Inflammatory mediators in childhood chronic diseases

A three-tiered approach

Genoveva C. E. Keustermans

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Inflammatory mediators in childhood chronic diseases
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Ontstekingsmediatoren bij kinderen met chronische ziektes
Een drieledige aanpak

(met een samenvatting in het Nederlands)

Proefschrift

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Genoveva Carolina Emmanuela Keustermans
geboren op 04 augustus 1989
te Blantyre, Malawi

Promotoren:	Prof. dr. A.B.J Prakken
	Prof. dr. G. Pasterkamp
Copromotoren:	Dr. W. de Jager
	Dr. H.S. Schipper

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Chapter 1

General Introduction

1 Inflammation

1.1 Definition

Inflammation is the bodies main means of combatting the pathogenic stimuli to which it is exposed on a daily basis. Various triggers can induce inflammation, ranging from extrinsic factors such as pathogens to intrinsic activators such as cell damage. The function of this immune response is to eliminate the initial harmful factor, remove damaged cells and tissue and induce repair through blood vessel involvement, immune cell recruitment and a plethora of protein mediators. Inflammation is typically accompanied by redness, heat, pain, swelling and loss of function (1). The initial (innate) response to a pathogen converts into an (adaptive) lymphocyte predominant response later on. Insufficient inflammation can result in progressive tissue damage by pathogens while chronic inflammation results in a plethora of diseases including atherosclerosis, rheumatoid arthritis and cancer (2, 3). Acute inflammation is the bodies short term primary response to detrimental stimuli however, if left unresolved, acute inflammation may enter a longer term and chronic phase: chronic inflammation.

1.2 Relevance

Within this thesis, all further mention of inflammation will refer to the chronic category. Characterized by the simultaneous destruction and repair of tissue, chronic inflammation is accompanied by symptoms such as pain, fatigue, depression, weight gain and an increased susceptibility to infection (2). A severe consequence of this type of inflammation is the development of chronic inflammatory systemic diseases (CISD) such as cardiovascular disease, rheumatoid arthritis, metabolic disorders, auto-immune disorders and neurological diseases, causing increased mortality and a costly burden to both the patient and society (2). CISDs share common pathways of activation that usually manifest with systemic responses including maladaptation's of the immune, endocrine, nervous and reproductive system (2, 4, 5, 6, 7).

The WHO reports that a large part of the disease burden in Europe is caused by non-communicable diseases where CISDs are key examples (8). As a result of their increasing prevalence (9), it is vital that the underlying mechanisms of these inflammatory phenomena are unravelled. This is especially pertinent to the paediatric population as CISDs result in life-long debilitating illness, increased mortality and increased financial burden, all factors that must be prevented in a generation that will soon become the main body of society.

1.3 Cytokines

During inflammation, a mixture of soluble factors regulate leukocyte recruitment, cellular activation, adhesion and systemic responses. These factors fall into four categories 1) inflammatory lipid metabolites 2) soluble proteases 3) nitric oxide and 4) cytokines (10). Cytokines are considered to orchestrate the inflammatory response by specifically influencing the interaction and communication between cells (11). T cells and macrophages are considered the predominant producers of cytokines however, many cell populations are able to manufacture cytokines as well. These molecules can act in an autocrine, paracrine, endocrine or juxtacrine manner and may have a pro- or anti-inflammatory role during inflammation (12). Every cytokine has a corresponding cell-surface receptor that upon binding induces an intracellular signalling cascade then alters gene regulation, cytokine production and receptor expression thus changing cellular activity (**Fig 1a**) (13). As previously mentioned, nomenclature often divides cytokines into the pro- or anti-inflammatory category. It must be noted however that this designation is not a sole characteristic of the cytokine but, must include the location and environment in which the cytokine is present (12, 13, 14, 15). Cytokines can thus express a suppressive or activating phenotype dependant on the milieu. For the purpose of this segment, the subsequent cytokine categorizations are based on the most common phenotype and activity these markers express during CISDs.

1.3.1 Pro-inflammatory cytokines

Pro-inflammatory cytokines are mainly produced by activated macrophages, helper T cells and neutrophils (12). These factors include IL-1β, IL-6, IL-12, IL-18, tumour necrosis factor (TNF), interferon gamma (IFN-gamma) and granulocyte-macrophage colony stimulating factor (GMCSF) (12, 13). These molecules are closely linked to pathological pain (12) and when left un-regulated play a central role in in CISD.

1.3.2 Anti-inflammatory cytokines

Anti-inflammatory cytokines have an immuno-regulatory role and are produced by T cells and antigen presenting cells. These cytokines include interleukin (IL)-1 receptor antagonist, IL-4, IL-6, IL-10, IL-11, and IL-13 (12). These molecules often act in congruence with cytokine receptors and inhibitors to impede pro-inflammatory cytokine activity (12). In a pathological state these cytokines may excessively inhibit the immune response or provide inadequate control over pro-inflammatory conditions (14, 16).

1.3.3 Chemokines

Chemokines, also known as chemotactic cytokines are a subfamily of cytokines that induce chemotaxis (17). These factors have a low molecular weight and are categorized according to their behavioural and structural characteristics (17). The principal function of these molecules is in the activation and migration of leukocytes, macrophages and other effector cells in order to induce an immune response or stimulate wound healing (13, 18). Within the inflammatory setting these factors are considered pro-inflammatory (19).

1.3.4 Technical Aspects

Due to their central role in immune regulation, cytokines are considered key systemic biomarkers in monitoring the status of the immune system and the (local) disease state. In order to be used as biomarkers, however, several key factors regarding sample handling and measuring must be kept under control in order to produce data that reflects the biological situation (20). The necessary controls when looking to generate and use cytokine data are as follows: 1) sample and collection tubes for donor material should be identical, when working with blood samples, different anti-coagulants can influence cytokine release, 2) keep samples on ice or at 4°C before sample processing 3) processing biological material should be executed as soon as possible 4) long term sample storage should be at -80°C and 5) collect samples at the same time of day to reduce the effect of circadian rhythms on analyte levels (20). Lastly, due to the abundance in technology available to measure cytokine levels, it is important to consider technology that a) has a high specificity, affinity and sensitivity b) is reproducible and reliable and c) allows the detection of multiple cytokines within a small sample volume (20).

1.4 Adipokines

Adipokines are cytokines and other immunologically active proteins secreted by adipocytes and/or adipose tissue (AT) resident immune cells that exert both a local and systemic effect (21, 22). Some conventional cytokines produced by adipocytes and immune cells residing in AT may also act under the term adipokine (21). Like cytokines, adipokines are often separated into pro- or anti-inflammatory mediators this is, however, dependant on a wide variety of factors including the hosts metabolic status, oxidative stress, systemic inflammation, age and sex (21). This group of proteins is known to play a central role in metabolism and the regulation of inflammatory responses during obesity, infection and systemic inflammation (23).

1.4.1 Pro-inflammatory adipokines

Adipokines associated with increased inflammation include leptin, resistin, retinol-binding protein 4 (RbP4), chemerin, lipocalin 2, angiopoietin-like protein 2 (ANGPTL2), CC-chemokine ligand 2 (CCL2), CXC-chemokine ligand 5 (CXCL5), nicotinamide phospho ribosyltransferase (NAmPT), IL-6, IL-18 and

TNF (21). Up-regulation of these factors bolsters a chronic inflammatory state, this exemplified by the fact, elevated leptin enhances the production of other pro-inflammatory chemokines and cytokines, including IL-6, IL-12, CXCL8, and TNF from monocytes and macrophages (24, 25). Furthermore, resistin receptor binding induces downstream signalling cascades that induce inflammatory cytokine production in monocytes (**Fig 1a**) (26) while RBP4 is involved in the regulation of glucose homeostasis (27) and is postulated to enhance T_H1 cell polarization by activating AT antigen presenting cells (28). Lastly, chemerin acts as a chemoattractant for monocytes and dendritic cells (29) while all the remaining afore mentioned adipokines enhance inflammation by influencing immune cell activation, function and cytokine release.

1.4.2 Anti-inflammatory adipokines

Adipokines secreted by AT that exhibit anti-inflammatory effects include adiponectin, C1q/TNF-related proteins, sFRP5 and omentin. Of these proteins, adiponectin is the most well-known and studied adipokine. Produced mainly by adipocytes, this protein can inhibit inflammation by blocking NFκB activation which intern reduces pro-inflammatory cytokine production (21, 30). Adiponectin has also been shown to diminish vascular inflammation by decreasing TNF induced expression of IL-8 and vascular endothelial cell adhesion molecules which subsequently reduces monocyte attachment (31, 32, 33). C1q/TNF-related proteins, sFRP5 and omentin further carry out their inhibitory role by reducing pro-inflammatory cytokine production and activity by altering receptor binding and cellular signalling pathways.

1.4.3 Technical Aspects

Like cytokines, adipokine measurements must be performed with a set of basal methodologies in order to produce data that is representative of the biological situation. Initial adipokine measurements were performed using ELISAs however, multiplex immune assays are now more commonly implemented due to small sample volumes, high-throughput and the ability to measure multiple cytokines in one assay (20). Analysis of healthy donor adipokine levels have also shown that adiponectin and leptin exhibit circadian rhythms (34, 35). Leptin levels are also drastically influenced by nutritional status (fasting/feeding) while adiponectin serum levels decrease at night. Although these variations in adipokine levels are no longer significant under disease conditions, it is important to keep these factors in mind when collecting and processing patient and control material in order to maintain absolute uniformity.

1.5 Extracellular Vesicles

Extracellular vesicles (EVs) are small membrane bound particles secreted by all human cell types, these intercellular communicators can thus be found in many biological fluids (36). The EV population is a heterogeneous mix of particles of varying sizes, these can range from 50-1000nm and can be generated in the endosomal compartments of cells (**Fig 1c**) or, are shed into the environment following the budding and fission of the plasma membrane (37, 38, 39). Although EVs can be found in varying size ranges, the function of these size differences has scarcely been assessed due to a lack of qualified methodologies (36). EVs carry parental cell specific proteins and, are additionally enriched with proteins involved in adhesion, membrane trafficking, heat shock proteins, cytoplasmic enzymes, cytokines, chemokines, mRNA and cell specific antigens just to name a few (**Fig 1c**) (37). EVs also contribute to cell signal regulation by removing plasma membrane receptors and membrane attack complexes, by releasing signalling proteins and by transferring microRNA (37, 40). It is this unique vesicular content and functionality that allows these particles to act as cell-cell communicators that are essential in immune regulation. EVs can influence immune stimulation and suppression and have been shown to advance the pathogenesis of inflammatory, auto-immune and infectious diseases (37, 40).

1.5.1 Technical Aspects

EV isolation is achieved through size exclusion methodologies of which high speed ultracentrifugation is most commonly used. Isolation through ultracentrifugation can be achieved through multiple steps of increasing speed or through the use of sucrose density gradients. In all, regardless of the isolation procedure, the resulting EV population is heterogeneous in nature. In order to separate and eventually investigate the different size groups within the EV population, many techniques have been applied however, due to overlapping size and buoyant densities, vesicles can, as of yet, not be classified according to their size. Classification according to the process of EV biogenesis is also difficult to apply due to the overlapping particle sizes. Various standardized techniques have, however, been implemented to allow vesicle isolation and characterization. Electron microscopy (EM) is used to create high resolution images of the small membrane bound particles. This technique yields images reflecting the typical cup shape of isolated particles (41). Of the many methods used in bulk isolate characterization, western-blotting is the most commonly accepted technique of vesicle identification. Although western-blotting gives clear data on the molecular composition of particles, the technique does not allow analysis of the individual vesicle subsets within the heterogeneous population. Flow cytometry has thus come forward as a robust technique for quantitative and qualitative analysis of vesicles and the various sub-populations (36). Flow cytometry analysis does, however, only yield relative information on vesicle sizes. Future methods should therefore be defined that allow vesicles to be characterized accurately in both quantity and size, as well as according to EV specific markers and marker patterns (42).

1.6 Immune cells

As previously mentioned, the innate immune response is triggered by signals from invading pathogens. Associated with this reaction is the recruitment and activation of innate immune cells that originate from the myeloid lineage (43). These cells include neutrophils, dendritic cells, macrophages, mast cells, and eosinophils. This initial immunological reaction can be categorized as an acute inflammatory response which is associated with increased blood flow, vascular permeability and immune cell migration. The process is fast and occurs within minutes to hours (44). In contrast to the broad initial response of the innate system, the adaptive immune response is more specific in targeting the pathogenic stimulus and results in the development of immunological memory. Lymphoid derived immune cells, namely B cells and T lymphocytes, play a critical role in this adaptive response (43). The ability of B and T cells to recognize antigens means they have key functions in sterile or pathogen induced inflammation as well as autoimmunity (44). If an acute immunological response is left unresolved a chronic condition arises. Monocytes, macrophages and T lymphocytes are the primary immune cells involved in chronic inflammation (45). These cell subsets produce copious amounts of cytokines and enzymes that induce cell damage and lead to disease propagation in a process that is slow and extends over a period of days to years (**Fig 1b**). Specific immune cell functionality and subtypes within chronic inflammation are elaborated on further on in this introduction.

1.6.1 Technical Aspects

Unlike cytokines, adipokines and EVs, immune cells have been examined for decades within the context of immunology. Starting of simply with microscope derived counts and histology, immune cell phenotyping and identification has reached its peak over the last two decades. By implementing technologies such as flow cytometry, genetic profiling, proteomics and immunoassays, immune cell function and activity within chronic inflammation can be analysed. Within the context of this thesis FACS, immunoassays and microarray techniques were used to investigate immune cell sub-populations.

2 Obesity

As previously mentioned, inflammation is a vital component in host defence and immune surveillance however, if left unresolved, a chronic inflammatory state can ensue. Obesity is a prime example of

chronic inflammation. With obesity becoming a worldwide epidemic that is rising at an alarming rate and is affecting the paediatric population at an ever-increasing rate, it is of vital importance that the mechanism, consequence and prevention of paediatric obesity is investigated in order to abate the inevitable future personal and economic burden of this disorder.

2.1 Role of cytokines

Important in obesity is the function of adipose tissue (AT). Adipocytes in adipose tissue act as immune cells (46, 47). During obese pathogenesis, these cells release copious amounts of pro-inflammatory cytokines including IL-1*β*, IL-6, IL-18, IL-33, CCL2, CXCL5 and TNF (47). Monocytes and lymphocytes present in AT also contribute to the cytokine inflammatory soup.

