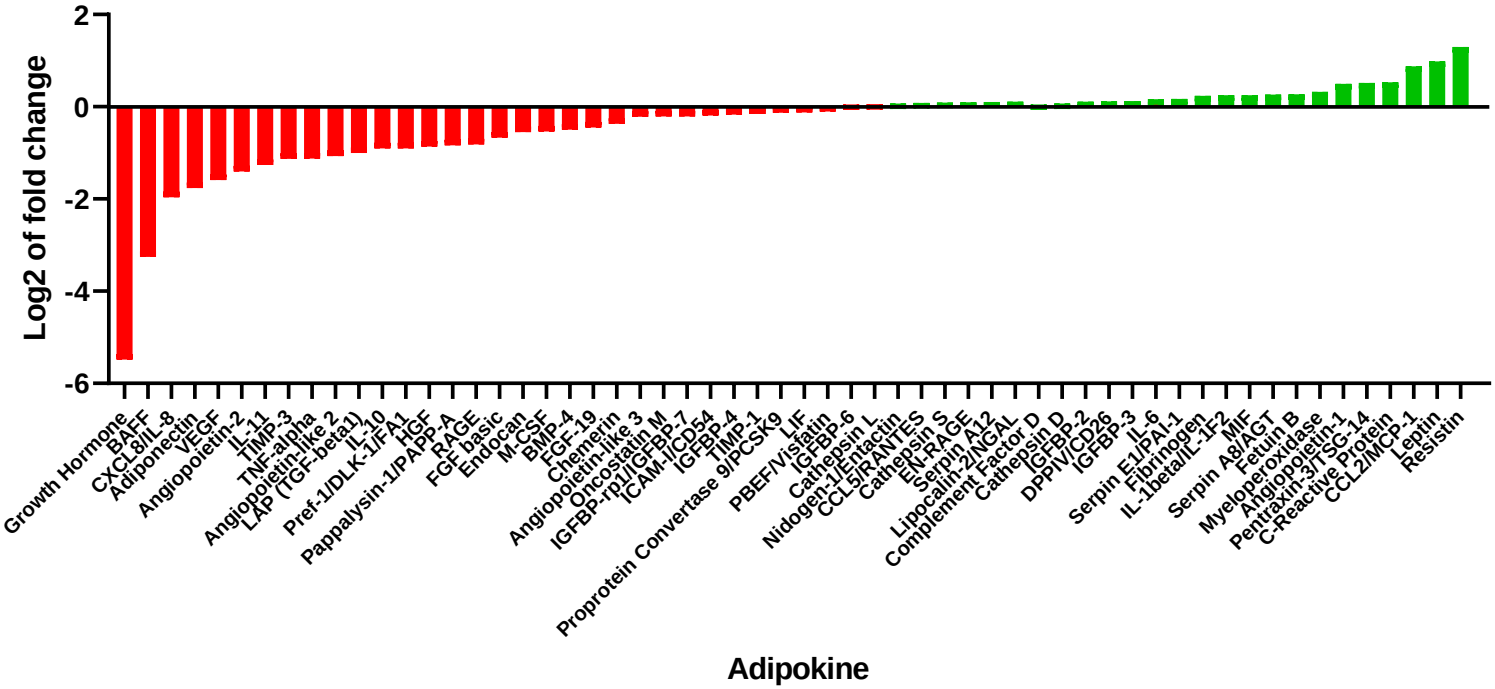

Figure 7.35: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 050. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in exp ression, red indicates a decrease and green an increase in expression. Patient age 6.6 years, male, induction regimen A, BMI percentile day 0=29, BMI percentile day 29=>95, Cytogenetic risk intermediate, MRD risk, % weight change +37.24, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
051	5.9	M	A	66	>95	Good	Low	+27.25

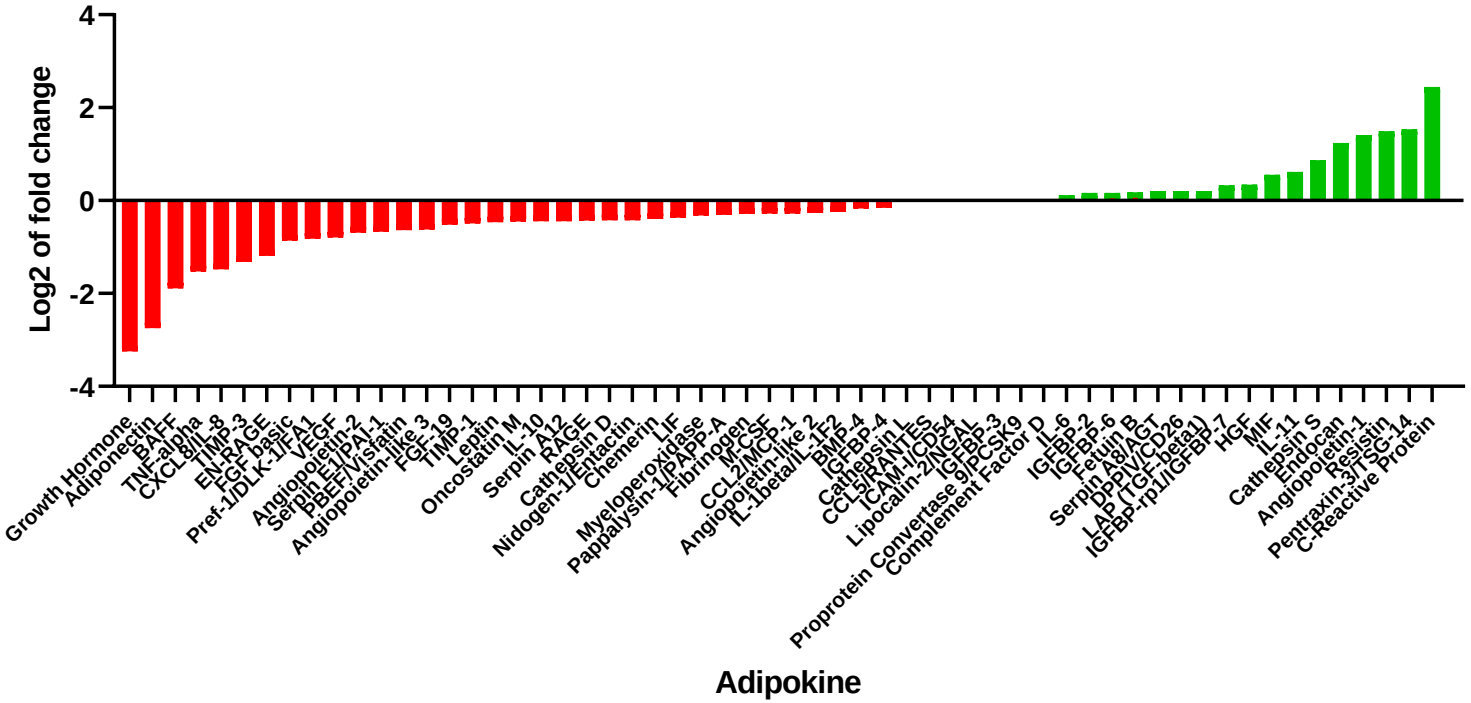

Figure 7.36: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 051. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 5.9 years, male, induction regimen A, BMI percentile day 0=66, BMI percentile day 29=>95, Cytogenetic risk good, MRD risk, % weight change +27.25, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
053	2.8	F	B	81	>95	Intermediate	Low	+14.84