Typical cytokine hallmarks in obesity include IL-6. IL-6 levels are elevated in obese individuals with one third of total circulating levels being produce by AT (21). IL-6 is also associated with insulin resistance and has been shown to be predictive of T2D development (48). It is postulated that IL-6 inhibits insulin signalling by inducing proteins that bind to the insulin receptor (21). TNF is elevated in the AT of experimental mouse models of obesity and type 2 diabetes (49). The neutralization of this cytokine through gene mutation or knock put leads to augmented insulin sensitivity (21, 50). IL-1*β* inhibits insulin signal transduction in AT (21, 51). Inhibiting IL-1*β* activity improves insulin signalling and is additionally accompanied by a decrease in the macrophage pro-inflammatory phenotype (52). Obesity is furthermore linked to elevated IL-18 levels with weight loss resulting in a subsequent decrease in cytokine concentration. Atherosclerotic lesions have also been shown to contain high levels of IL-18 which may play a role in CVD pathology by influencing plaque stability (21). CCL2 levels rise in AT during glucose deprivation and CXCL5 secreted by macrophages has been associated with AT inflammation and insulin resistance (21). In all evidence clearly indicates the importance of cytokine activity in obese pathology.

2.2 Role of adipokines

Similar to cytokines, pro-inflammatory adipokine levels increase during obesity. Leptin expression is increased in obese AT, inducing insulin resistance and inflammation. Leptin levels have furthermore been linked to disease severity in CVD (21, 53, 54). Increased resistin levels are associated with insulin resistance and CVD by influencing CCL2 production by AT and subsequent monocyte recruitment or, by stimulating monocyte foam cell development (21, 53). Chemerin levels are associated with metabolic syndrome and obesity while RBP4 expression is increased in congruence to increased BMI and waist circumference and, has been shown to contribute to insulin resistance (21, 53). Lastly, lipocalin 2 levels are elevated in atherosclerotic plaques and may thus influence obesity associated CVD pathology (53). Unlike the afore mentioned adipokines, adiponectin levels are decreased in obese AT (21). Adiponectin is known to have protective functions against obesity associated metabolic disorders and CVD. It has furthermore been shown that plasma adiponectin levels are decreased in patients with T2D (21). Data thus indicates that metabolic dysfunctions associated with obesity are influenced by the disparity in pro- and anti-inflammatory adipokines expression.

2.3 Role of extracellular vesicles

EVs secreted by mouse AT have been associated with systemic insulin resistance (55). Human AT also secretes EVs that affect local insulin resistance in adipocytes by causing macrophage activation (55). EV production by visceral adipose tissue has also been shown to influence insulin resistance on a systemic level (55). Previous research has elegantly shown that AT derived EVs specifically impede insulin signalling in liver and muscle cells (55). Differences in downstream insulin signalling pathways thus allow AT derived EVs to differentially influence liver and skeletal muscle activity with reduced insulin signalling in muscles resulting in impaired glucose uptake while, in liver cells, affecting gluconeogenesis and glucose and lipid glycogen storage which could all contribute to typical obesity related pathologies such as non- alcoholic fatty liver disease. Lastly, in patients with clinically

manifested CVD, EV cystatin C levels are positively associated with obese metabolic pathologies while EV CD14 expression is inversely associated with male visceral adiposity and a reduced risk for T2D (56).

2.4 Role of immune cells

AT contains almost all immune cell populations. These include macrophages, mast cells, monocytes, dendritic cells, invariant natural killer T-cells (iNKTs), B-cells, T-cells, neutrophils, and eosinophils (57). During obesity there is a significant increase in AT macrophages (58). Chemokines cause monocytes to migrate from peripheral blood into the AT where they are subsequently polarized towards an inflammatory phenotype (**Fig 1b**) (59). In mice, an important AT macrophage sub-population expresses the integrin CD11c. All macrophage populations increase with adiposity however the CD11c positive is predominant in this elevation (57). These CD11c positives cells are considered to be key participants in AT inflammation and metabolic dysfunction (57). In humans, $CD11c^{+}$ AT macrophages are associated with insulin-resistance and express a pro-inflammatory phenotype (60). Similar to CD11c expressing macrophages, mast cell numbers are elevated during obesity (57). Eosinophil IL-4 production is reduced during obesity thereby influencing macrophage phenotype (57). Similar to macrophages, CD3 positive T cells are a predominant immune cell population within AT (57). As adiposity increases so too does the size of the CD3 positive T cell population. CD8+ T cells, IFNγ+ T helper 1 (TH1) cells and regulatory T cells (T regs) are found in human AT. Research with obese mice indicates an increase in CD4 and CD8 positive T cell numbers while elevated TH1 numbers and reduced T reg numbers contribute to the inflammatory phenotype in AT. Finally, iNKT cells are important mediators of glucose tolerance and these cell numbers decrease in AT during obesity (57).

3. Juvenile Idiopathic Arthritis

Juvenile idiopathic arthritis (JIA) is another ideal illustration of chronic inflammation. It is the most common example of chronic rheumatic disease in children (61). JIA onset occurs before 16 years of age (61). The disease exhibits clinical heterogeneity manifested in several classifications as result of varying clinical and immunological compositions. Patients can exhibit local or systemic inflammation in acute, recurrent or extended episodes (62). Patients in clinical remission, furthermore, still express higher levels of pro-inflammatory cytokines when compared to healthy controls (63). The cause of this disease is difficult to decipher as a result of this complex manifestation (61, 62). Exhibiting a high prevalence and causing both short and long-term disability, it is thus important to understand every facet of this disease (62). For the purpose of this thesis, JIA will be used to study chronic inflammation in the paediatric population.

3.1 Role of cytokines

Although JIA onset and disease course is manifested in various forms, each subtype of JIA exhibits chronic inflammation of the joint accompanied by immune cell infiltration and elevated cytokine production (63). Previous research has shown that irrespective of the JIA subtype, patients exhibit elevated CCL2, CCL3, CCL11, CCL22, CXCL9, TNF and macrophage inhibitory factor (MIF) when compared to healthy controls (63). Furthermore, patient synovial fluid exhibits increased levels of IL-6, IL-15, CCL2, CCL3, CXCL8, CXCL9 and CXCL10 when compared to donor matched peripheral blood samples (63). Lastly, data from cluster analyses indicates that during active diseases, patients exhibit a pro-inflammatory cytokine profile while, during remission, the profile becomes more regulatory and anti-inflammatory. This data collectively indicates the central role played by cytokines in JIA pathogenesis, exemplified by the successful use of anti-TNF therapy in treating polyarthritic JIA patients.

3.2 Role of adipokines

Previous research on adult RA patients shows that serum and synovial fluid adiponectin levels are linked to radiographic damage and elevated when compared to healthy donors (64). Data further suggests that adiponectin may exert a pro-inflammatory role and mediate joint degradation (64).

Within the same patient population, some research has suggested an association between leptin levels and disease activity however, other groups were unable to measure any changes in this adipokine (64). Chemerin has been shown to have a pro-inflammatory effect on fibroblast-like synoviocytes while visfatin levels are increased in RA patient synovial fluid (64). Finally, resistin secretion is also increased in RA derived synovial fluid (64). Although much work has been carried out in the adult population, little data is present for adipokine functionality in JIA. What is known, however, is that the decreased BMI in JIA patients may be caused by patient ghrelin and leptin levels. A positive correlation has been recorded for the relationship between ghrelin and leptin levels and patient BMI, with patients exhibiting active disease showing significantly lower levels of ghrelin when compared to those with inactive disease (65).

3.3 Role of extracellular vesicles

Similar to adipokines, much work on the role of EVs in arthritis has focused on RA patients. In RA patients, both local and systemic increased EV numbers are associated with increased disease severity (37, 40). These EVs have, furthermore, been shown to contain pro-inflammatory cytokines and are able to cause fibrin deposition by inducing coagulation (37, 40). RA synovial fluid derived vesicles increase IL-6, CXCL8, CCL2, CCL5, and ICAM-1 production while RA platelet derived vesicles carry IL-1ß and induce CXCL8 production by synovial fibroblasts (37). T cell and monocyte derived vesicles have furthermore been shown to activate various signalling pathways within synovial fibroblasts. In all, this data indicates the key involvement of EVs in local inflammation and joint destruction. Of the limited data available on EVs in JIA, one body of work has shown that DEK autoantigen secretion from macrophages in a subtype of JIA patients occurs through extracellular vesicles which may contribute to joint inflammation by generating immune complexes (37, 66).

3.4 Role of immune cells

JIA pathogenesis is accompanied by the exuberant infiltration of immune cells into the joint. Monocytes, T cell, B cells, macrophages, dendritic cells infiltration and subsequent cellular activation leads to profound joint inflammation (61, 67). T cells within the joint are skewed towards a TH1 phenotype while CD4+ cells exhibit an activated memory phenotype along with elevated CCR5 and CXCR3 receptor expression (68). Differences in regulatory T cell frequencies within JIA subtypes contribute to the heterogeneity in disease pathology (61, 67, 69). It is furthermore known that an imbalance between pro-inflammatory and regulatory T cell subtypes contributes to synovial inflammation (70). Monocytes in addition to T cells are known to contribute to JIA pathology. Within the systemic JIA subtype, elevated blood monocyte numbers accompany disease flare while DC and γδ T cell numbers are reduced. Systemic JIA patients also exhibit reduced NK cell function as a result of defective IL-18Rβ phosphorylation. Finally, B cells have been proposed to augment chronic inflammation in JIA by expressing various costimulatory molecules and secreting cytokine that can activate and polarize T cells (71).

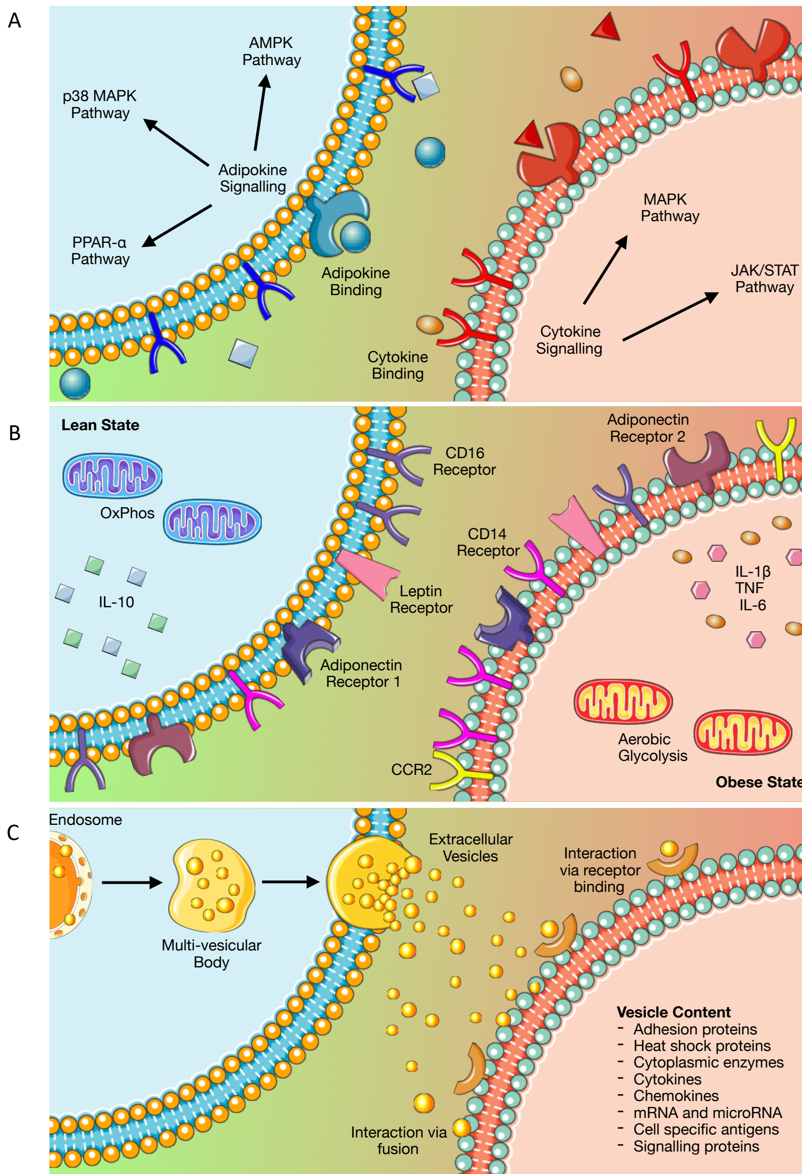

Fig 1: Here obesity is used as an example of chronic inflammation. A- illustrates the downstream signalling cascades induced by adipokines and cytokines upon immune cell binding. B- In this panel, monocytes are used to illustrate the changes that occur in immune cells during inflammation, this includes alterations in receptor expression, pro-inflammatory cytokine production and immune cell metabolism (from oxidative phosphorylation to aerobic glycolysis). Lastly, C- demonstrates intraluminal extracellular vesicle production, illustrates subsequent vesicle-immune cell interactions through receptor binding or fusion and shortly describes the complex extracellular vesicle content that contributes to their immune activity.

Thesis Outline

For the purpose of this thesis we took a 3-tier approach. The first tier (**chapter 2**) focuses on cytokines as the smallest factors involved in inflammation. This chapter highlights the methodological steps that are necessary when implementing cytokines as biomarkers of chronic inflammation. The second tier (**chapter 3**) looks to analyse the role of EVs in chronic inflammation. In the context of our experiments we were able to show that in paediatric chronic inflammation, vesicles can be successfully isolated and implemented in functional assays. In the last tier of this thesis we take a step toward the cellular level by focusing on the role of immune cells within paediatric chronic inflammation. In **chapter 4 and 5** chronic inflammation in the form of childhood obesity comes to the forefront. By looking at adipokine receptor expression on various cellular subsets in combination with circulating adipokine levels we were able to provide insight into a previously unexplored section of paediatric obesity (**Chapter 4**). In **chapter 5** we delve into the influence of obesity on monocyte gene expression and, the subsequent association of these genes with circulating adipokines and clinical variables within paediatric obesity as well as coronary atherosclerosis within the adult population. In **chapter 6**, the role of cytokines, adipokines, EVs and immune cells within the context of chronic paediatric inflammation will be discussed. By combining previous work with the findings in this thesis, this final chapter will look to the future of dissecting and understanding chronic childhood inflammation in the hopes of providing tools towards combatting and resolving the deleterious outcomes of this disease.

References

1. Abbas A.K, Lichtman A.H.. Basic immunology : functions and disorders of the immune system . [book auth.] Abul K. Abbas. *Basic immunology : functions and disorders of the immune system .* 3rd Edition. London : Saunders, 2009, p. Ch.2.

2. Straub R. H, Schradin C. *Chronic inflammatory systemic diseases.* Evol Med Public Health. 2016, Vol. 1, pp. 37-51.

3. Prasad S, Aggarwal B.B.*Chronic Diseases Caused by Chronic Inflammation Require Chronic Treatment: Anti-inflammatory Role of Dietary Spices.* J Clin Cell Immunol. 2014, Vol. 5.

4. Straub R.H, Besedovsky H.O. *Integrated evolutionary, immunological, and neuroendocrine framework for the pathogenesis of chronic disabling inflammatory diseases.* FASEB J. 2003, Vol. 15, pp. 2176-2183.

5. Soares P.M, Borba E.F, Bonfa E, Hallak J, Corrêa A.L, Silva C.A. *Gonad evaluation in male systemic lupus erythematosus.* Arthritis Rheum. 2007, Vol. 56, pp. 2352-2361.

6. Straub R. *The Origin of Chronic Inflammatory Systemic Diseases and their Sequelae.* 1st Edition. Oxford : Elsevier LTD, 2015. 9780128033210.

7. Wallenius M, Skomsvoll J.F, Irgens L.M, Salvesen K.Å, Nordvåg B.Y, Koldingsnes W, Mikkelsen K, Kaufmann C, Kvien T.K. *Fertility in women with chronic inflammatory arthritides.* Rheumatology (Oxford). 2011, Vol. 50, pp. 1162-1167.

8. World Health Organization Europe. *WHO website.* [Online] 2017. http://www.euro.who.int/en/health-topics/noncommunicable-diseases.

9. World Health Organization Europe . *euro.who.int.* [Online] 2017. http://www.euro.who.int/en/health-topics/noncommunicable-diseases/noncommunicable-diseases.

10. Feghali C.A, Wright T.M. *Cytokines in Acute and Chronic Inflammation.* Frontiers in Bioscience . 1997, Vol. 2, pp. 12-26.

11. Lackie J. *A Dictionary of Biomedicine.* s.l. : Oxford University Press, 2010. 9780199549351.

12. Zhang J, An J. *Cytokines, Inflammation and Pain.* Int Anesthesiol Clin. 2007, Vol. 45, pp. 27-37.

13. Turner M. D, Nedjai B, Hurst T, Pennington D.J. *Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease.* Biochimica et Biophysica Acta. 2014, Vol. 1843, pp. 2563-2582.

14. Opal S.M, DePalo V.A. *Anti-Inflammatory Cytokines.* CHEST. 2000, Vol. 117, pp. 1162-1172.

15. Cavaillon, J.M. *Pro- versus anti-inflammatory cytokines: myth or reality.* Cell Mol Biol. 2001, Vol. 47, pp. 695-702.

16. Donnelly S.C, Strieter R.M, Reid P.T, Kunkel S.L, Burdick M.D, Armstrong I, Mackenzie A, Haslett C. *The association between mortality rates and decreased concentrations of interleukin-10 and interleukin-1 receptor antagonist in the lung fluids of patients with the adult respiratory distress syndrome.* Ann Intern Med. 1996, , Vol. 125, pp. 191-196.

17. Le Y, Zhou Y, Iribarren P, Wang J. *Chemokines and Chemokine Receptors: Their Manifold Roles in Homeostasis and Disease.* Cell & Molec Immunol. 2004, Vol. 1, pp. 95-104.

18. Ono S.J, Nakamura T , Miyazaki D, Ohbayashi M, Dawson M, Toda M. *Chemokines: Roles in leukocyte development, trafficking, and effector function.*J Allergy & Clin Immun. 2003, Vol. 11, pp. 1185-1199.

19. Fernandez E.J, Lolis E. *Structure, function, and inhibition of chemokines.* Annu Rev Pharmacol Toxicol. April, Vol. 42, pp. 469-499.

20. Keustermans G.C.E, Hoeks S.B, Meerding J.M, Prakken B.J, de Jager W. *Cytokine assays: an assessment of the preparation and treatment of blood and tissue samples.* Methods. 2013, Vol. 61, pp. 10-17.

21. Ouchi N, Parker J.L, Lugus J.J, Walsh K. *Adipokines in inflammation and metabolic disease.* Nat Rev Immunol. 2011, Vol. 11, pp. 85-97.

22. Berg A.H, Scherer P.E. *Adipose tissue, inflammation, and cardiovascular disease.* Circ Res. 2005, Vol. 96, pp. 939-949.

23. Mancuso P. *The role of adipokines in chronic inflammation.* Immuno Targets & Therapy. 2016, Vol. 5, pp. 47-56.

24. Santos-Alvarez R, Goberna V, Sanchez-Margalet J. *Human leptin stimulates proliferation and activation of human circulating monocytes.* Cell Immunol. 1999, Vol. 194, pp. 6-11.

25. Kiguchi N, Maeda T, Kobayashi Y, Fukazawa Y, Kishioka S. *Leptin enhances CC-chemokine ligand expression in cultured murine macrophage* Biochem Biophys Res Commun. 2009, Vol. 384, pp. 311-315.

26. Bokarewa M, Nagaev I, Dahlberg L, Smith U, Tarkowski A. *Resistin, an adipokine with potent proinflammatory properties.* J Immunol. 2005, Vol. 174, pp. 5789-5795.

27. Graham T.E, Yang Q, Blüher M, Hammarstedt A, Ciaraldi T.P, Henry R.R, Wason C.J, Oberbach A, Jansson P.A, Smith U, Kahn B.B. *Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects*. N Engl J Med. 2006,Vol. 354.

28. Moraes-Vieira P.M, Yore M.M, Dwyer P.M, Syed I, Aryal P, Kahn B.B. *RBP4 activates antigen-presenting cells leading to adipose tissue inflammation and systemic insulin resistance.* Cell Metab. 2014, Vol. 19, pp. 512-526.

29. Bondue B, Wittamer V, Parmentier M. *Chemerin and its receptors in leukocyte trafficking, inflammation and metabolism.* Cyto & Growth factor Rev. 2011, Vol. 22, pp. 331-338.

30. Ouchi N, Kihara S, Funahashi T, Matsuzawa Y, Walsh K. *Obesity, adiponectin and vascular inflammatory disease.* Curr Opin Lipidol. 2003, Vol. 14, pp. 561-566.

31. Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H , Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. *Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin.* Circulation. 1999, Vol. 100, pp. 2473-2476.

32. Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H , Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. *Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway.* Circulation. 2000, Vol. 102, pp. 1296-1301.

33. Kobashi C, Urakaze M, Kishida M, Kibayashi E, Kobayashi H, Kihara S, Funahashi T, Takata M, Temaru R, Sato A, Yamazaki K, Nakamura N, Kobayashi M. *Adiponectin inhibits endothelial synthesis of interleukin-8.* Circ Res. 2005, Vol. 97, pp. 1245-1252.

34. Hill M.J, Kumar S, McTernan P.G. *Adipokines and the clinical laboratory: what to measure, when and how?* J Clin Pathol. 2009, Vol. 62, pp. 206-211.

35. Shea S.A, Hilton M.F, Orlova C, Ayers R.T, Mantzoros C.S. *Independent Circadian and Sleep/Wake Regulation of Adipokines and Glucose in Humans.* J Clin Endocrinol & Metab. 2005, Vol. 90, pp. 2537-2544.

36. van der Vlist E.J, Nolte-'t Hoen E.N.M, Stoorvogel W, Arkesteijn G.J.A. Wauben M.H.M. *Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry.* Nat prot. 2012, Vol. 7.

37. Buzas E.I, Gyorgy B, Nagy G, A Falus, Gay S. *Emerging role of extracellular vesicles in inflammatory diseases.* Nat Rev Rheumatol. 2014, Vol. 10, pp. 356-364.

38. Simons M, Raposo G. *Exosomes- vesicular carriers for intercellular communication.* Curr Opin Cell Biol. 2009, Vol. 21, pp. 575-581.

39. Cocucci E, Racchetti G, Meldolesi J. *Shedding microvesicles: Artefacts no more.* Trends in cell biol. 2009, Vol. 19, pp. 43-51.

40. Robbins P.D, Morelli A.E. *Regulation of immune responses by extracellular vesicles.* Nat Rev. 2014, Vol. 14, pp. 195-208.

41. van der Pol E, Böing A.N, Harrison P, Sturk A, Nieuwland R. *Classification, Functions, and Clinical Relevance of Extracellular Vesicles.* Pharmacological Rev. 2012, Vol. 64, pp. 676-705.

42. áñez-Mó M, Siljander P.R.M, Andreu Z, Zavec A.B, Borràs F.E, Buzas E. I, … De Wever O. *Biological properties of extracellular vesicles and their physiological functions.* J Extracell Vesicles . 2015, Vol. 4.

43. Kuby J, Kindt T.J. *Kuby Immunology.* 6th Revised Edition. New York : W.H.Freeman &Amp; Co Ltd. 0716767643.

44. Luster A.D, Alon R , von Andrian U.H. *Immune cell migration in inflammation: present and future therapeutic targets.* Nat Immunol. 2015, Vol. 6, pp. 1182-1190.

45. Rubin E, Reisner H. *Essentials of Rubin's Pathology.* 6th Edition. Baltimore : Lippincott Williams And Wilkins, 2009. 978-1451110234.

46. Schäffler A, Schölmerich J, Salzberger B. *Adipose tissue as an immunological organ: Toll-like receptors, C1q/TNFs and CTRPs.* Trends Immunol. 2007, Vol. 28, pp. 393-399.

47. Monteiro R, Azevedo I. *Chronic Inflammation in Obesity and the Metabolic Syndrome.* Mediators Inflamm. 2010.

48. Pradhan A.D, Manson J.E, Rifai N, Buring J.E, Ridker P.M. *C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus.* JAMA. 2001, Vol. 286, pp. 327-334.

49. Hotamisligil G.S, Shargill N.S, Spiegelman B.M. *Adipose expression of tumor necrosis factor-α: direct role in obesity-linked insulin resistance.* Science. 1993, Vol. 259, pp. 87–91.

50. Hotamisligil G.S, Budavari A, Murray D, Spiegelman B.M. *Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes. Central role of tumor necrosis factor-α J.* Clin Invest. 1994, Vol. 94, pp. 1543–1549.

51. Gao D, Madi M, Ding C, Fok M, Steele T, Ford C, Hunter L, Bing C. *Interleukin-1β mediates macrophage-induced impairment of insulin signaling in human primary adipocytes.* Am J Physiol Endocrinol Metab. 2014, Vol. 307.

52. Bing C. *Is interleukin-1β a culprit in macrophage-adipocyte crosstalk in obesity?* Adipocyte. 2015, Vol. 4, pp. 149–152.

53. Mancuso P. *The role of adipokines in chronic inflammation.* Immuno Targets & Therapy. 2016, Vol. 5, pp. 47–56.

54. Koh K.K, Park S.M, Quon M.J. *Leptin and Cardiovascular Disease.* Circulation. 2008, Vol. 117, pp. 3238–3249.

55.. Kranendonk M.E.G, Visseren F.L.J, van Herwaarden J.A, Nolte-'t Hoen E.N.M, de Jager W, Wauben M.H.M , Kalkhoven E. *Effect of Extracellular Vesicles of Human Adipose Tissue on Insulin Signaling in Liver and Muscle Cells*. Obesity. 2014, Vol. 22.

56. Kranendonk M.E.G, de Kleijn D.P.V, Kalkhoven E, Kanhai D.A, Uiterwaal C.S.P.M, van der Graaf Y, Pasterkamp G, Visseren F.LJ. *Extracellular vesicle markers in relation to obesity and metabolic*

complications in patients with manifest cardiovascular disease. Cardiovascular Diabetology. 2014, Vol. 13.

57. Ferrante A.W Jr. *The Immune Cells in Adipose Tissue.* Diabetes Obes Metab. 2013, Vol. 15, pp. 34-38.

58. Weisberg S.P, McCann D, Desai M, Rosenbaum M, Leibel R.L, Ferrante A.W Jr. *Obesity is associated with macrophage accumulation in adipose tissue.* J Clin Invest. 2003, Vol. 112, pp. 1796-1808.

59. Lumeng C.N, Bodzin J.L, Sal el A.R. *Obesity induces a phenotypic switch in adipose tissue macrophage polarization.* J Clin Invest. 2007. Vol. 117, pp. 175-184.

60. Wentworth J.M, Naselli G, Brown W.A, Doyle L, Phipson B, Smyth G.K, Wabitsch M, O'Brien P.E, Harrison L.C. *Pro-Inflammatory CD11c+CD206+ Adipose Tissue Macrophages Are Associated With Insulin Resistance in Human Obesity.* Diabetes. 2010, Vol. 59, pp. 1648-1656.

61. Prakken B, Albani S, Martini A. *Juvenile idiopathic arthritis.* Lancet. 2011,Vol. 377, pp. 2138-2149.

62. Ravelli A, Martini A. *Juvenile idiopathic arthritis.* Lancet. 2007, Vol. 369, pp. 767-778.

63.. de Jager W, Hoppenreijs E.P.A.H, Wulffraat N.M, Wedderburn L.R, Kuis W, & Prakken B.J. *Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study.* Ann Rheum Dis. 2007,Vol. 66, pp. 589–598.

64. Del Prete A, Salvi V, Sozzani S. *Adipokines as potential biomarkers in rheumatoid arthritis.* Mediators of Inflammation. 2014, Vol. 2014, pp. 1-11.

65.. Selim Z.I, Abdel Aziz N.H.R, Sherif T, Monzea E.M.M. *Serum levels of ghrelin, adipokines, and tumor necrosis factor-a (TNF-a) in patients with juvenile idiopathic arthritis in Assuit University Hospitals: Relation to nutritional status and disease activity*. Egyptian Ped Assoc Gazette. 2015, Vol. 63, pp. 52-57.

66. Mor-Vaknin N, Kappes F, Dick A.E, Legendre M, Damoc C, Teitz-Tennenbaum S, Kwok R, Ferrando-May E, Adams B.S, Markovitz D.M. *DEK in the synovium of patients with juvenile idiopathic arthritis: characterization of DEK antibodies and posttranslational modification of the DEK autoantigen.* Arthritis Rheum. 2011, Vol. 63, pp. 556-567.

67. Macaubas C, Nguyen K, Deshpande C, Phillips C, Peck A, Lee T,Park J.L, Sandborg C, Mellins E.D. *Distribution of circulating cells in systemic juvenile idiopathic arthritis across disease activity states*. Clin Immunol. 2010, Vol. 134, p. 206.

68. Prelog M, Schwarzenbrunner N, Sailer-Höck M, Kern H, Klein-Franke A, Ausserlechner M.J, Koppelstaetter C, Brunner A, Duftner C, Dejaco C, Strasak A.M, Müller T, Zimmerhackl L.B, Brunner J. *Premature aging of the immune system in children with juvenile idiopathic arthritis.* Arthritis Rheum. 2008, Vol. 58, pp. 2153-2162.

69. Macaubas C, Nguyen K, Milojevic D, Park J.L, Mellins E.D. *Oligoarticular and polarticular JIA: epidemiology and pathogenesis.* Nat Rev Rheumatol. 2009, Vol. 5, pp. 616–626.

70. Nistala K, Wedderburn L.R. *Th17 and regulatory T cells: rebalancing pro- and anti-inflammatory forces in autoimmune arthritis*. Rheumatology. 2009, Vol. 48, pp. 602-606.