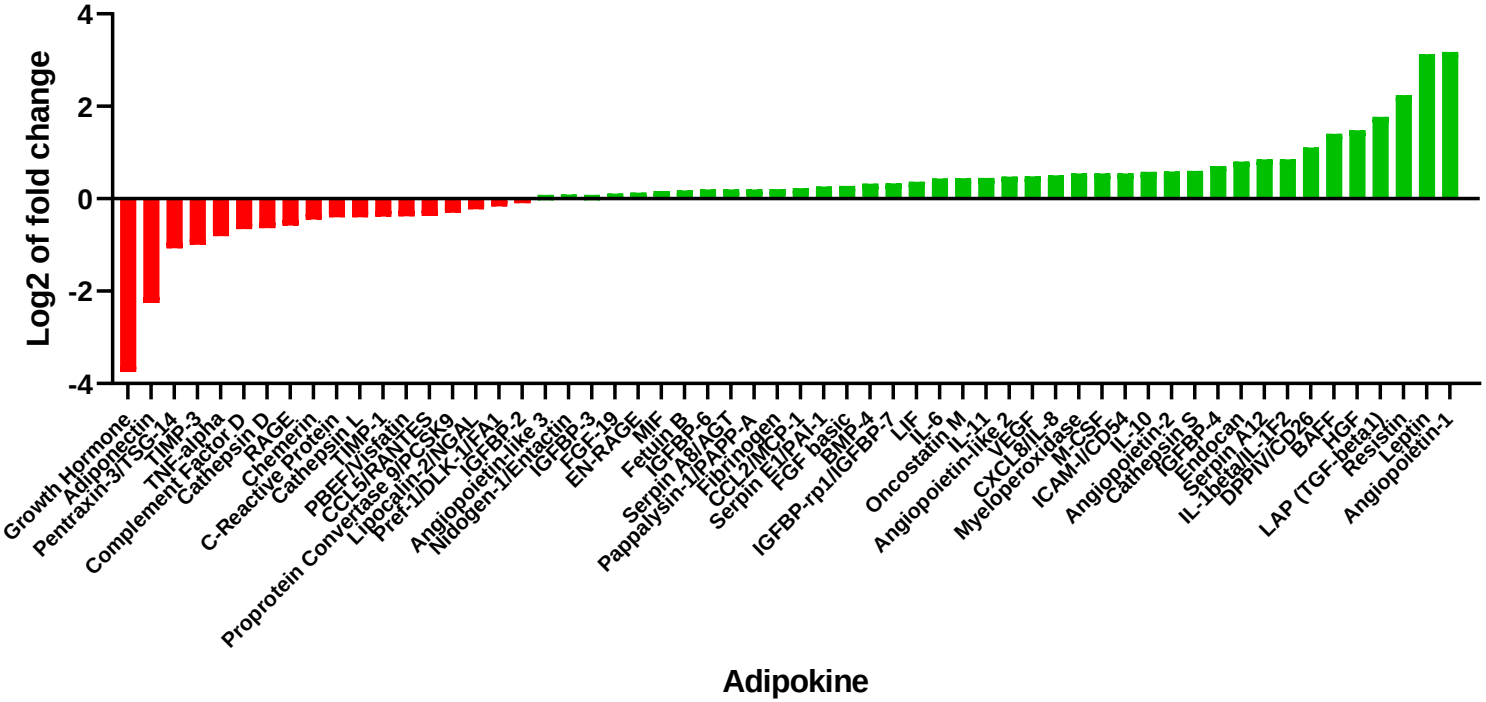

Figure 7.37: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 053 . Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 2.8 years, female, induction regimen B, BMI percentile day 0=81, BMI percentile day 29=>95, Cytogenetic risk intermediate, low risk, % weight change +14.84, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
055	2.9	M	B	40	84	Good	MRD	+10.07

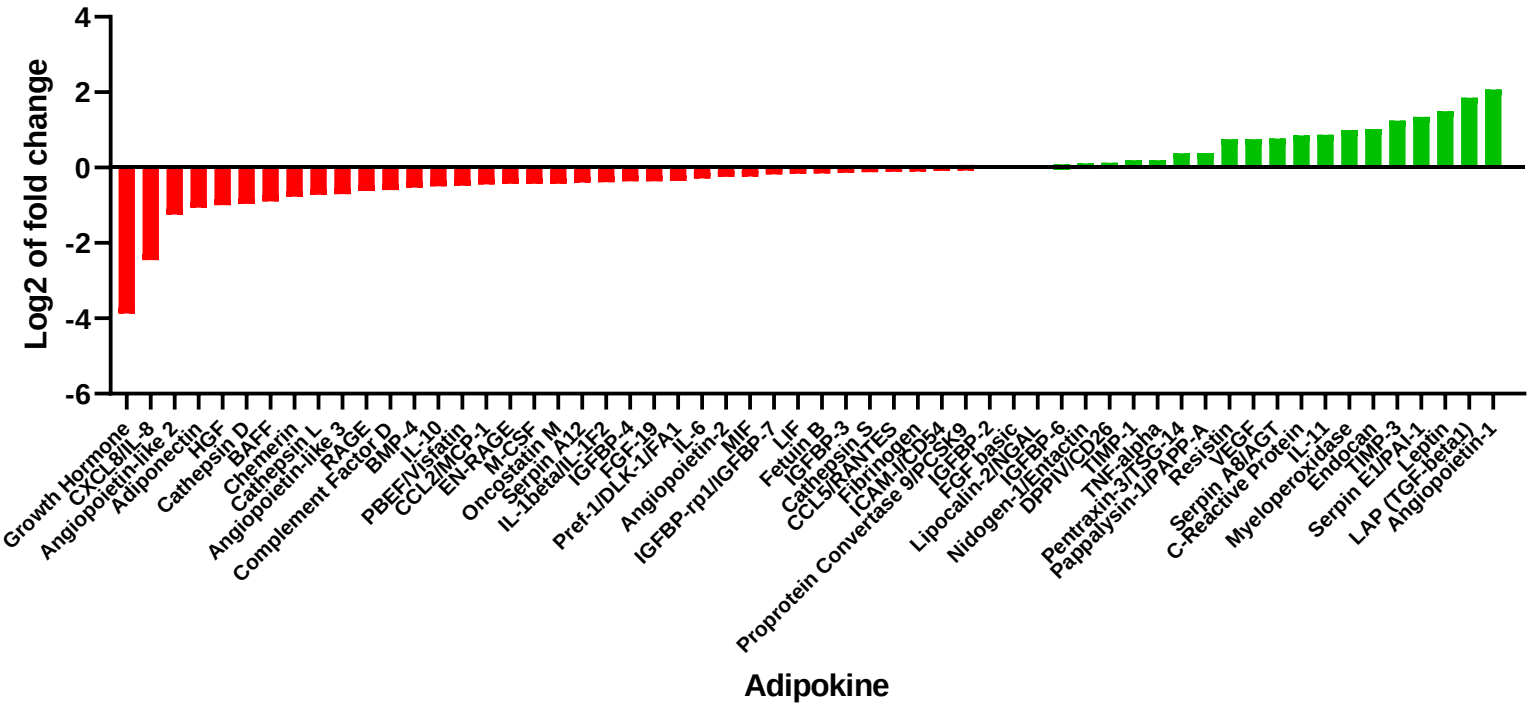

Figure 7.38: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 055 . Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 2.9 years, male, induction regimen B, BMI percentile day 0=40, BMI percentile day 29=84, Cytogenetic risk good, MRD risk, % weight change +10.07, n=1.

ID	Age	Sex	Induction Regimen	BMI % at day 0	BMI % at day 29	Cytogenic risk	MRD risk	% weight change
057	11.2	M	B	50	54	High	MRD	+1.43

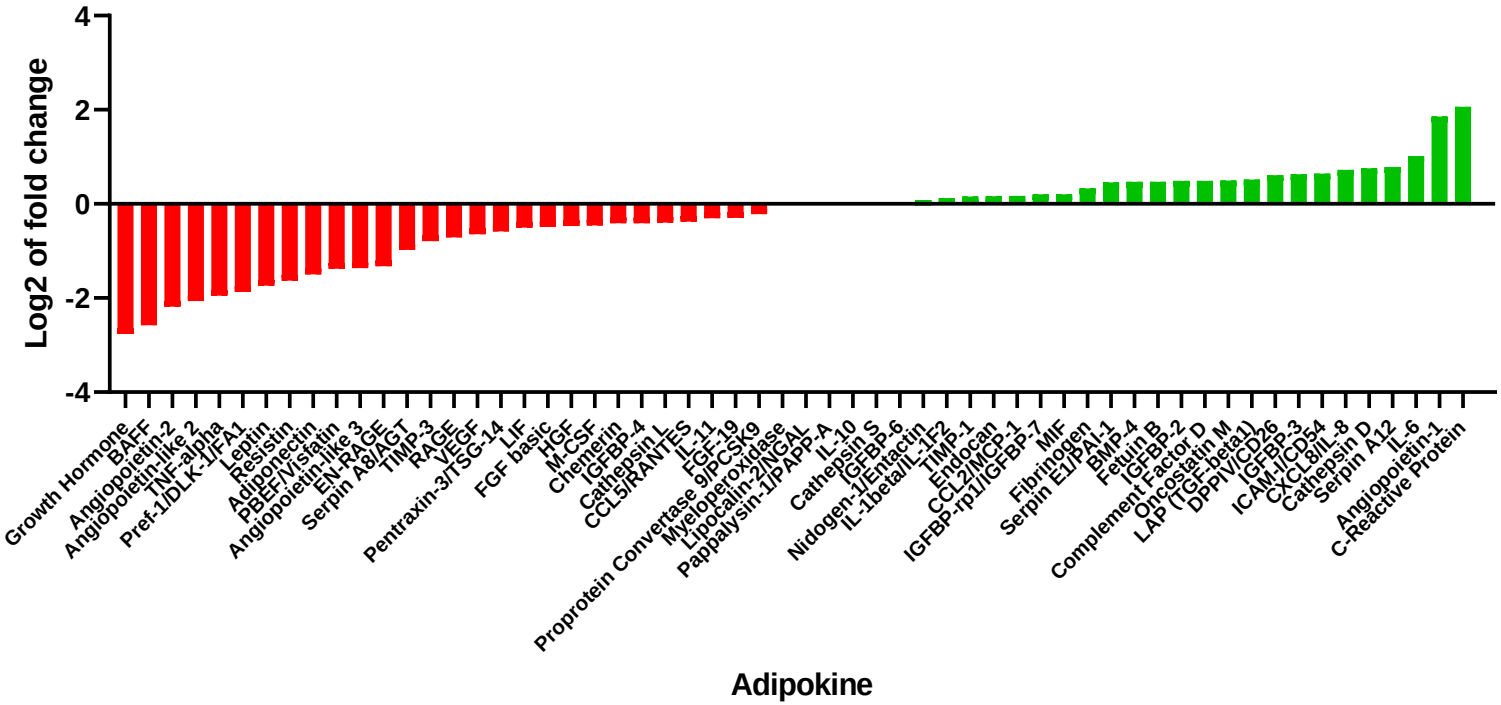

Figure 7.39: Adipokine expression at diagnosis (day 0) and after induction phase of treatment (day 29) for patient 057. Adipokine expression plotted against Log2 of the fold change of pixel density, zero is day 0 and day 29 is shown as an increase or decrease in expression, red indicates a decrease and green an increase in expression. Patient age 11.2 years, male, induction regimen B, BMI percentile day 0=50, BMI percentile day 29=54, Cytogenetic risk high, MRD risk, % weight change +1.43, n=1.

7.2 Individual patient data discussion

The following section will discuss the set of patients who are MRD risk at the end of induction, following this the patients who are low risk at the end of induction, along with the clinical data the highlights from the patients adipokine profile will be discussed to gain insight into adipokine expression and its possible links with MRD risk and weight. This study has reported 15 patients who are MRD risk, which is 39% of the cohort of patients, this is a higher percentage compared to other studies where the percentage of patients reported as MRD risk were 24.37% (Meleshko et al., 2011) although the Meleshko study has a higher percentage of T-cell patients and 27.31% for the following study findings (Laughton et al., 2005). The MRD risk patients within this cohort have a range of weight change percentages from the lowest (-11.93%) to the highest (37.24%), also as stated in the clinical data results in this study MRD risk is associated but not significantly with a lower weight at the end of induction. MRD risk patients will be discussed first starting with patients presenting with T cell disease, followed by MRD risk patients who lost weight moving to MRD risk patients who gained weight, then patients who are low risk will be discussed. Finally, the top five significant adipokines for weight gain and MRD risk followed by a summary of overall trends observed in expression.

7.2.1 Patient 031

Patient 031 is one of two T-cell patients within the cohort, this is less than the expected diagnostic rate of T-ALL within an ALL cohort which is usually between 12 and 15% (Raetz and Teachey, 2016). T-cell disease comes with the highest cytogenetic risk and can explain the MRD risk status without any other additional risks in the clinical data or the adipokine profile. It carries the highest risk genetic profile and is notoriously difficult to treat (Teachey and Pui, 2019). Many of the underlying genetic mutations interfere and activate several pathways including NOTCH1, JAK/STAT, PI3K/Akt/ mTOR and also MAPK (Raetz and Teachey, 2016). The patient is a 12.7 year old male who received induction regimen B. The patient stayed in the normal weight range at the beginning and the end of induction and gained 1.66% of their starting body weight. The patients highest recorded adipokine increase was serpin A12. Information regarding serpin A12 is limited in ALL, although information on its target receptor

GPR78 is more prevalent. GRP78, which is an important component of the unfolded protein response (UPR) signalling network, was abundantly expressed in relapsed B ALL and vincristine resistance was reversed by using the GRP78 inhibitor epigallocatechin gallate, which targets the ATP binding domain of GRP78 and inhibits its anti-apoptotic function (Uckun et al., 2011). Leptin was the second highest change, which would complement the gained weight and this would account for the higher expression compared to a patient who lost weight as leptin correlates with body fat percentage (Fujita et al., 2019). The lowest expression was growth hormone, which is known to be reduced after ALL chemotherapy treatments as discussed previously, and angiopoietin-2, which acts as an partial agonist and antagonist for Angiopoietin-1 in the Tie2 signalling pathways and has been shown to be lowered in response to treatment in patients with ALL at day 33 of treatment (Karakurt et al., 2016). Angiopoietin-1 which fully activates the Tie2 pathway is raised in this patient; it is possible that without the antagonistic effect of angiopoietin-2 due to lower risk, the downstream proliferative pathways may be activated.

7.2.2 Patient 048

Patient 048 is the second T-cell disease patient within our cohort. The patient is a 9 year old male who received induction regimen B. Males have been linked to increased diagnosis of T-ALL (Pui et al., 1999), their BMI at diagnosis was 45 decreasing to 18 by the end of induction therapy, the patient lost 7.14% of their starting body weight. The largest adipokine increases for the patient were DPPIV/CD26 and angiopoeitin-1 and their largest decreases were BAFF and RAGE. Angiopoietin-1 has been implicated in the pathogenesis of T-cell ALL and its overexpression linked to increased cell proliferation (Castro et al., 2010). Similar to patient 031 there is decreased expression of angiopoietin-2 which has increased the Ang1/Ang2 ratio and is associated with poorer outcome (Schneider et al., 2011). DPPIV/CD26 was increased on cell membranes of the ALL cell lines CEM-CCRF and Molt3 in response to chemotherapy and has been suggested as a target for its potential role in chemotherapy resistance (Dourado et al., 2007). The patients low BAFF may contribute to the poor patient outcome at day 29, BAFF signalling differs in B-ALL cells and treatment with BAFF promoted cell death of primary BCR-ABL^{+} cells (Sevdali et al., 2019).