71. Wiegering V, Girschick H.J, Morbach H. *B-Cell Pathology in Juvenile Idiopathic Arthritis.* Arthritis. 2010.

Chapter 2

Cytokine assays: An assessment of the preparation and treatment of blood and tissue samples

Genoveva C.E. Keustermans, Sanne B.E. Hoeks, Jenny M. Meerding, Berent J. Prakken, Wilco de Jager

Department of Paediatric Immunology (KC01.069.0), Centre for Molecular and Cellular Intervention, University Medical Centre Utrecht, Utrecht, The Netherlands

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ABSTRACT

Cytokines are key components of the innate and adaptive immune system. As pivotal players in the progression or regression of a pathological process, these molecules provide a window through which diseases can be monitored and can thus act as biomarkers. In order to measure cytokine levels, a plethora of protocols can be applied. These methods include bioassays, protein microarrays, high-performance liquid chromatography (HPLC), sandwich enzyme-linked immunosorbent assay (ELISA), Meso Scale Discovery (MSD) electrochemiluminescence and bead based multiplex immunoassays (MIA). Due to the interaction and activity of cytokines, multiplex immunoassays are at the forefront of cytokine analysis by allowing multiple cytokines to be measured in parallel. However, even with optimized protocols, sample standardization needs to occur before these proteins can optimally act as biomarkers. This review describes various factors influencing the levels of cytokines measured in plasma, serum, dried blood spots and tissue biopsies, focusing on sample collection and handling, long term storage and the repetitive use of samples. By analysing how each of these factors influences protein levels, it is concluded that samples should be stored at low temperatures in order to maintain cytokine stability. In addition, within a study, sample manipulations should be kept the same, with measurement protocols being chosen for their compatibility with the research in question. By having a clear understanding of what factors influence cytokine levels and how to overcome these technical issues, minimally confounded data can be obtained and cytokines can achieve optimal biomarker activity.

1. Introduction

Cytokines are small protein, glycoprotein or peptide molecules that, through cell signalling, allow intricate cellular communication. These molecules are produced by cells of various embryological origin and are classified according to structure or function (1). Structural classification is used mainly for cytokines that do not display a high level of redundancy, allowing these members to be separated into distinct groups. In general, however, cytokines are divided into functional families based on the enhancement of immunological responses, these include interleukin-1 (IL-1), IL-6, IL-10, IL-12, IL-17, TNF, IFN, TGF, PDGF, gamma chain (IL-2, IL-4, IL-7, IL- 9, IL-15, TLSP), and beta chain (IL-3, IL-5) families and chemokines (C, CC, CX and CXC3). They exhibit a wide variety of activities but are most often seen as effector molecules that can alter the behaviour of the immune system instantly during an immune response. Cytokines can act in an autocrine, paracrine, endocrine or juxtacrine manner (Fig. 1). Upon binding with their cell surface receptor, these molecules initiate an intracellular signalling cascade that may result in the up regulation and/or down regulation of gene expression or may take on transcription factor activity all subsequently inducing further cytokine production or curbing their own activity via feedback inhibition. The effect exerted by a cytokine depends on its extracellular levels, the expression of its complementary receptor and the type of signalling cascade initiated by this receptor binding, with cell type particularly influencing receptor expression and downstream signalling. These molecules exhibit redundancy in their activity as well as dual functionality. This is exemplified by their ability to act in one case as a receptor ligand and in another as a transcription factor (2). Cytokines produced by cells of the innate immune system play an essential role in influencing the immune response towards protective immunity and act as a key link between the innate and adaptive immune system. In response towards pathogens these molecules are secreted by immune cells and act as first danger signals, alerting, and initiating immunological pathways. Disturbance in this balance, however, plays a role in chronic disease progression by influencing auto-inflammatory or auto-immune pathways (3,4).

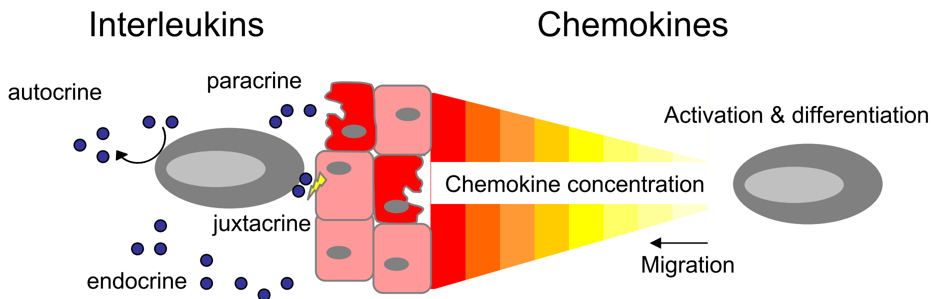

Fig. 1: General function of cytokines. When secreted cytokines (interleukins) can act on cells or tissue that secrete them (autocrine), surrounds them (paracrine), travel to distant cells (endocrine) or remain cell bound and activate neighbouring cells (juxtacrine). On the other hand chemokines are regulatory proteins which induce migration, activation and differentiation of cells to inflamed or damaged tissue.

The specific role of cytokines in these pathological disorders has been the subject of extensive research over the past few decades with molecular cloning, specific blockage of activity, gene deletion and receptor identification as well as the testing of recombinant cytokines illustrating how essential cytokines are to disease pathogenesis (5,6,7). As a result of this work, the use of new drugs which modulate the inflammatory processes of the immune system for many disease types in the field of oncology and immunology have been shown to result in major changes in the downstream cytokine milieu. The most elegant illustration of the functionality of cytokines comes from the blockage of TNFa in rheumatoid arthritis, this specific blockage results in the effective reduction of disease activity and joint destruction (8,9). Contradictory to these positive intervention strategies, in 2006, a phase 1

clinical trial involving a CD28 receptor monoclonal antibody agonist TGN1412 resulted in a severe cytokine storm in healthy volunteers (10,11). These positive and negative cytokine mediated events illustrate that cytokines are key components of effector phase immunity. Thus, monitoring these molecules can provide key insight into disease progression or regression, in essence, cytokines can be looked at as biomarkers of a disease process. Per definition, biomarkers are ''A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention'' (12). Biomarkers can be discovered and isolated locally (biopsies) or systemically (e.g. blood or urine) and can either be drug-related or disease-related with cytokines falling into the latter of these categories. Disease related biomarkers provide insight into the possible effect of treatment on a patient (predictive marker), the disease state of a patient (diagnostic marker) or future disease development with regards to a certain outcome but irrespective of treatment (prognostic marker) (13). In order for cytokines to take on this biomarker role however, a key hurdle needs to be overcome, namely that of a lack of standardization in cytokine analysis. This standardization process is independent of cytokine structure or biology but dependant on protocols and sample handling. This review aims to pro- vide a comprehensive overview on current methods for cytokine analysis in an effort to provide a means to standardization that will be applicable to biomarker discovery and validation in a clinical context.

2. Sample standardization

Various methodologies are being optimized and designed to al- low the precise measurement of cytokine levels within biological fluids however, even in the presence of assay improvement, a key factor needs to be monitored to achieve accurate cytokine measurements, that of sample standardization. The fact cytokines have a short-half life, are released by cells during storage as well as the possibility of degradation during sample handling make it of vital importance that we understand how to treat and store these samples for optimal analysis (14). As a whole, unless standardization is achieved, data produced by these analytical proto- cols will remain incomparable and will not represent the true nature of the biological processes at hand.

Various types of samples have been used for cytokine analysis. For most patients, these samples can comprise of blood (plasma or serum) (15), urine (16), saliva (17), synovial fluid (15), cerebrospinal fluid (18), bronchoalveolar fluid (19), aqueous humour eye fluid (20), intestinal fluid (21), exhaled breath condensates (22), middle ear effusion (23) or lysed biopsies of a diseased organ (24,25) thus, for each of these sample types, standardization needs to occur. The following section will illustrate which factors influence cytokine levels within common clinical sample types (blood and biopsies), outlining which conditions allow maximum cytokine measurement, stability and representation within a biological process.

2.1. Sample collection and handling

Sample handling and storage are pivotal in biomarker discovery as mishandling of these samples can drastically alter experimental outcomes and produce data that are not reflective of the biological situation. It has been shown for plasma collected in EDTA, sodium heparin (heparin) and sodium citrate (citrate) tubes (see de Jager et al. for methodology (26)) that, in healthy donors, cytokines are expressed at low levels while chemokines are expressed at higher levels (Fig. 2). In addition, data did not seem to indicate a difference in cytokine levels between anticoagulant effects, as cytokine levels were expressed in a similar range. However, following a consecutive measurement from the same donor (matched data), the general total recovery of spiked cytokines was more stable for heparin and EDTA tube samples, with EDTA tubes exhibiting decreased recovery of IL-15 and IL-18 (26). Citrate tubes showed lower levels of recovery for IL-1b, IL-2 and IL-6 while serum samples had significantly higher levels of CXCL8 and lower IL-6 (26). In addition to this data, it is known that platelet associated chemokines CCL3, CCL5, CXCL4 and TGF-b increase in serum as a result of ex vivo platelet degranulation, this activity could explain the higher levels of CXCL8 recorded in serum samples (27,28).

Along with platelet degranulation, white blood cells release IL-1b during the clotting process resulting in increased levels of this cytokine in serum samples (29).

For many clinical trials, experiments do not require the separation of blood components thus, most sample handling errors occur during the culturing of whole blood samples. To prevent the introduction of manipulation errors under these conditions a standardized culture system such as the True Culture© syringe can be used (30). In this culture model, blood is collected directly from donors into a specially designed syringe tube. These tubes can be stored stably at 20 °C and can easily be thawed prior to use. The culture system creates a sealed environment in which freshly drawn blood can mix with a pre-prepared nutrient and stimulant solution. Samples can easily be incubated at 37 °C and once this incubation period is over, sedimented cells can be separated rapidly from the supernatant, preventing any further contact between the two environments. The syringes can be stored at 20 °C until further analysis, further minimizing analyte degradation. Data shows that cytokine levels measured using this system have a high degree of stability, with similar levels being measured in consecutive healthy donor blood draws (30). Thus, in a clinical setting, this system facilitates stable and comparable culture conditions with minimal methodological error.

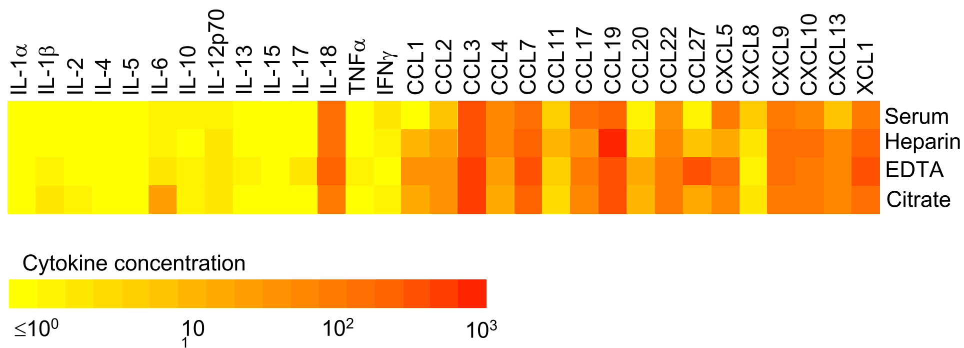

Fig. 2: Cytokine expression in various blood drawing tubes. Blood samples were obtained from healthy individuals using various blood collection tubes. After centrifugation cell free plasma (sodium heparin, citrate EDTA) and serum was measured using a multiplex immunoassay as described elsewhere (15). Colour profiles were generated using geometric mean values as previously described (61). In general all different tubes types can be used for this kind of assay, though slight variations are observed between various blood collection tubes.

2.2. Temperature and time delay of processing

It is known that artefacts in cytokine measurements are affected by the duration of contact between serum or plasma and blood cells (31). When plasma or serum cannot be isolated from whole blood or, if there is a delay in this separation procedure, it is important to understand cytokine dynamics under these conditions as changes here may greatly affect the experimental outcome and data analysis. For whole blood it has been shown that cytokine production can already occur within a 2-h period following blood draw (32). When whole blood is stored at 4 °C, room temperature (RT) and 35 °C before separation into serum and plasma, some poignant changes in cytokine levels can be seen (33). From a panel of inflammatory markers, keeping blood at 4 °C before the removal of plasma was sufficient to maintain cytokine levels when compared to control plasma samples (plasma immediately isolated and store at frozen until analysis). After 4h at 4 °C however, cytokine levels already began to increase (33). Samples stored at RT before separation showed a greater increase in cytokine levels than those seen at 4 °C. After 4h at RT most analytes significantly increased and levels continued to rise significantly over the 48 h. In order to expand on these data, we chose to analyse the effect of temperature, time and tube type on the level of a set of cytokines during a 48h period. Our analysis revealed that plasma storage temperature (RT or 4 °C), time and collection tube type have an influence of total cytokine

levels. In general, it was seen that at 4 °C there is less fluctuation in cytokine levels with minimal variation between plasma collection tubes while serum exhibited a partially higher total marker level compared to plasma samples stored at 4 °C. The data seems to indicate that there is no difference in anticoagulant tube effect or time at 4 °C even after 48h (Fig. 3). The partially higher levels seen in serum samples can be explained by higher chemokine levels known to occur in serum samples when compared to plasma (26). At RT data showed that, over a 48h period, cytokine levels increase drastically with heparin tubes showing the greatest increase (89 ± 28%) followed by serum (56 ± 16%), EDTA (45 ± 13%) and finally citrate (30 ± 12%). The substantial increase in heparin tubes illustrates that although this sample tube type is ideal in multiple measurements for recovery, it is less than optimal when used to collect samples that are left at RT for longer than 4h. If do- nor plasma samples are to be left at RT for up to 48 h citrate tubes would be the best for blood collection, showing the smallest change over time. The general increase in analyte levels after 48h at RT can be explained by the fact that at RT, soluble receptors may begin to release bound analytes into solution resulting in an increase in the measurable amounts of these compounds (34). It has been shown that blood samples stored at 35 °C have a lower mean concentration of inflammatory markers than those stored at RT however, these levels are higher than those measured in samples stored at 4 °C (33). Serum isolated from clotted blood stored at 4 °C shows the lowest increase in inflammatory marker concentration while at 35 °C samples have the greatest increase, exhibiting a mean fold increase of 320 when compared to control serum samples. At RT most inflammatory markers are significantly increased and after 48h general levels are significantly larger ranging from a 15 to 1700 fold increase. As a whole, serum samples do not show a significant decrease in analyte concentration. In addition to this, it is known that activation of the coagulation cascade occurs in serum tubes and that above RT, large amounts of chemokines are re- leased from the blood cells (33,35).