7.2.3 Patient 017

Patient 017 is an MRD risk patient at the end of induction, this patients has an increased amount of risk factors within their clinical data alone which could explain the MRD risk outcome. The patient is 12 which is over 10 years old which puts them at higher risk due to increasing age (Kakaje et al., 2020) and also received induction regimen B. In our study having induction B treatment was not significantly associated with MRD risk, but this does exclude that the 4 drug regimen may have contributed to the MRD risk status. The patient lost 11.93% of their starting body weight during induction which is a known risk factor which contributes to MRD risk; previously patients who lost >5% of weight during induction therapy were at identified as increased risk (Hill et al., 2020). The patient's weight loss is reflected in their BMI percentile change which decreased from 40 to 11 by the end of induction, their cytogenics were intermediate risk.

The patient's highest expression of adipokines were serpinA12 (discussed for patient 031), pentraxin-3 and IL-8. Increased pentraxin-3 expression is consistent with infection, particularly mold infections and sepsis (Brunel et al., 2018) and may explain the MRD status in itself, as this is associated with poor outcome. IL-8 is known to have an a suppressive effect on normal blood cell development and a positive effect on the proliferation of ALL cells (de Vasconcellos et al., 2011). Compared to the other patients' profiles, when we look at the profile for patient 017, appendix figure 7.9, the majority of their adipokines apart from seven had decreased expression compared to levels at induction, this is different to the other profiles in the cohort where there is more balanced expression changes which are increased and decreased. This observation could indicate that the patients weight loss has severely impacted adipokine secretions and fat cell function and a possible indication of cancer cachexia which can occur in advance cancer and chronic infection, (Loprinzi, 1995).

The patient also indicated higher levels of IL-6 is associated with the inflammation involved with cancer cachexia which is also increased at day 29 in patient 017, this could also be linked with the raised pentraxin-3 level. Both leptin and resistin were decreased at day 29 which are likely to be related to weight loss as both adipokines are associated and increased with weight gain, this would fit in as the patient lost weight and this would account for the lower expression compared to a patient who gained weight as leptin correlates with body fat

percentage (Fujita et al., 2019). The patient's largest decreases in adipokine expression was growth hormone, lower levels are linked to ALL treatment which has been discussed previously. Angiopoietin-1 which has been implicated in the pathogenesis of T-cell ALL and its overexpression linked to increased cell proliferation (Castro et al., 2010) is reduced in this patient so this may not be contributing to the disease process which is the opposite to the T-cell patients, this patient did have the majority of adipokines levels reduced from diagnosis by day 29.

7.2.4 Patient 018

Patient 018 is an MRD risk patient who lost weight during induction, see appendix figure 7.10. The patient is female and over 10 years of age at 11.8 years and received induction regimen B because of this. Their cytogenics were intermediate risk and they lost 2.63% of their starting body weight, the patient recorded BMI percentile numbers in the normal range at the start and end of induction which were 18 dropping to 13 by day 29. The adipokine profile for patient 018 showed the highest expressed adipokines were CRP and BMP-4. CRP is a known indicator of bacterial septicaemia in children with ALL (Peltola et al., 1983). BMP-4 is raised which has been shown to be increased in ALL development and progression (Valencia et al., 2019). Patient 018 has HGF as their lowest expressed adipokine. TIMP-3 was the second lowest. HGF is known to positivity correlate with obesity and in this case the patient lost weight so this may explain the change without effecting disease processes (Oliveira et al., 2018). TIMP-3 information is lacking in ALL and has mixed reports in other cancers. In a study experimenting with AML cell lines increasing TIMP-3 inhibited growth of the K562 cell line (Yu et al., 2006). TIMP-3 is also known to inhibit angiogenesis (Su et al., 2019), low levels here maybe contributing to drivers of angiogenesis and indicating that these processes are activated.

7.2.5 Patient 025

Patient 025 lost weight during induction and was classed at MRD risk at day 29, see appendix figure 7.15. Patient 025 was a younger female at age 6 who received induction regimen A, their cytogenics were good and they lost 6.1% of their starting body weight, the patients recorded BMI percentile numbers in the normal range at the start and end of induction. The

patients highest increased adipokines were IL-11, IL-11 which is often linked to poor prognosis in cancer at higher expression levels (Nishina et al., 2020) further to this finding research into IL-11 and ALL where targeting the IL-11 receptor IL-11R was shown to reduce cell viability in the ALL cell lines Molt4 and CEM-CCRF (Karjalainen et al., 2015). Visfatin was also increased, a study using an endogenous NAMPT inhibitor OT-82 confirmed significant antileukemic effect in patient derived samples (Somers et al., 2020) The lowest growth hormone and BAFF which has been previously discussed.

7.2.6 Patient 049

The clinical data for patient 049 confirms they are a 4.8 year old female who received induction treatment A, the patient lost weight during induction, their BMI was 77 at diagnosis decreasing to 59 at day 29 losing 4.73% of the starting body weight. Their cytogenic risk was good even though they were classed as MRD risk by the end of induction. The largest adipokine increases were IGFBP-2 which has been linked to relapse in paediatric ALL (Mohnike et al., 1998). TIMP-1 was raised and has been associated with a significantly lower 5-year EFS in paediatric ALL. The (Scrideli et al., 2010) lowest decreases were resistin and growth hormone by day 29 which have been previously discussed.

7.2.7 Patient 015

The clinical data for patient 015 confirm that they developed treatment acquired obesity (TAO) and that their risk factors in the clinical data were low. The patient is under 10 years of age and has good cytogenetics, the patient is male and received induction regimen A. By day 29 at the end of induction the patient has moved from a normal BMI percentile category into the obese category and gained 22.22% of their starting body weight. This weight gain is reflected in their adipokine profile results with the largest increased adipokine being leptin and also myeloperoxidase, see appendix figure 7.8. Leptin is known to be increased in obesity (Soliman et al., 2012) and also during ALL treatment (Argyrou et al., 2019). High levels of myeloperoxidase are also present which is associated with inflammation, infection in cancer also has antibacterial activities in neutrophils (Khan et al., 2018) this could indicate infection. Patient 015 has lowest decreases in adipokine expression of MCP-1 and growth hormone (Haddy et al., 2006) which has known to be decreased as a side effect of chemotherapy

treatment in ALL.. MCP-1 is significantly implicated in CNS involvement (Eisenkraft et al., 2006), patient 015 has decreased levels which may indicate that the disease has not progressed to the CNS in this case.

7.2.8 Patient 026

Clinical data for patient 026 indicates the patient is male and 4.2 years old. Their cytogenic risk was intermediate and they received induction regimen A. Patient 026 was in the underweight percentile category before treatment commenced and by the end of induction it had increased to 34 which is in the normal range. The patient gained 16.26% of their starting body weight. It is possible that the patient starting underweight contributed to them being MRD risk as the weight loss may have occurred in the initial phase of the disease before diagnosis, this can be correlated with disease progression as patients who were underweight at diagnosis have a higher risk of relapse (den Hoed et al., 2015). The patients weight gain during induction could actually have benefitted them as shown by (Ethier et al., 2012). The patient was still MRD risk at the end of induction so it may be possible that their intermediate cytogenic risk contributed to this. It is interesting that their adipokine profile has CRP as the second highest expression which may indicate increased inflammation and infection (Peltola et al., 1983). Patient 025 highest adipokine expressed is leptin which again reflects their 16.22% weight gain during induction. Their lowest expression was growth hormone. Patient 026 also has an increased ANG1/ANG2 ratio.

7.2.9 Patient 035

Patient 035 is a 12.1 year old female who received induction regimen B, the patients BMI percentile was 9 at the start of induction and rose to 16 by the end of induction which was a 3.68 % weight change which in the normal BMI percentile category albeit at the lower end of the weight percentile category which can indicate increased risk as previously discussed. The patients cytogenic risk is classed at intermediate. The highest levels of adipokines secreted were pappalysin A and IL-10. There is very little information regarding pappalysin A and ALL, it has been shown to be expressed at higher levels in Ewing's Sarcoma as a member of the IGF family, targeting this protein in transgenic T cells has shown to be effective at treating this bone cancer (Kirschner et al., 2017). IL-10 is a known anti-inflammatory cytokine with anti-

angiogenic functions (Sheikhpour et al., 2018) but it has a somewhat paradoxical role in cancer due to its suppression of the immune response and also activation of pathways associated with cancer progression (Chen et al., 2019), low levels of IL-10 have been associated with poorer outcomes in ALL (Fitch et al., 2016). The lowest levels expressed were BAFF, the implications of low BAFF levels have been discussed for patient 025, patient 035 also has a high ANG1/ANG2 ratio.

7.2.10 Patient 037

The clinical data for patient 037 indicates that the patient became overweight during treatment from the normal weight range at the start of induction. Being overweight carries risk during ALL treatment and could explain the patients increased risk (Orgel et al., 2014b). Their BMI was 25 and raised to 86 and the patient gained 16.77% of their starting body weight. The patient is aged 4.6 years and female and received induction regimen A which is all low risk, their cytogenic risk was intermediate in comparison. The largest increases in the patients adipokine profile was leptin and also angiopoietin-1, leptin has been discussed previously for patient 015 at increased levels. Angiopoietin-1 has been implicated in the pathogenesis of T-cell ALL and its overexpression linked to increased cell proliferation (Castro et al., 2010). The lowest expression of adipokines were adiponectin which is well documented in ALL and expressed at lower levels in disease (Argyrou et al., 2019) and BAFF which ties in with previous patients.

7.2.11 Patient 046

The adipokine profile data for patient 046 displayed similarities to 037 where the largest increases were leptin and VEGF, leptin has been discussed in patient 015. VEGF at increased levels has been shown to be predictive of poorer EFS in paediatric ALL (Avramis et al., 2006). The lowest expression included IL-8, BAFF. IL-8 and at lower levels have been discussed for patient 026 and BAFF patient 035. The patient's clinical data was mainly low risk, the patient was male and 6.2 years old and received induction regimen A although their cytogenic risk was intermediate. The patients BMI percentile started at 47 and rose to 73, both readings in the normal range, their body weight increased by 6.47%.

7.2.12 Patient 047

The clinical data for patient 049 confirms they are a 4.7 year old male who received induction treatment A, the patient gained weight during induction, their BMI was 22 at diagnosis decreasing to 80 at day 29 and gaining 12.93% of the starting body weight. Their cytogenic risk was intermediate and they were classed as MRD risk by the end of induction the patients largest adipokine expression increases were BMP-4 and CRP, increases in both of these adipokines have been explained previously in this section. The lowest expression recorded was for BAFF and EN-RAGE, BAFF has already been discussed. EN-RAGE is known as a local bio-inflammatory marker (Brenner and Bruserud, 2018), little is known about its implications at low levels in ALL and cannot be linked with clinical parameters.

7.2.13 Patient 050

The clinical data for patient 050 confirm that they developed treatment acquired obesity (TAO). The patient is under 10 years of age and has intermediate cytogenetics, the patient is male and received induction regimen A. By day 29 at the end of induction the patient has moved from a normal BMI percentile category into the obese category and gained 37.24% of their starting body weight in 29 days. Unremarkably the patient's highest increases in adipokines were leptin and resistin both shown to be raised in weight gain in paediatric ALL (Moschovi et al., 2010a). The largest decreases in adipokines were for growth hormone and BAFF which have previously been discussed.

7.2.14 Patient 055

The clinical data for patient 055 confirms they are a 2.9 year old male who received induction treatment B and the patient gained weight during induction, their BMI was 40 at diagnosis increasing to 84 at day 29 losing 10.07% of the starting body weight. Their cytogenic risk was good even though they were classed as MRD risk by the end of induction. The largest increases in adipokines were for angiopoietin-1 and LAP(TGF-beta1) and the largest decreases growth hormone and IL-8. Lap(TGF-beta1) is a tumour suppressor protein when expressed at higher levels in ALL (Downing, 2004), the low levels in this patient could reflect higher tumour burden, as we have limited clinical data we are unable to confirm this.

7.2.15 Patient 057

The clinical data for patient 057 confirms they are a 11.2 year old male who received induction treatment B, the patient gained a small amount of weight during induction, their BMI was 50 at diagnosis increasing to 54 at day 29 gaining 1.43% of the starting body weight. Their cytogenic risk was high which could explain their MRD risk status without other factors. Their largest increased expression of adipokines was for angiopoietin-1 and CRP and the lowest growth hormone and BAFF which have all been discussed previously in patients.

The combination of findings in the MRD risk patients who lost weight during induction treatment, patients 017, 018, 025 and 049 indicate clues from their adipokine profiles which may add to the drivers of increased risk. High levels of CRP and increased levels of pentraxin-3 could indicate underlying inflammation and infection which could further contribute to MRD risk and poor outcome and it is likely this rather than their clinical data which has contributed to MRD risk and the weight loss that has occurred. Other findings such as increased ANG1/ANG2 ratio could be driving disease by activating downstream proliferative pathways, changes in adipokines that are implicating drivers of angiogenesis or increased leptin levels in weight gain MRD risk patients, patients 015 and 050, at this stage we do not know the impact of these results on disease progression.

7.2.16 Low MRD risk patients

In comparison to the 15 MRD risk patients there were 22 low risk patients who either lost or gained weight ranging from losing 11.56% of their body weight to gaining 37.24% of their starting body weight. One noticeable observation is that we didn't have any patients in the MRD risk category that recorded a BMI percentile of overweight or obese at the start of induction. A possible explanation for this may be that starting treatment overweight or obese may be a protective factor against MRD risk, we have shown that being a lower weight at the end of induction is linked to increased risk and having pointed out previously gaining a small amount of weight may be protective compared to losing weight during treatment and be a protective factor against MRD risk at any stage of treatment and poorer outcomes (Ethier et al., 2012). Five patients were classed as overweight or obese at diagnosis, patient 010, 028, 030, 040 and 044 were all low MRD risk so their weight didn't contribute to MRD risk at this

stage of treatment. A further observation in the low risk profiles is the correlation with weight gain and the noticeable increase of Leptin expression as with the MRD risk patients. Three of the patients with the largest weight gain were low risk and gained more than 10% of their starting body weight excluding 051 and recorded Leptin as high in the expressed adipokines, these patients were 011, 036, 045, 051 and 053. Patient 011 gained 13.23% of their starting body weight and logged CRP as and increased adipokine with leptin. The relationship between CRP and outcome is reported the literature as associated with increased inflammation and infection (Peltola et al., 1983), this study confirms that CRP is correlated with increased expression in many patients at the end of induction but not associated with MRD risk in this cohort of patients during induction treatment, there are reported high levels in MRD risk and low risk patients. Two questions of this research were to investigate if adipokine expression correlated with MRD risk and weight gain, it helps to answer this by observing and comparing the patients who gained weight and were MRD risk or low risk by the end of induction. Patient 015 see appendix figure 7.8 was the first patient in the cohort who became obese with treatment in 29 days and went onto be MRD risk. In comparison patient 045, see figure 4.39 gained 24.43% of their starting body weight and did not go onto be MRD risk. Both patients recorded high leptin, but there were other noticeable differences in their profiles being low serpin A12 in patient 045 where it is high in patient 015, also lower ANG1/ANG2 ratio in patient 045 but in patient 015 it is high indicating higher risk. The patterns emerging in the adipokine profiles of the MRD risk patients support the idea that particular adipokines maybe associated with MRD risk and weight gain. It is clear that many risk factors contribute to this. The clinical data alone may contribute to MRD risk without adding the complexity of the adipokine profiles. Preliminary observations indicate that leptin is expressed at higher levels in patients who gain weight which is consistent with other studies (Argyrou et al., 2019). In patients who lose weight the opposite can be seen. At this stage it is difficult to predict if particular adipokines or adipokine profiles contribute to weight gain or MRD risk across the whole cohort, this is examined in Chapter 4 .