Taking all this data into consideration, it is of vital importance that whole blood be kept 4 °C until separation into plasma or serum and that this separation occur within 4h in order to prevent the absorption, release or degradation of cytokines (36). If procedure specific tube types cannot be used then, any tube can be used as long as it is the same type within an experiment. Healthy donor samples that are stored at the same temperature and for an equal amount of time as patient samples should also be used as comparative controls. Samples should also be collected at the same time of day as, it is known that analyte levels are influenced by circadian rhythms. This is exemplified in Rheumatoid arthritis where pro-inflammatory cytokines (TNF and IL-6) released at night contribute to morning disease related symptoms (37). If all these steps can be taken then artificial anlayte changes can be circumvented.

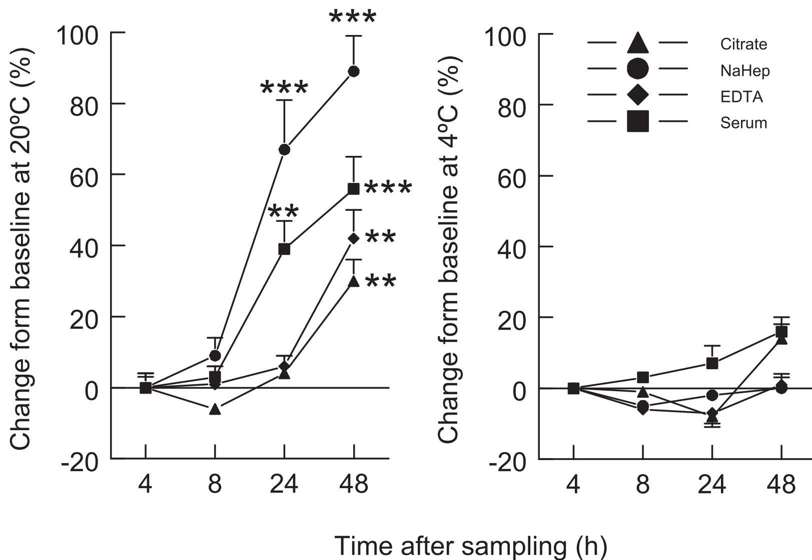

Fig. 3: Influence of time and temperature after blood draw. Blood was collected from healthy donors in EDTA (EDTA), Sodium Heparin (NaHep), Sodium Citrate (Citrate) and clotting (serum) tubes. Subsequent to centrifugation, cell free plasma or serum was collected and stored for 4, 8, 24 and 48 h at room temperature or at 4 °C. All the plasma samples were then analysed by multiplex immunoassay as described elsewhere (15). The figure illustrates the level of inflammatory cytokines for various tube types when samples were left at room temperature (left) and 4 degrees (right). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

2.3. Long term stability and repetitive use

Previous work of our group has shown that most cytokines re- main stable within a two year period however degradation of some cytokines can already be seen within one year of storage at 80 °C (26). In general a decrease over time is seen losing approximately 10–20% each year after 2 years of storage (Fig. 4). Eventually, a five- fold increase or decrease in different cytokine levels can occur after 5 years as a result of cross-reactivity between protein epitopes (38). In addition to this storage effect, repetitive freeze thawing cycles also influence cytokine recovery. The levels of these molecules can either be stable, increased or decreased after multiple freeze– thawing cycles, depending on each cytokine. In general, cumulative levels significantly decrease (Fig. 3). Thus, in order to optimally measure cytokine levels, samples should be analysed within 2 years and undergo minimal freeze–thawing procedures so as to maintain stable cytokine levels (26,38,39). If the two year time period is however, not conducive to the experimental set up, individual changes in cytokine/chemokine levels should be assessed before conducting these long term storage studies, or bio-banking.

Several long term stored samples used in experiments or housed in biobanks are, in some cases, not originally collected for the experiments they are used in. A prime example of this is the use of dried blood spots (DBS) in neonatal screening. Excess DBS samples from these procedures are often stored in biobanks for use in other research as the Guthrie card on which samples are stored is an easy and appropriate medium for transport and long term storage (33,34,40). The use of these samples in research and particularly inflammatory marker research means that a comprehensive understanding of sample storage, handling and analyte stability is required. Skogstrand et al. performed experiments in which DBS were stored for several days at various temperatures. Analysis of these samples revealed that the levels of inflammatory markers remained stable for up to 7 days when compared to control DBS samples stored at 20 °C. Any changes seen in analyte levels at this stage were smaller than those observed in plasma and serum from whole blood stored for shorter periods of times (33). After 30 days

however, some pro-inflammatory markers showed a significant change in concentration. In addition to this, at RT or 35 °C, there was no specific trend for individual inflammatory markers but rather a trend towards increased analyte levels with protection of samples against humidification at 4 °C having no positive influence. Even though measurable levels of pro-inflammatory markers were lower in DBS, these levels were more stable than those observed for liquid blood samples. Sample storage for several years, however, still results in varied levels of individual compounds. It is suggested in this study that faster drying of DBS and storage at low temperatures would be the optimal method by which to conserve analyte levels (33). DBS thus seem to be a possible reliable alternative method for whole blood, serum or plasma sample storage. Requiring a small volume of blood, (approximately 15µl), on a stable and portable filter paper (41), these samples are less vulnerable to biological degradation. How- ever, even with higher analyte stability, the sample extraction pro- cess for DBS results in the dilution of low abundant proteins. This dilution factor greatly influences the cytokine panel being measured and results in the loss of delicate disease associated cytokines (42).

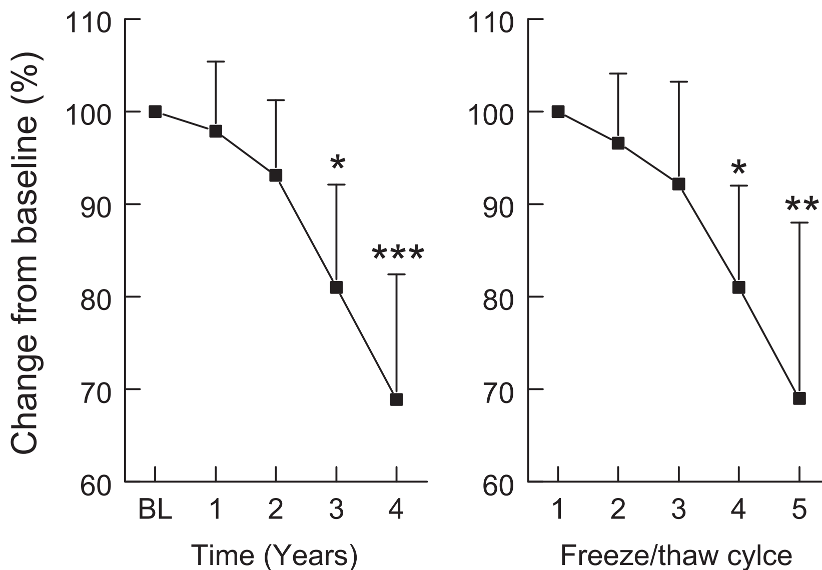

Fig. 4: Influence of long time storage and multiple freeze thawing cycles. Blood samples of healthy individuals were stored at 80 °C and measured at baseline and various time points. As shown in the left panel cytokine levels are subject to change even frozen at 80 °C after approximately 2 years of storage. Next blood samples were repeatedly thawed and cytokine profiles were assed. After several cycles cytokine levels are subjected to breakdown, resulting in lower levels (right panel). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

2.4. Lysed tissue (biopsies)

In order to analyse diseased tissue, biopsies must be taken. Due to the fact that not all cytokine mediated pathological responses can be measured systemically, measuring cytokine levels within the diseased tissue can provide a better understanding of the cellular pathological process. In addition to this, cytokine levels that can be measured systemically only provide a reflection of what is occurring at the sight of inflammation whilst biopsies provide a key primary representation of the tissue specific diseases process. As with all samples, the processing of this tissue is important. When analysing cytokine levels in whole cells, samples need to initially be lysed. We thus performed an experiment to determine if the type of lysis buffer used could alter cytokine levels measured due to increased background levels. For these experiments, peripheral blood human mononuclear cells where used to

mimic biopsy tissue due to the fact that tissue samples where not readily avail- able. Cells were stimulated with LPS (4h, 37 °C). Following stimulation, cells were lysed with various lysis buffers; Roche Complete Lysis M, Cell Signalling Technology (CST) or Bio-Rad Lysis buffers. Data showed that the Roche lysis buffer had the highest noise/ background levels and resulted in the lowest cytokine measurements, Bio-Rad background levels were lower than those of Roche with partially higher cytokine levels while the CST lysis buffer resulted in the least background activity and produced the highest cytokine measurements, resulting in a better signal to noise ratio (Fig. 5). Our data clearly indicates that depending on the lysis buffer used, cytokine levels measured within tissue can be drastically altered, indicating that lower lysis buffer background levels result in higher cytokine measurements. It is thus important to under- stand the functionality of any lysis buffer used in tissue sample analysis as this may influence the discovery of cytokine networks that cannot be measured systemically but rather have a localized tissue specific role (24).

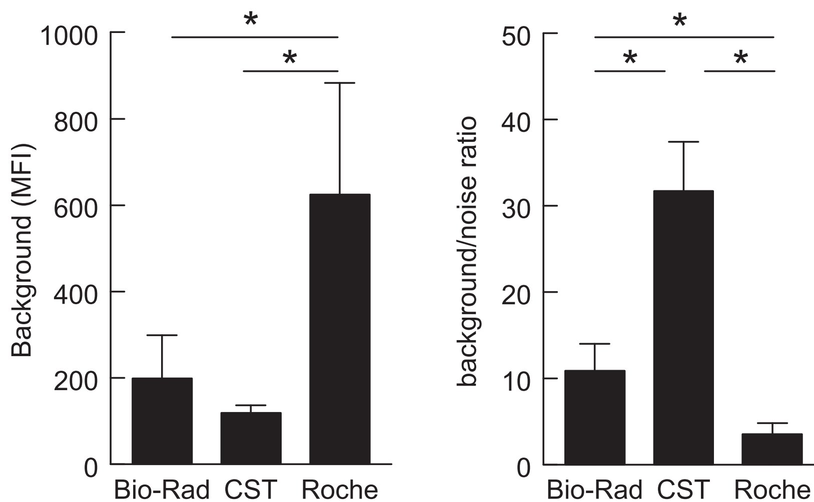

Fig. 5: Influence of lysis buffer on assay performance. Peripheral blood human mononuclear cells from healthy donors were isolated using Ficoll-gradient centrifugation. Cells were then stimulated with a concentration 10 ng/ml of LPS for 4 h. Following the incubation period supernatant was removed and cells were washed with PBS. Next cells were lysed according to the lysis protocols for Complete lysis M (Roche), Cell signalling Technologies lysis buffer (CST) and Bio-Rad. The samples were then analysed by multiplex immunoassay for various markers. The graphs illustrate (left) background levels, only lysis buffer and (right) background – noise ratio for the complete cytokine panel. *indicates all p values <0.05.

3. Methods to analyse cytokines

Cytokines can be measured at various levels. mRNA can be detected by real time PCR (43) and intracellular proteins can be detected by flowcytometric assays of permeabilized cells (44). Due to the range and location of cytokine activity, there are a plethora of methods by which to measure cytokine levels. Irrespective of the chosen methodology, several factors are key to creating the optimal protocol by which to analyse cytokines as biomarkers. Parameters should include (a) a high specificity, affinity and sensitivity (b) make use of a simple protocol (c) be reproducible and reliable (d) be time and cost effective and (e) allow the detection of multiple cytokines within a small sample volume (45,46). This review focuses on the measurement of cytokines at a protein level describing various techniques that can be used for their detection. Pro's and con's of the various methodologies are summarized in Table 1.

3.1. Bioassays

Bioassays look at cytokines and their activity within a system by focusing on their biological activity and using this as a read out. In a bioassay the activity of a sample is tested on a sensitive cell line and the results of this activity are compared to a standard cytokine preparation. This assay is highly sensitive and permits the detection of bioactive molecules however, it is also semi-quantitative, low in specificity, shows a narrow analytical range, is time consuming and requires a large sample size (45). In addition to these factors, cell lines respond to different molecules present within a sample thus, any changes observed may be the result of one or multiple compounds (47). This combination of disadvantageous factors leads to the innovation of more specific and sensitive assays by which to measure cytokines directly.

3.2. Protein microarrays

Protein microarrays analyse the interaction, function and activity of proteins on a large scale (48). This sensitive, high-throughput method allows large numbers of proteins to be measured rapidly, economically and in parallel (49). The protein chip used in this procedure is comprised of a support surface to which a range of capture proteins are bound. Fluorescently labelled probe molecules are then added to the array and upon interaction with the bound capture protein, a fluorescent signal is released and read by a laser scanner (50). Analytical (capture) microarrays use antibodies, aptamers or affibodies bound to the chip surface in order to bind the specific and desired protein within a complex protein solution, commonly cell lysate. The subsequent protein interactions provide information on the expression levels, binding affinity and or specificity of the proteins within the solution permitting comparisons of protein expressions between various solutions. The protein microarray system is applicable for biomarker detection by allowing protein expression profiling however, some challenges do exist for this procedure. Downfalls come in the form of difficulties manufacturing chips with stable proteins holding the necessary primary or tertiary structure as these are vital in their interactive ability and biological activity. Protein array shelf life is relatively short due to protein denaturation and difficulties still exist in finding and isolating capture molecules for the wide range of proteins within the human genome (51). A complex balance between quantifying amounts of bound protein, maintaining sensitivity and reducing background noise is difficult to obtain especially due to the low affinity or low specificity of capture agents. Most poignant however, is the inability of the chip to provide a complete view of the proteome with abundant proteins overpowering the detection of less abundant proteins whose levels are also key in therapeutic analysis (52).

3.3. High-performance liquid chromatography

Unlike protein microarrays, high-performance liquid chromatography (HPLC) does not identify cytokines using protein–protein interactions but rather, as compounds with specific weights, hydrophobicity, protonating abilities, ligand affinity and ion ex- change (53). In short, HPLC uses the specific chemistry of each compound as a method of identification and separation. This meth- od allows the quantification and purification of compounds by loading a sample onto a separation column containing solid particles under pressure. The sample is then separated into individual compounds according to their interaction with the column particles. The separation is in itself influenced by the liquid solvent condition and the chemical interactions between sample and solvent. HPLC has successfully been used to purify and separate cytokines such as IL-1 derived from various cells such as macrophage and epidermal cells (53). However, even with the ability to achieve better separation than ordinary liquid chromatography, HPLC is a less than optimal method of analysis. Some disadvantages of this process include a high cost and complexity, the coelution of compounds with similar structure and polarity (54), the irreversible absorption of compounds which then remain undetected and the low sensitivity of the apparatus to certain compounds as a result of the speed of the process (55).