Table 7.2: Summary of all adipokines tested in relation to weight gain at diagnosis. Patients were grouped into those that presented as overweight or obese at the start of induction therapy, n=5 and those that were in the normal weight range, n=33, adipokine mean pixel intensity levels were compared using an unpaired t-test for each patient group. Adipokines ranked by p value, significant to non-significant and indicated in green if the adipokine increased or red if decreased at day 0, n=38.

Adipokine	P value		Adipokine	P value	
Leptin	0.0013		IL-6	0.5795	
Angiopoietin-2	0.0230		IGFBP-6	0.5944	
Endocan	0.0495		LAP (TGF-beta1)	0.5994	
RAGE	0.0699		Angiopoietin-like 3	0.6046	
M-CSF	0.1229		IGFBP-3	0.6151	
Pappalysin-1/PAPP-A	0.2090		TNF-alpha	0.6158	
BAFF	0.2369		CCL2/MCP-1	0.6341	
HGF	0.2427		Pref-1/DLK-1/FA1	0.6501	
Lipocalin-2/NGAL	0.2511		Angiopoietin-like 2	0.6669	
IGFBP-rp1/IGFBP-7	0.2513		Serpin A12	0.7128	
IL-10	0.2523		Pentraxin-3/TSG-14	0.7188	
LIF	0.3812		IGFBP-4	0.7279	
Chemerin	0.3829		CCL5/RANTES	0.7364	
PBEF/Visfatin	0.3912		Proprotein Convertase 9	0.7421	
FGF basic	0.3922		Cathepsin L	0.7437	
Serpin A8/AGT	0.4079		Fibrinogen	0.7479	
FGF-19	0.4167		IGFBP-2	0.7787	
TIMP-1	0.4261		VEGF	0.8043	
Oncostatin M	0.4265		EN-RAGE	0.8184	
DPPIV/CD26	0.4316		BMP-4	0.8485	
CXCL8/IL-8	0.4396		ICAM-I/CD54	0.8595	
Adiponectin	0.4449		Cathepsin S	0.8602	
IL-1beta/IL-1F2	0.4456		Serpin E1/PAI-1	0.8832	
Cathepsin D	0.4506		IL-11	0.9037	
Fetuin B	0.4563		TIMP-3	0.9046	
Myeloperoxidase	0.4870		Nidogen-1/Entactin	0.9088	
MIF	0.5317		Angiopoietin-1	0.9120	
C-Reactive Protein	0.5408		Growth Hormone	0.9843	
Complement Factor D	0.5747		Resistin	0.9987	

Table 7.3: Summary of all adipokines tested for prediction of biomarkers in relation to MRD risk at day 29. Mean pixel intensity for each adipokine value were compared at day 0 for those patients who went onto to be MRD risk, n=15, and low risk n=22. Groups were compared using an unpaired t-test. Adipokines ranked by p value, significant to non-significant and indicated in green if the adipokine increased or red if decreased at day 0, n=37.

Adipokine	P value		Adipokine	P value	
Pappalysin-1/PAPP-A	0.1054		IGFBP-rp1/IGFBP-7	0.5015	
RAGE	0.1229		Serpin A8/AGT	0.5215	
TIMP-3	0.1259		Fibrinogen	0.5357	
Serpin E1/PAI-1	0.1286		TNF-alpha	0.5815	
IL-10	0.1514		FGF basic	0.6359	
CXCL8/IL-8	0.1716		IL-6	0.6476	
FGF-19	0.1729		Lipocalin-2/NGAL	0.6529	
Oncostatin M	0.2026		Angiopoietin-2	0.6633	
Myeloperoxidase	0.2089		Cathepsin L	0.6637	
M-CSF	0.2142		BMP-4	0.6682	
MIF	0.2196		ICAM-I/CD54	0.7149	
Resistin	0.2315		Complement Factor D	0.7253	
EN-RAGE	0.2409		Cathepsin D	0.7264	
Angiopoietin-like 3	0.2617		Growth Hormone	0.7293	
Serpin A12	0.2870		Pref-1/DLK-1/FA1	0.7471	
IL-1beta/IL-1F2	0.3093		Proprotein Convertase 9	0.7496	
DPPIV/CD26	0.3273		IL-11	0.7526	
CCL5/RANTES	0.3496		IGFBP-2	0.7534	
IGFBP-3	0.3547		Angiopoietin-1	0.7870	
BAFF	0.3755		PBEF/Visfatin	0.8155	
Adiponectin	0.4100		Cathepsin S	0.8189	
LAP (TGF-beta1)	0.4341		IGFBP-4	0.8383	
TIMP-1	0.4504		VEGF	0.8776	
CCL2/MCP-1	0.4697		Angiopoietin-like 2	0.9082	
Pentraxin-3/TSG-14	0.4823		Endocan	0.9132	
HGF	0.4858		C-Reactive Protein	0.9340	
Leptin	0.4924		LIF	0.9399	
IGFBP-6	0.4939		Nidogen-1/Entactin	0.9615	
Fetuin B	0.4981		Chemerin	0.9815	

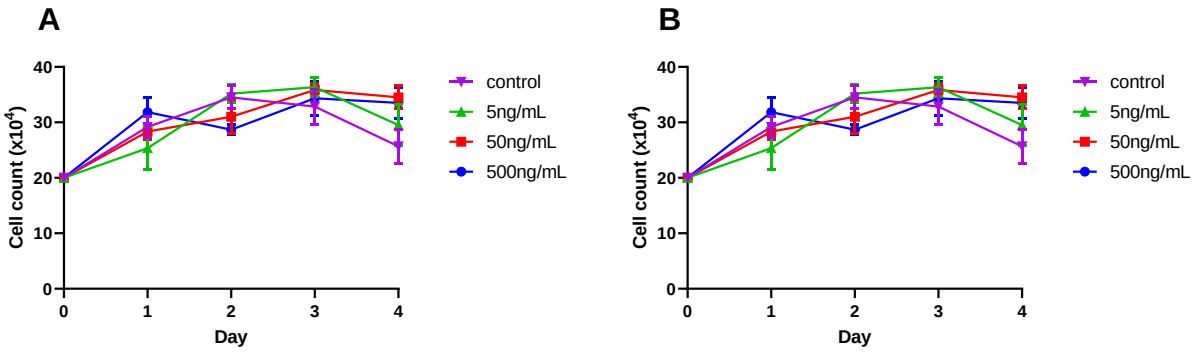

Figure 7.40: Leptin stimulates Molt4 ALL cancer cell viability by activating the PI3K/Akt pathway. Molt4 cells were incubated for 5 days in increasing concentrations of leptin. A, Trypan blue exclusion assay live cell counts results. B Trypan blue exclusion assay % cell viability results, n=3.

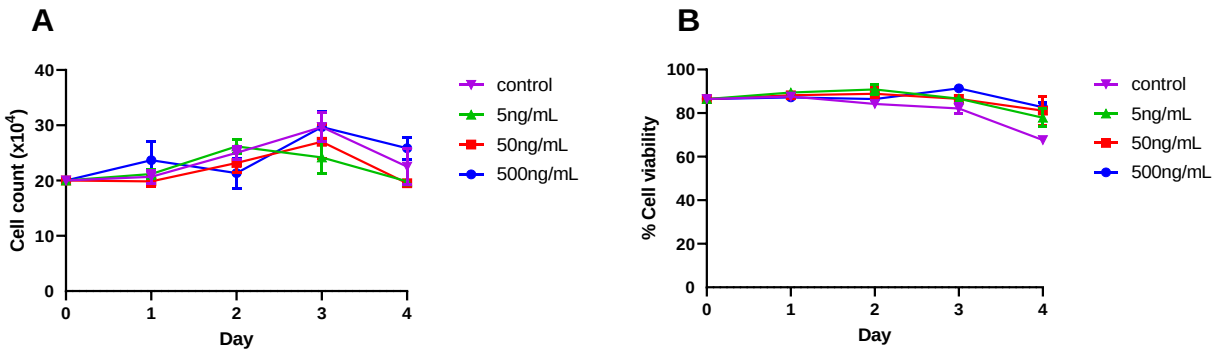

Figure 7.41: IL-8 stimulates Molt4 ALL cancer cell viability. Molt4 cells were incubated for 5 days in increasing concentrations of IL-8. A, Trypan blue exclusion assay live cell counts results. B, Trypan blue exclusion assay % cell viability results, n=3.

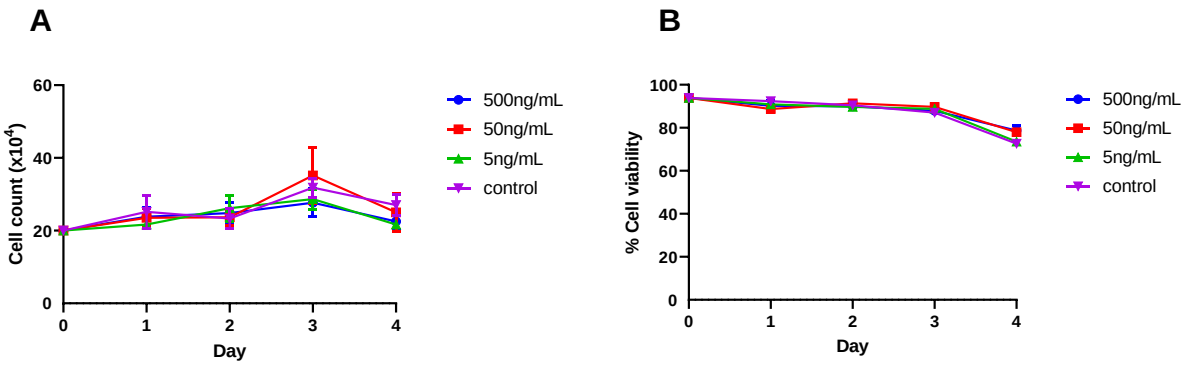

Figure 7.42: Resistin has minimal impact on Molt4 cell growth and viability. Molt4 cells were incubated for 5 days in increasing concentrations of resistin. A, Trypan blue exclusion assay live cell counts results. B Trypan blue exclusion assay % cell viability results, n=3.

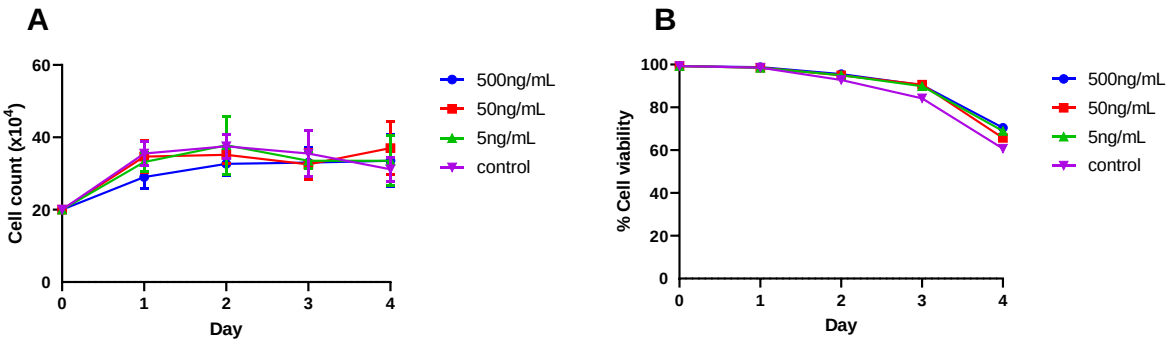

Figure 7.43: Serpin A12 stimulates Molt4 cell viability. Molt4 cells were incubated for 5 days in increasing concentrations of serpin A12. A, Trypan blue exclusion assay live cell counts results. B Trypan blue exclusion assay % cell viability results, n=3.

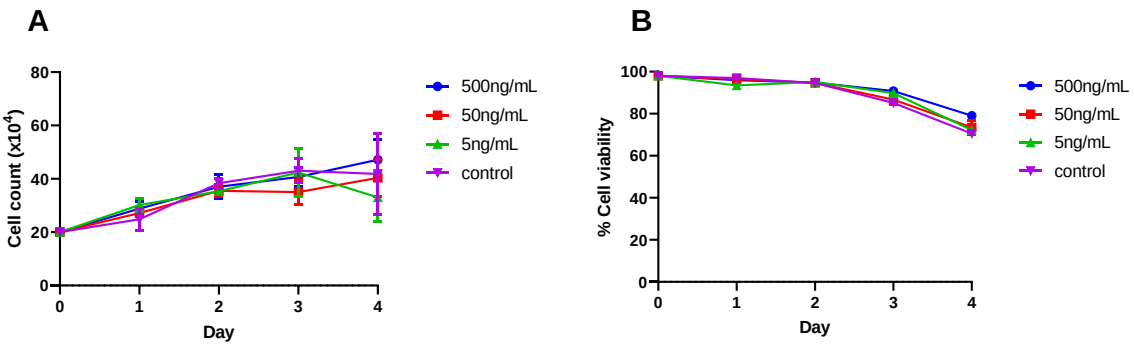

Figure 7.44: Angiopoietin-2 increases Molt4 cell viability. Molt4 cells were incubated for 5 days in increasing concentrations of angiopoietin-2. A, Trypan blue exclusion assay live cell counts results. B, Trypan blue exclusion assay % cell viability results, n=3.

Table 7.4: Adipokines tested, coordinates on the array and alternate nomenclature of the mouse adipokine array ARY013.