3.4. Sandwich antibody assays

It is known that several parameters must be met in order for a protocol to be optimal for cytokine biomarker discovery. As seen in bioassays, protein microarrays and HPLC, parameters are often either suboptimal or conflicting within an assay. Brining each of these factors close to or within optimal range will, however, give way to the perfect assay. There are two types of sandwich antibody assays, those that are plate-based and those that are bead-based. Plate-based assays such as Sandwich Enzyme-linked immunosorbent assay (ELISA) and Meso Scale Discovery electrochemiluminescence (MSD) as well as bead-based assays such as multiplex immunoassays (MIA) are currently on the forefront of achieving the parameter goals required for cytokine biomarker discovery. In essence, these sandwich assays work with the principle of sandwiching a cytokine between two specific antibodies that intern bind to two none competing epitopes of that cytokine (45). For bead based analysis, the antibodies are either coated to a solid carrier (bead), acting as the capture antibody and or, in the case of the second antibody, bound to a labelled reporter. In plate-based assays however, the capture antibodies are bound in distinct positions within the wells of 96 well plate (56).

Table 1
pro's and con's of various assays.

	Pros	Cons
Bioassay	Sensitive detection of bioactive molecules	Semi-quantitative Narrow analytical range Low specificity Time consuming Large sample size
Protein microarray	Sensitive High through put Rapid Parallel measurement of multiple proteins	Low Protein detection system stability (denaturation) Non-specific activity of capture protein Masking of low protein levels by higher protein levels Matrix/Heterophlic (auto-) antibody interference
HPLC	Relatively rapid Low false positives	High cost and complexity Co-elution of compounds Irreversible absorption of compounds Low sensitivity
ELISA	High specificity High sensitivity Wide analytical range Reproducibility	Unable to distinguish between bioactive and inactive molecules Varying binding affinity of antibodies Large sample volume High reagent costs Narrow dynamic range Only measure one protein at a time Matrix/Heterophlic (auto-) antibody interference
MSD	Quantitative and qualitative analysis High sensitivity Low background Detection of multiple cytokines	Unable to distinguish between bioactive and inactive molecules Performance of assay in various Matrices is unknown
Bead based multiplex immunoassay	High specificity High sensitivity Broad analytical and dynamic range Reproducibility Rapid Small sample volume	Unable to distinguish between bioactive and inactive molecules Matrix/Heterophlic (auto-) antibody interference

3.4.1. Enzyme-linked immunosorbent assay

The ELISA procedure encompasses the detection of an analyte within a liquid sample in a liquid environment within a reaction chamber. In lieu with heterogeneity of the assay, the desired component is separated from the analytical mixture by binding to an immobilized solid phase, usually the bottom of a transparent plate. Following this binding, substrate is added which is enzymatically converted, resulting in an optical change (coloured or fluorescent) that allows the quantitative and qualitative measurement of the desired compound. The ELISA protocol allows for high specificity and sensitivity as well as a wide analytical range and reproducibility, all of which are dependent on the type of biological fluid and cytokine being measured (57,58). Downfalls in this procedure, however, include the inability to distinguish between bioactive and inactive compounds, varying binding affinity of antibodies as a result of differences in the internal structure of recombinant proteins used to generate these antibodies, large sample volumes, high reagent costs, a narrow dynamic range and the fact the protocol only permits the measurement of one cytokine at a time in a specified sample volume (58). In order to overcome the inability to detect multiple cytokines simultaneously, the ELISA protocol was

advanced to include a sequential ELISA analysis and ELISPOT assays however, these assays are time consuming, laborious and limited in their ability to detect a spectrum of cytokines (45). Taking into account the complex interaction between multiple cytokines during a disease process, an assay had to be developed that combated one of the major pitfalls of the ELISA protocol, that of an inability to sufficiently and accurately measure multiple cytokines. At present, some of the most common multiplex assays used are the bead- based Luminex multi-analyte profiling (xMAP) technology [Luminex, Austin Texas, USA] and the cytometric bead array (BD Biosciences, San Diego California, USA) (57) or, the plate-based MSD array (Meso Scale Discovery, Gaithersburg, USA) (56).

3.4.2. Meso scale discovery

The principle of MSD is based on a reaction in which an electron transfer in electrochemically generated intermediates causes these molecules to enter an excited state. Once excited these molecules can emit a photon of light when re-entering a lower energy level (59). Initially, capture antibodies are coated onto the surface of a plate. Samples are then incubated on the plate followed by the addition of an electrochemiluminescent tagged antibody. Analysis of this plate reveals fluorescent regions in which specific interactions have occurred between the antibodies and analyte, allowing both a quantitative and qualitative analysis of the desired com- pound. This method is highly sensitive, has low background, does not incorporate washing steps and most importantly, allows the detection of multiple analytes at the same time. What remains unknown however is, how this assay will perform in various sample matrices (56).

3.4.3. Multiplex immunoassays

In bead-based multiplex immunoarrays, identifiable bead sets are stably coated with desired and specific capture antibodies. These beads are then incubated with a small sample volume allowing the capture of the analyte that binds specifically to the capture antibody. Following this, labelled detection antibodies bind to the analyte-capture antibody-bead complex to make a four member solid phase sandwich that when passed through the detection sys- tem allows the identification and quantification of the desired compound. In comparison to ELISA, multiplex assays in general are more sensitive, show a broad analytical and dynamic range – measuring a few pg/ml, are highly specific, are rapid, require smaller sample volumes (57) and allow the simultaneous measurement of up to 500 different proteins (xMAP technology, Luminex, Austin Texas, USA). Even in the presence of all these advantageous features however, these multiplex assays, similar to other antibody based technologies, are affected by the presence of heterophilic and auto- antibodies. These antibodies cause false positive and false negative signals by binding to either the capture antibody, detection antibody or to the antigen (34). In order to combat this phenomenon three methods can be undertaken. The first involves heterophillic/auto- antibody blockage with animal serum (ineffective when antibody titres are high- as seen in a diseased state) (57). The second is through the use of internal assay markers that allow the interfering antibodies to be monitored. These internal markers can clearly indicate when heterophillic or auto-antibodies are influencing data and can thus allow the exclusion of unfit samples during assay analysis (60,61). The third and most preferable meth- od is the removal of these antibodies by incubating samples with either protein-L (61), or with antibodies cocktail such as Hetero- Block (62).

4. Conclusions

By understanding how factors can manipulate cytokine levels and plugging in the necessary controls or methodology, an objective view of cytokine levels within a disease process can be reached, enabling clear results and definitions that will all culminate in achieving optimal biomarker function of these molecules. We recommend that, especially in the context of clinical trials, sample handing and processing should be done in a standardized way with all individuals undergoing identical practical and handling procedures. In general, different blood sampling tubes can be used however, the tube type

within a study should remain the same. Furthermore, directly after sample collection, we recommend keeping samples on ice or at 4 °C before sample separation. The process of sample separation should then be performed as quickly as possible and the products of this separation should be stored at -80 °C for long term storage. When performing cytokine analyses, depending on the sample origin, the matrix interference can be dealt with by removing impeding substances, such as auto antibodies, or by choosing reagents which result in the lowest interference during the assay. By following this general outline, data produced within a study will provide a clearer representation of cytokine levels in patients and allow maximum data mining to be achieved.

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References

1. Gilman A, L.S. Goodman J.G, Hardman L.E, Limbird. *Goodman and Gilman's the Pharmacological Basis of Therapeutics*. McGraw-Hill, New York, 2001.

2. Dinarello C.A. *Historical insights into cytokines.* Eur J Immunol. 2007, Vol. 37, S34–S45.

3. Hoffman H.M, Mueller J.L, Broide D.H, Wanderer A.A, Kolodner R.D. *Mutation of a new gene encoding a putative pyrin-like protein causes familial cold auto inflammatory syndrome and Muckle–Wells syndrome.* Nat Genet. 2001, 29, Vol 29, pp 301–305.

4. Jin Y, Mailloux C.M, Gowan K, Riccardi S.L, LaBerge G, Bennett D.C, Fain P.R, Spritz R.A. *NALP1 in Vitiligo-Associated Multiple Autoimmune Disease.* N Engl J Med. 2007, Vol. 356, pp. 1216–1225.

5. Feldmann M, Maini R.N, Bondeson J, Taylor P, Foxwell B.M, Brennan F.M. *Cytokine Blockade in Rheumatoid Arthritis*. Adv Exp Med Biol. 2001, Vol. 490, pp. 119–127.

6. Netea M.G, Joosten L.A, Lewis E, Jensen D.R, Voshol P.J, Kullberg B.J, Tack C.J. *Deficiency of interleukin-18 in mice leads to hyperphagia, obesity and insulin resistance*. Nat Med. 2006, Vol.12, pp. 650-656

7. Sims J.E, March C.J, Cosman D, Widmer M.B, MacDonald H.R, McMahan C.J, Grubin C.E, Wignall J.M, Jackson J.L, Call S.M. *cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily.* Science. 1988, Vol. 241, pp. 585–589.

8. Elliott M.J, Maini R.N, Feldmann M, Long-Fox A, Charles P, Katsikis P, Brennan F.M, Walker J, Bijl H, Ghrayeb J. *Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor α.* Arthritis Rheum. 1993, Vol. 36, pp. 1681–1690.

9. Feldmann M, Maini R.N. *Anti-TNFα Therapy of Rheumatoid Arthritis: What Have We Learned?* Annu Rev Immunol. 2001, Vol. 19, pp. 163–196.

10. Mayor S. *Severe adverse reactions prompt call for trial design changes.* BMJ. 2006, Vol. 332, pp.683.

11. Goodyear M. *Learning from the TGN1412 trial.* BMJ. 2006, 332 Vol. 332, pp.677–678.

12. *Biomarkers and surrogate endpoints: preferred definitions and conceptual framework*. Clin Pharmacol Ther. 2001, Vol. 69, pp. 89–95.

13. Sapsford K.E, Tezak Z, Kondratovich M, Pacanowski M.A, Zineh I, Mansfield E. *Biomarkers to improve the benefit/risk balance for approved therapeutics: a US FDA perspective on personalized medicine.* Ther Deliv. 2010, Vol. 1, pp. 631–641.

14. Panicker G, Meadows K.S, Lee D.R, Nisenbaum R, Unger E.R. *Effect of storage temperatures on the stability of cytokines in cervical mucous.* Cytokine. 2007, Vol. 37, pp. 176–179.

15. de Jager W, Hoppenreijs E.P, Wulffraat N.M, Wedderburn L.R, Kuis W, Prakken B.J. *Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study.* Ann Rheum Dis. 2007, Vol. 66, pp. 589–598.

16. Corcoran A.T, Yoshimura N, Tyagi V, Jacobs B, Leng W, Tyagi P. *Mapping the cytokine profile of painful bladder syndrome/interstitial cystitis in human bladder and urine specimens*. World J Urol. 2013, Vol. 31, pp. 241–246,

17. Thorman R, Lundahl J, Yucel-Lindberg T, Hylander B. *Inflammatory cytokines in saliva: Early signs of metabolic disorders in chronic kidney disease. A controlled cross-sectional study*. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010, Vol. 110, pp. 597–604.

18. Brandsma D, Voest E.E, de Jager W, Bonfrer H, Algra A, Boogerd W, Korse T, Reijneveld J. C, Verbeek M.M, Rijkers G, Taphoorn M.J.B. *CSF protein profiling using Multiplex Immuno-assay*. J Neurol. 2006, Vol. 253, pp. 1177–1184.

19. Switalla S, Lauenstein L, Prenzler F, Knothe S, Forster C, Fieguth H.G, Pfennig O, Schaumann F, Martin C, Guzman C.A, Ebensen T, Muller M, Hohlfeld J.M, Krug N, Braun A, Sewald K. *Natural innate cytokine response to immunomodulators and adjuvants in human precision-cut lung slices*. Toxicol Appl Pharmacol. 2010, Vol. 246, pp. 107–115.

.20. Ricker L.J, Kijlstra A, Kessels A.G, Louwerenburg J.W, Liem A.T, Hendrikse F, La Heij E.C. *Interleukin and Growth Factor Levels in Subretinal Fluid in Rhegmatogenous Retinal Detachment: A Case-Control Study.* PLoS One. 2011, Vol. 6, e19141.

21. Kreiner F, Langberg H, Galbo H. *Increased muscle interstitial levels of inflammatory cytokines in polymyalgia rheumatic***.** Arthritis Rheum. 2010, Vol. 62, pp. 3768–3775.

22. Robroeks C. M, Robroeks H. H. T, van Vliet D, Jöbsis Q, Braekers R, Rijkers G. T, Wodzig W. K. W. H, Bast A, Zimmermann L. J. I, Dompeling E. *Prediction of asthma exacerbations in children: results of a one-year prospective study***.** Clin Exp Allergy. 2012, Vol. 42, pp. 792–798.

23. Granath A, Norrby-Teglund A, Uddman R, Cardell L.O. *Reduced iNOS expression in adenoids from children with otitis media with effusion.* Pediatr Allergy Immunol. 2010, Vol. 21, pp. 1151–1156.

24. Kan A.A, de Jager W, De Wit M, Heijnen C, van Zuiden M, Ferrier C, van Rijen P, Gosselaar P, Hessel E, van Nieuwenhuizen O, de Graan P.N. *Protein expression profiling of inflammatory mediators in human temporal lobe epilepsy reveals co-activation of multiple chemokines and cytokines.* J Neuroinflamm. 2012, Vol. 9, pp. 207.

25. de Kleijn D.P, Moll F.L, Hellings W.E, Ozsarlak-Sozer G. *Local Atherosclerotic Plaques Are a Source*

of Prognostic Biomarkers for Adverse Cardiovascular Events. Arterioscler Thromb Vasc Biol. 2010, Vol. 30, pp. 612–619.

26. de Jager W, Bourcier K, Rijkers G.T, Prakken B.J, Seyfert-Margolis V. *Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays.* BMC Immunol. 2009, Vol. 10, pp. 52.

27. Picker S.M, Steisel A, Gathof B.S. *Evaluation of White Blood Cell- and Platelet-Derived Cytokine Accumulation in MIRASOL-PRT-Treated Platelets.* Transfus Med Hemother. 2009, Vol. 36, pp. 114– 120.

28. Apelseth T.O, Hervig T.A, Wentzel-Larsen T, Bruserud O. *Cytokine accumulation in photochemically treated and gamma-irradiated platelet concentrates during storage.* Transfusion. 2006, Vol. 46, pp. 800–810.

29. Cannon J.G, van der Meer J.W, Kwiatkowski D, Endres S, Lonnemann G, Burke J.F, Dinarello C.A. *Interleukin-1β in human plasma: Optimization of blood collection, plasma extraction, and radioimmunoassay methods*. Lymphokine Res. 1988, Vol. 7, pp. 457–467.

30. Mueller S.C, Marz R, Schmolz M, Drewelow B. *Intra-individual long term stability and response corridors of cytokines in healthy volunteers detected by a standardized whole-blood culture system for bed-side application.* BMC Med Res Methodol. 2012, Vol. 12, pp. 112.