Coordinate	Adipokine/Control	Alternate Nomenclature
A1, A2	Reference Spots	----------
A23, A24	Reference Spots	----------
B1, B2	Adiponectin	Acrp30/AdipoQ
B3, B4	AgRP	ART
B5, B6	ANGPT-L3	----------
B7, B8	C-Reactive Protein	CRP
B9, B10	DPPIV	CD26/DPP4
B11, B12	Endocan	ESM-1
B13, B14	Fetuin A	AHSG
B15, B16	FGF acidic	FGF-1
B17, B18	FGF-21	----------
B19, B20	HGF	----------
B21, B22	ICAM-1	CD54
B23, B24	IGF-I	Somatomedin C
C1, C2	IGF-II	Somatomedin A
C3, C4	IGFBP-1	----------
C5, C6	IGFBP-2	----------
C7, C8	IGFBP-3	----------
C9, C10	IGFBP-5	----------
C11, C12	IGFBP-6	----------
C13, C14	IL-6	----------
C15, C16	IL-10	----------
C17, C18	IL-11	----------
C19, C20	Leptin	OB
C21, C22	LIF	----------
C23, C24	Lipocalin-2	NGAL
D1, D2	MCP-1	CCL2/JE

D3, D4	M-CSF	CSF-1
D5, D6	Oncostatin M	OSM
D7, D8	Pentraxin 2	PTX2/SAP
D9, D10	Pentraxin 3	PTX3/TSG-14
D11, D12	Pref-1	DLK-1/FA1
D13, D14	RAGE	----------
D15, D16	RANTES	CCL5
D17, D18	RBP4	----------
D19, D20	Resistin	ADSF/FIZZ3
D21, D22	Serpin E1	PAI-1
D23, D24	TIMP-1	----------
E1, E2	TNF-α	TNFSF1A
E3, E4	VEGF	VEGF-A
F1, F2	Reference Spots	----------
F23, F24	PBS (Negative Control)	Control (-)

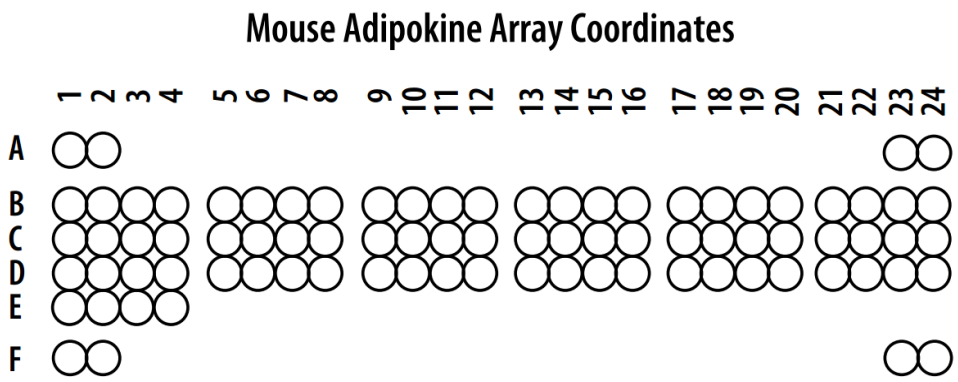

Figure 7.45: Mouse Adipokine Array Coordinates.

Table 7.5: Adipokines tested, coordinates on the array and alternate nomenclature of the Human Adipokine Array ARY024.

Coordinate	Adipokine/Control	Alternate Nomenclature
A1, A2	Reference Spots	----------
A5, A6	Adiponectin/Acrp30	AdipoQ, ApM1, GBP28
A7, A8	Angiopoietin-1	ANGTP1
A9, A10	Angiopoietin-2	ANGTP2
A11, A12	Angiopoietin-like 2	ANGTPL2, ANGRP2
A13, A14	Angiopoietin-like 3	ANGTP5, Ang-5, Angiopoietin-5, ANGTPL5
A15, A16	BAFF/BLyS/TNFSF13B	CD257, TALL1, THANK, ZTNF4
A17, A18	BMP-4	----------
A19, A20	Cathepsin D	CTSD
A23, A24	Reference Spots	----------
B1, B2	Cathepsin L	CTSL, CTSL1
B3, B4	Cathepsin S	CTSS
B5, B6	Chemerin	RARRES2, TIG-2
B7, B8	Complement Factor D	Adipsin, AND, AMBP-1, CFD, PFD
B9, B10	C-Reactive Protein/CRP	----------
B11, B12	DPPIV/CD26	DPP4
B13, B14	Endocan	ESM1, ESM-1, IGFBP-rp6
B15, B16	EN-RAGE	CAAF-1, CAAF-I, CAGC, CAGCS100, Calgranulin C, CGRP, MRP6, S100A12
B17, B18	Fetuin B	FETUB
B19, B20	FGF basic	FGF-2, FGF2AS, GFG1, HBGH-2, NUDT6, Prostatropin
B21, B22	FGF-19	----------
B23, B24	Fibrinogen	----------
C1, C2	Growth Hormone	GH1, Somatotropin
C3, C4	HGF	Hepatopoietin A, SF
C5, C6	ICAM-1/CD54	----------
C7, C8	IGFBP-2	----------

C9, C10	IGFBP-3	----------
C11, C12	IGFBP-4	----------
C13, C14	IGFBP-6	----------
C15, C16	IGFBP-rp1/IGFBP-7	IGFBP7, Mac25, PSF
C17, C18	IL-1β/IL-1F2	IL1B
C19, C20	IL-6	BSF-2, IFN-β2, MGI-2A
C21, C22	CXCL8/IL-8	GCP1, LAI, MDNCF, NAP1, NCF, TCF, TSG1
C23, C24	IL-10	CSIF
D1, D2	IL-11	AGIF
D3, D4	LAP (TGF-β1)	----------
D5, D6	Leptin	LEP, OB
D7, D8	LIF	----------
D9, D10	Lipocalin-2/NGAL	LCN2, Siderocalin
D11, D12	CCL2/MCP-1	MCAF
D13, D14	M-CSF	CSF1, CSF-1
D15, D16	MIF	----------
D17, D18	Myeloperoxidase	Lactoperoxidase, MPO
D19, D20	Nidogen-1/Entactin	NID1
D21, D22	Oncostatin M (OSM)	----------
D23, D24	Pappalysin-1/PAPP-A	ASBABP2, DIPLA1, IGFBP-4ase, PAPA, PAPPA, PAPP-A1
E1, E2	PBEF/Visfatin	NAmPRTase, NAMPT, PBEF1
E3, E4	Pentraxin-3/TSG-14	PTX3
E5, E6	Pref-1/DLK-1/FA1	DLK1, DLK-1, FA1, pG2, ZOG
E7, E8	Proprotein Convertase 9/PCSK9	FH3, HCHOLA3, NARC-1, PC9
E9, E10	RAGE	AGER
E11, E12	CCL5/RANTES	SISd
E13, E14	Resistin	ADSF; FIZZ3; RETN
E15, E16	Serpin A8/AGT	Angiotensin II, Angiotensinogen, Aogen, PAT
E17, E18	Serpin A12	OL-64, Vaspin

E19, E20	Serpin E1/PAI-1	Nexin, PLANH1
E21, E22	TIMP-1	----------
E23, E24	TIMP-3	----------
F1, F2	Reference Spots	----------
F5, F6	TNF-α	Cachetin, DIF, TNF, TNFA, TNFSF1A, TNFSF2
F7, F8	VEGF	VAS, Vasculotropin, VEGFA, VPF
F23, F24	Negative Controls	----------

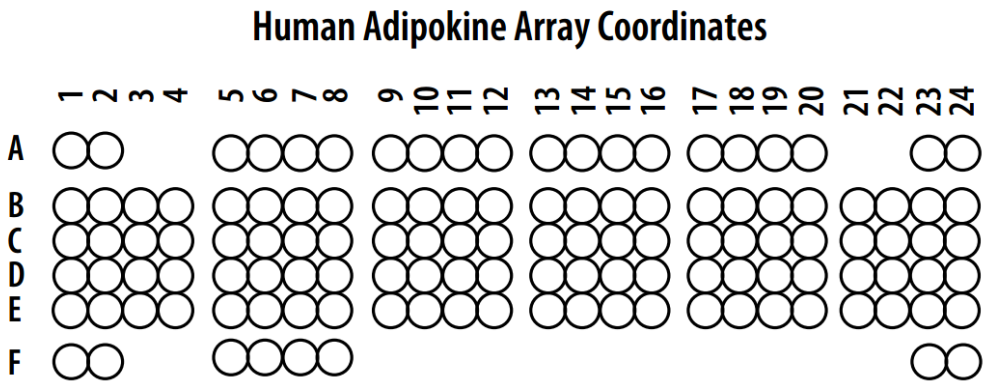

Figure 7.46: Human Adipokine Array Coordinates.

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