31. Banks R.E. *Measurement of Cytokines in Clinical Samples Using Immunoassays: Problems and Pitfalls.* Crit Rev Clin Lab Sci. 2000, Vol. 37, pp. 131–182.

32. Riches P, Gooding R, Millar B.C, Rowbottom A.W. *Influence of collection and separation of blood samples on plasma IL-1, IL-6 and TNF-α concentrations.* J Immunol Methods. 1992, Vol. 153, pp. 125–131.

33. Skogstrand K, Ekelund C.K, Thorsen P, Vogel I, Jacobsson B, Norgaard- Pedersen B, Hougaard D.M. *Effects of blood sample handling procedures on measurable inflammatory markers in plasma, serum and dried blood spot samples.* J Immunol Methods. 2008, Vol. 336, pp. 78–84.

34. Skogstrand K. *Multiplex assays of inflammatory markers, a description of methods and discussion of precautions – Our experience through the last ten years.* Methods. 2012, Vol. 56, pp. 204–212.

35. Tammen H, Hess R, Rose H, Wienen W, Jost M. *Peptidomic analysis of blood plasma after in vivo treatment with protease inhibitors—A proof of concept study*. Peptides. 2008, Vol. 29, pp. 2188–2195.

36. Bienvenu J.A, Monneret G, Gutowski M.C, Fabien N. *Cytokine assays in human sera and tissues.* Toxicology. 1998, Vol. 129, pp. 55–61.

37. Straub R.H, Cutolo M. *Circadian rhythms in rheumatoid arthritis: Implications for pathophysiology and therapeutic management.* Arthritis Rheum. 2007, Vol. 56, pp. 399–408.

38. Butterfield L.H, Potter D.M, Kirkwood J.M. *Multiplex serum biomarker assessments: technical and biostatistical issues.* J Transl Med. 2011, Vol. 9, pp. 173.

39. de Jager W, Prakken B, Rijkers G.T. *Cytokine multiplex immunoassay: methodology and (clinical) applications.* Methods Mol Biol. 2009, Vol. 514, pp. 119-133.

40. Klamer A,Skogstrand K, Hougaard D.M, Norgaard-Petersen B, Juul A, Greisen G. *Adiponectin levels measured in dried blood spot samples from neonates born small and appropriate for gestational age.*

Eur J Endocrinol. 2007, Vol. 157, pp. 189–194.

41. Skogstrand K, Thorsen P, Norgaard-Pedersen B, Schendel D.E, Sorensen L.C, Hougaard D.M. *Simultaneous Measurement of 25 Inflammatory Markers and Neurotrophins in Neonatal Dried Blood Spots by Immunoassay with xMAP Technology*. Clin Chem. 2005, Vol. 51, pp. 1854–1866.

42. Pfleger C, Schloot N, ter V.F. *Effect of serum content and diluent selection on assay sensitivity and signal intensity in multiplex bead-based immunoassays.* J Immunol Methods. 2008, Vol. 329, pp. 214–218.

43. Halminen M, Sjoroos M, Makela M.J, Waris M, Terho E, Lovgren T, Ilonen J. *Simultaneous detection of IFN-γ and IL-4 mRNAS using RT-PCR and time-resolved fluorometry.* Cytokine. 1999, Vol. 11, pp. 87–93.

44. Prussin C, Metcalfe D.D. *Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies*. J Immunol Methods. 1995, Vol. 188, pp. 117–128.

45. de Jager W, Rijkers G.T. *Solid-phase and bead-based cytokine immunoassay: A comparison*. Methods. 2006, Vol. 38, pp. 294–303.

46. Kellar K.L, Douglass J.P. *Multiplexed microsphere-based flow cytometric immunoassays for human cytokines*. J Immunol Methods. 2003, Vol. 279, pp. 277–285.

47. Gillis S, Smith K.A. *Long term culture of tumour-specific cytotoxic T cells.* Nature. 1977, Vol. 268, p.p 154–156.

48. Melton L. *Protein arrays: Proteomics in multiplex.* Nature. 2004, Vol. 429, pp. 101–107.

49. Mitchell P. *A perspective on protein microarrays*. Nat Biotechnol. 2002, Vol. 20, pp. 225–229.

50. Espina V, Woodhouse E.C, Wulfkuhle J, Asmussen H.D, Petricoin III E.F. *Protein microarray detection strategies: focus on direct detection technologies.* J Immunol Methods. 2004, Vol. 290, pp. 121–133.

51. Talapatra A, Rouse R, Hardiman G. *Protein microarrays: challenges and promises.* Pharmacogenomics. 2002, Vol. 3, pp. 527–536.

52. Fung E.T, Thulasiraman V, Weinberger S.R, Dalmasso E.A. *Protein biochips for differential profiling.* Curr Opin Biotechnol. 2001, Vol. 12, pp. 65–69.

53. Kock A, Luger T.A. *High-performance liquid chromatographic separation of distinct epidermal cell-derived cytokines*. J Chromatogr. 1985, Vol. 326, pp. 129–136.

54. Sviridov D, Meilinger B, Drake S.K, Hoehn G.T, Hortin G.L. *High-performance liquid chromatographic separation of distinct epidermal cell-derived cytokines*. Clin Chem. 2006, Vol. 52, pp. 389–397.

55. Sadek P.C, Carr P.W, Bowers L.D, Haddad L.C. *A radiochemical study of irreversible adsorption of proteins on reversed-phase chromatographic packing materials*. Anal Biochem. 1986, Vol. 153, pp. 359–371.

56. Dabitao D, Margolick J.B, Lopez J, Bream J.H. *Multiplex measurement of proinflammatory cytokines*

in human serum: Comparison of the Meso Scale Discovery electrochemiluminescence assay and the Cytometric Bead Array. J Immunol Methods. 2011, Vol. 372, pp. 71–77.

57. Leng S.X, McElhaney J.E, Walston J.D, Xie D, Fedarko N.S, Kuchel G.A. *ELISA and Multiplex Technologies for Cytokine Measurement in Inflammation and Aging Research.* Gerontol A Biol Sci Med Sci. 2008, Vol. 63, pp. 879–884.

58. Bird C, Wadhwa M, Thorpe R. *Development of immunoassays for human interleukin 3 and interleukin 4, some of which discriminate between different recombinant DNA-derived molecules.* Cytokine. 1991, Vol. 3, pp. 562–567.

59. Forster R.J, Bertoncello P, Keyes T.E. Electrogenerated Chemiluminescence. Annu Rev Anal Chem. 2009, Vol. 2, pp. 359–385.

60. Hansen H.J, LaFontaine G, Newman E.S, Schwartz M.K, Malkin A, Mojzisik K, Martin E.W, Goldenberg D.M. *Solving the problem of antibody interference in commercial 'sandwich'-type immunoassays of carcinoembryonic antigen.* Clin Chem. 1989, Vol. 35, pp. 146–151.

61. de Jager W, Prakken B.J, Bijlsma J.W, Kuis W, Rijkers G.T. *Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies*. J. Immunol. Methods. 2005, Vol. 300, pp. 124–135.

62. Todd D.J, Knowlton N, Amato M, Frank M.B, Schur P.H, Izmailova E.S, Roubenoff R, Shadick N.A, Weinblatt M.E, Centola M, Lee D.M. *Erroneous augmentation of multiplex assay measurements in patients with rheumatoid arthritis due to heterophilic binding by serum rheumatoid factor.* Arthritis Rheum. 2011, Vol. 63, pp. 894–903.

Chapter 3

Isolating extracellular vesicles from juvenile idiopathic arthritis synovial fluid; a feasibility study for functional assays

Genoveva Keustermans[1], Nienke Kettelarij[2], Rianne Scholman[1], Belinda van't land[2], Henk S. Schipper[1,3,4], Berent Prakken[1,3,] Wilco de Jager[1,3]

1 Laboratory for Translational Immunology, University Medical Centre Utrecht, Utrecht, the Netherlands, **2** Nutricia Research, Utrecht, The Netherlands, **3** Division of Paediatrics, Wilhelmina Children's Hospital, University Medical Centre Utrecht, Utrecht, The Netherlands, **4** Department of Paediatric Cardiology, Wilhelmina Children's Hospital, University Medical Centre Utrecht, The Netherlands

Manuscript in Preparation

ABSTRACT

Objective

The aim of this study was to ascertain the feasibility of extracellular vesicle isolation from small volumes of JIA synovial fluid plasma and investigate the functionality of said JIA derived vesicles.

Methods

Vesicles were isolated using differential centrifugation. Electron microscopy, western blot and FACS analysis were used to assess the vesicle isolation and a multiplex immune assay was used as a read out for biological activity.

Results

Data indicates that vesicles can successfully be isolated from small plasma sample volumes with vesicles exhibiting typical marker expression and morphology. When testing vesicle biological activity, multiplex data indicated that irrespective of the source fluid, EVs can increase IL-6 production when compared to unstimulated conditions.

Conclusion

Our pilot study shows that vesicle isolation from sparse JIA synovial fluid plasma volumes is feasible and that these particles can be implemented in biological assays.

Key Words
Extracellular vesicles, synovial fluid, juvenile idiopathic arthritis, cytokine

List of Abbreviations

JIA	- Juvenile Idiopathic Arthritis
EVs	- Extracellular vesicles
EM	- Electron microscopy
ILVs	- Intraluminal vesicles
MVBs	- Multivesicular bodies
RA	- Rheumatoid arthritis
SF	- Synovial fluid
PB	- Peripheral blood
PBMC	- Peripheral blood mononuclear cell
HC	- Healthy control
SG	- Sucrose gradient
FACS	- Fluorescence activated cell sorting

1. Introduction

Extracellular vesicles (EVs) are membrane bound particles found in all biological fluids. Vesicles range in size and can be generated as intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) or formed as a result of the outward budding and fission of the plasma membrane (1). EVs carry parental cell specific proteins, targeting and adhesion proteins, mRNA and miRNA, signal transduction proteins and cytoplasmic enzymes (1). Although possessing distinct information (1), it is difficult to classify vesicles upon release due to overlapping sizes, buoyant densities and marker expression (2, 3). Cell-EV interactions can nevertheless produce a plethora of biological outcomes as a result of the unique vesicle content and varying status of target cells.

Previous research has shown that vesicles exhibit immune activating and suppressing characteristics (4). The role of EVs in tumor and microorganism immunology has been thoroughly investigated (4) however, their function within autoimmunity remains unclear. In autoimmunity, work has centered on Rheumatoid arthritis (RA) (1). Indeed, in RA, increased EV counts are associated with disease severity and attributed to the deposition of fibrin and induction of angiogenesis (1). T cell and monocyte derived EVs upregulate enzyme activity in synovial fibroblasts and synovial fibroblasts produce vesicles that contain a membranous form of TNF-α (1). Taken together, data indicates that EVs participate in arthritic pathogenesis.

Although investigations have been performed for RA, little is known about the function of EVs within Juvenile idiopathic arthritis (JIA). JIA pathology involves the activation and migration of innate cells into the joint (5). These activated cells produce pro-inflammatory cytokines which intern amplify the infiltration and activation of more immune cells (5). This propagating feedback loop results in bone degradation and tissue damage which drives cellular activation and results in EV release. We therefore focused on determining the feasibility of vesicle isolation and identification using small volumes (1ml) of JIA synovial fluid plasma and next analyzed the biological effects of JIA derived EVs in a peripheral blood mononuclear cell (PBMC) culture system.

2. Methods

Patients

Healthy control and JIA samples were collected under approval of the local ethical committee. The SF donors were a pool of various oligoarthritic patients.

Vesicle isolation

Peripheral blood (PB) or SF derived plasma was diluted 1:5 with cold sterile PBS (Gibco). Samples were spun for 30mins, 2000g at 4°C and the supernatant was harvested. The supernatant was then spun for 30mins, 10,000g at 4°C, harvested and transferred to polyallomer tubes (Beckman Coulter). Tubes were filled with PBS and centrifuged for 1hour, 100,000g at 4°C. The supernatant was discarded and the pellet underwent either sucrose gradient ultracentrifugation according to the protocol of Kranendonk M et al. (6) or the tube was refilled with a 0.2% vesicle free BSA/PBS solution and centrifuged for 1hour, 100,000g at 4°C. Following this final spin, the vesicle pellet was resuspended in either culture medium comprising of RPMI1640 supplemented with L-glutamate and 25 mM HEPES (Gibco), containing 10% vesicle free FCS (Biowest) and penicillin/streptomycin (100 U/mL) (Invitrogen) for stimulation experiments, 0.2% FCS/PBS for flowcytometry and EM analysis or lysed for western blot.

For the western blots, pools of plasma were used (N=3). For the stimulation assays (N=3), EM (N=2) and flowcytometry (SG effect N=2, EV counts N=4) 1ml of plasma was used per donor (unpooled). All reagents were made vesicle free by ultracentrifugation for 16hours, 100,000g at 4°C.

Peripheral blood mononuclear cell Isolation and Stimulation
PBMCs were isolated using Ficoll-Paque density gradient centrifugation and resuspended in vesicle free culture medium. Cells were placed in a plate coated with aCD3 (Bio-Rad) and stimulated with vesicles derived from healthy control (HC) plasma or JIA SF for 4 days.

Electron Microscopy
Vesicles were isolated and resuspended in 0.2% FCS/PBS and visualized using the methodology of Slot et al. (7).

Western blot analysis
Samples were lysed in Laemmli Sample Buffer(BioRad) 10% Betamecaptoethanol (Sigma-Aldrich) with 1% Triton (Sigma-Aldrich). Equal amounts of protein were loaded and run on a 4–20% Mini-PROTEAN® TGX™ Gel (BioRad). Blots were incubated with the following antibodies: anti-Calnexin (Abcam), anti-CD9 (Biolegend), anti-MHCI (Acris), anti-HSP70 (Enzo life sciences) and anti-CD81 (Santa Cruz). Blots were analyzed using ECL Prime Western Blotting Detection Reagents (Amersham).

Vesicle flowcytometry
Vesicles were stained as described by van der Vlist et al. (8). Stained EVs were resuspended in 0.2% FCS/PBS and spun down for 1hour, 100,000g at 4°C. Prior to flowcytometry samples were diluted 1:50 in cold filtered PBS. Vesicle analysis was performed on a 3-laser Aria III flowcytometer (Becton Dickinson) equipped with a 100µm nozzle and sample pressure was set at 10 PSI.

Multiplex immune assay
Supernatants were analyzed undiluted using Luminex xMAP technology (xMAP, Luminex Austin TX USA) as previously described (9).

Statistics
A paired samples T- test was used to compare SG dependent and independent isolation. Vesicle count data and cytokine data was analyzed using a Kruskal-Wallis test. IBM SPSS 23 statistics software was used.

3. Results

Vesicle isolation and identification
Current methods of vesicle isolation use ultracentrifugation based on a sucrose gradient (SG) dependent or independent protocol. Imaging techniques are combined with western blots or omics to provide evidence of successful vesicle isolation.

Electron microscopy (EM) allows for clear EV illustration. Using both a SG dependent (Fig 1A and C) and independent technique (Fig 1B and D), we were able to demonstrate the successful isolation of EVs from both healthy control PB plasma (Fig1 A and B) and JIA SF plasma (Fig 1 C and D). These images illustrate typical EV morphology (10).

Next, using flowcytometry, we compared vesicle numbers in healthy control PB samples that underwent SG dependent or independent isolation. Fig 1E shows that SG isolation resulted in a decrease in vesicle numbers (p=0.241). Our data corroborates studies in which SG isolation causes major vesicle loss (11). In order to preserve EV numbers we chose to continue our study using the SG independent technique.

When comparing PB derived vesicles, healthy controls and JIA patients had similar EV counts (Fig 1F). SF plasma vesicle numbers were, however, higher than PB plasma EV counts (Fig 1F, p=0.359). Visualization of the EVs through flowcytometry also showed a difference in vesicle population distribution (Fig 1H). The difference in vesicle populations was affirmed by western blot analysis (Fig 1H). Using typical EV markers (HSP70, MHCI, CD9, CD81), we demonstrated that SF derived vesicles

contained higher levels of MHCI and CD81 while healthy donor EVs contained greater amounts of HSP70 and CD9. Note, as expected, samples were positive for debris (calnexin) due to non-SG isolation. In all, the data shows that we could successfully isolate and identify EVs from JIA patient samples.

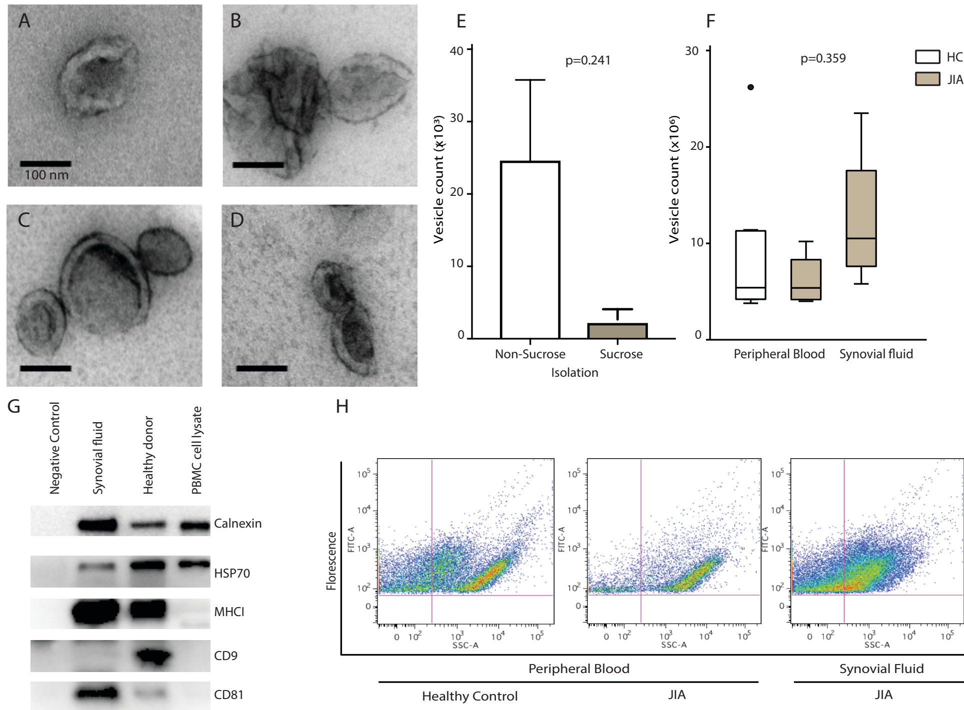

Fig 1: Vesicle Isolation and Identification: Fig 1A-D EM imaging technology was used to visualize vesicles isolated using sucrose and non-sucrose gradient based ultracentrifugation. EM images of healthy control peripheral blood plasma (A -sucrose, B- non-sucrose B) and JIA synovial fluid plasma (C- sucrose, D- non-sucrose) derived vesicles depict a typical cup shaped morphology with scale bars (black) representing 100nm respectively. FACS technology allowed vesicle numbers to be tallied and compared between Fig1 E Non-sucrose and sucrose isolated vesicles, error bars shown as SD. Using non-sucrose gradient based ultracentrifugation in congruence with FACS technology, vesicle numbers and population distribution were compared between healthy control peripheral blood, JIA peripheral blood and synovial fluid plasma derived populations Fig 1F and H error bars shown as SD. Fig 1G Western blot analysis was implemented as a third method of vesicle identification. Vesicles were isolated using non-sucrose gradient based ultracentrifugation from pooled synovial fluid plasma samples and compared to vesicles from pooled healthy control peripheral blood plasma samples. Western blot analysis was performed using typical extracellular vesicles markers (HSP70, MHC I, CD9, CD81) and Calnexin was used to control for sample contamination.

Vesicle Functionality

To test the biological effects of JIA derived EVs, vesicles were used to stimulate healthy donor derived PBMCs for 4 days. EVs influence cytokine production in immune cells (12), we thus measured cytokine levels in order to determine the effect of JIA EVs on PBMC activity. It is known that PB and SF of JIA patients exhibit higher levels of IL-6, IL-17, TNFα and IFNγ [9]. Here we show that the administration of EVs to PBMCs results in a significant increase only in IL-6 ($p=0.001$) when compared to the unstimulated PBMC condition, regardless of the vesicle source (figure 2). When comparing SF plasma derived vesicles to healthy control PB EVs however, we do not observe any difference in effect on cytokine production. Taken together this data indicates that, although EVs have been known to alter

immune cell cytokine production, in the context of our analysis JIA EVs do not seem to cause a robust change in PBMC activation.

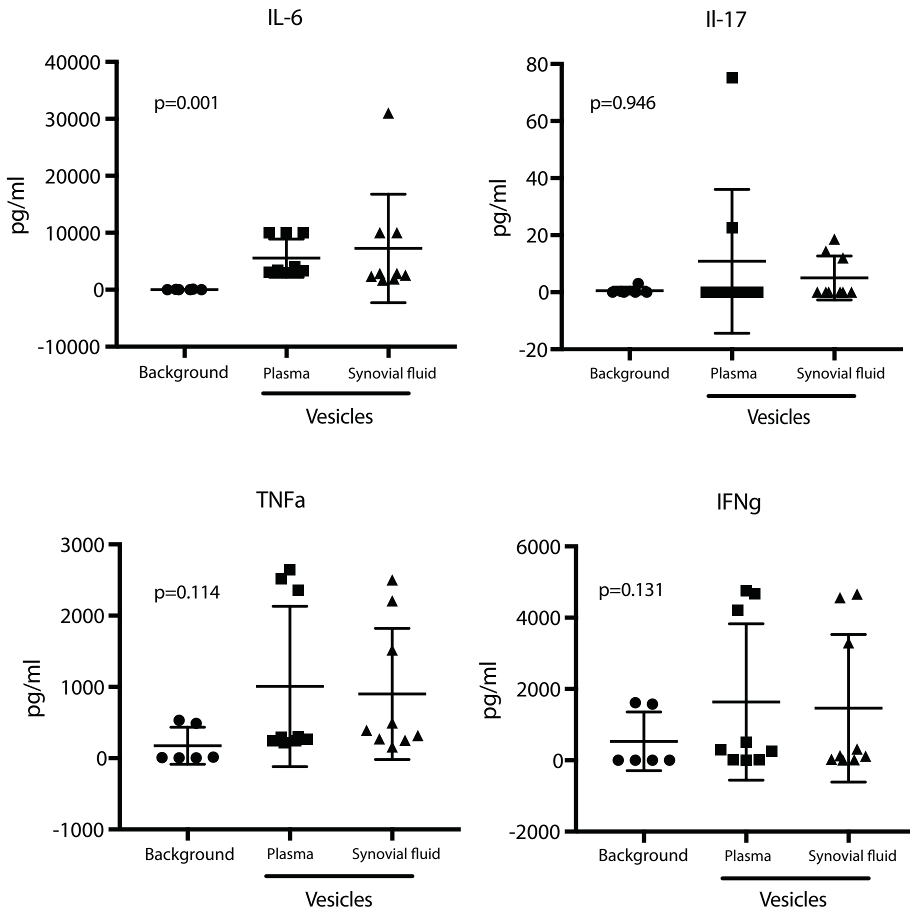

Fig 2: Vesicle functionality: Healthy control plasma vesicles and synovial fluid vesicles were used to stimulate peripheral blood derived mononuclear cells. Luminex technology was implemented as a read-out system. A panel of cytokines typically associated with JIA was tested: IL-6, IL-17, TNFα and IFNγ.

4. Discussion

In this study, we aimed to determine the feasibility of vesicle isolation from small volume JIA SF plasma samples and investigate the biological effects of JIA EVs on PBMC activity. Our data indicates that EVs can successfully be isolated from PB and SF plasma using only 1 ml of biological fluid (Fig 1). Furthermore, irrespective of the source fluid, EVs can moderately alter JIA associated cytokine production when compared to unstimulated conditions (Fig 2). Data also indicates a difference in MHCI and tetraspanin expression depending on the vesicle source. (Fig 1H). EVs function as intercellular communicators through lipid modulation, receptor ligand interaction or fusion and transfer of luminal content, (1, 4, 13, 14). Differences in activity as a result of differences in vesicle fusion capabilities and

content could thus be proposed as a factor influencing the observed heterogeneity in JIA disease course amongst patients. EV methods of direct and indirect cellular interaction remain, however, difficult to distinguish and until techniques are optimized this role will remain un-investigated.

Methodologically, three elements may have influenced our results. Firstly, due to small sample aliquots and vesicle loss after SG isolation, we used non-SG isolation. We thus acknowledge that our vesicle enriched isolates may contain other debris. Secondly, to normalize our stimulation assays, equal starting volumes were used (1ml). As demonstrated by our flowcytometry data, 1ml of sample may contain different amounts of vesicles depending on the source. It would thus have been optimal if EV counts were used for normalization. Popular techniques to count EVs exist (15) however, methodology remains inaccurate (15) and would have resulted in a reduction in EV numbers from an already small sample. Lastly, we know that EVs have suppressive activity (4) which may have influenced the level of cytokines we measured. Ascertaining the optimal time of cytokine measurement may therefore provide better insight into the effects of EVs.

5. Conclusions

In all, data indicates that upon successful isolation, JIA SF derived EVs can be implemented in biological assays. Although in this pilot study no disease specific effects were observed, future experiments could provide possible insight into EVs as potential key players in JIA disease activity.

6. Declarations

Ethics approval and consent to participate
Sample collection and handling was approved by the Medical Research Ethics Committee UMC Utrecht. Healthy control samples were collected under the approval number 07-125/C and JIA samples under SF 11-499/C.

Consent for publication
No individual data has been represented in this publication.

Availability of data and material
The data used in this study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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Authors Contributions
GCK and WdJ designed the study. GCK, NK and RS did the experimental work. GCK, NK, RS collected data. GCK and NK analyzed the data, supervised by WdJ, HS and BL. GCK and WdJ drafted the manuscript, and all authors revised it critically for intellectual content. All authors have approved the final version.

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References

1.Buzas E.I, György B, Nagy G, Falus A, Gay S. *Emerging role of extracellular vesicles in inflammatory diseases*. Nat Rev Rheumatol. 2014, Vol. 10, pp. 356–364.

2. van der Vlist E.J, Arkesteijn G.J.A, van de Lest C.H.A, Stoorvogel W, Nolte-'t Hoen E.N.M, Wauben M.H.M. *CD4+ Tcell activation promotes the differential release of distinct populations of nanosized vesicles*. JEV. 2012, Vol. 1.

3. Bobrie A, Colombo M, Krumeich S, Raposo G, Thery C. *Diverse subpopulations of vesicels secreted by different intracellular mechanisms are present in exosome preparations obtained by differnetial ultracentrifugation.* JEV. 2012, Vol. 1.

4. Robbins P.D, Morelli A.E. *Regulation of immune responses by extracellular vesicles.* Nat Rev. 2014, Vol. 14, pp. 195-208.

5. Prakken B, Albani S, Martini A. *Juvenile Idiopathic Arthritis.* Lancet. 2011, Vol. 377, pp. 2138-2149.

6. Kranendonk M.E.G, Visseren F.L.J, van Balkom B.W.M, Nolte-'t Hoen E.N.M, van Herwaarden J.A, de Jager W, Schipper H.S, Brenkman A.B, Verhaar M.C, Wauben M.H.M ,Kalkhoven E. *Human adipocyte extracellular vesicles in reciprocal signaling between adipocytes and macrophages.* Obesity. 2014, Vol. 22, pp. 1296-1308.

7. Slot J.W, Geuze H.J. *Cryosectioning and immunolabeling*. Nature protocols. 2007, Vol. 2.

8. van der Vlist E.J, Nolte-'t Hoen E.N.M, Stoorvogel W, Arkesteijn G.J.A, Wauben M.H.M, *Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry*. Nature protocols. 2012, Vol. 7.

9. de Jager W, Hoppenreijs E.PA.H, Wulffraat N.M, Wedderburn L.R, Kuis W, Prakken B.J.*Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study.* Ann Rheum Dis. 2007, Vol. 66, pp. 589–598.

10.van der Pol E, Böing A.N, Harrison P, Sturk A, Nieuwland R. *Classification, Functions, and Clinical Relevance of Extracellular Vesicles.* Pharmacological Reviews. 2012, Vol. 64, pp. 676-705.

11.Muller L, Hong C, Stolz D.B, Watkins S.C and Whiteside T.L. *Isolation of Biologically-Active Exosomes from Human Plasma.* J Immunol Methods. 2014, Vol. 411, pp. 55–65.

12. Scanu A, Molnarfi N, Brandt K.J, Gruaz L, Dayer J.M, Burger D. *Stimulated T cells generate microparticles, which mimic cellular contact activation of human monocytes:Differential regulation of pro- and anti-inflammatory cytokine production by high intensity lipoproteins*. J Leukoc Biol. 2008, Vol. 83, pp. 921-927.

13. Choi A.M.K, Ryter S.W, Levine B. *Autophagy in human health and disease*. New Eng J Med. 2013, Vol. 368, pp. 651–662.

14. Simons M, Raposo G. *Exosomes-vesicular carriers for intercellular communication*. Curr Opin Cell Biol. 2009, Vol. 21, pp. 575-581.

15. Akers J.C, Ramakrishnan V, Nolan J.P, Duggan E, Fu C, Hochberg F.H ,Chen C.C. *Comparative Analysis of Technologies for Quantifying Extracellular Vesicles (EVs) in Clinical Cerebrospinal Fluids (CSF).* PLOS one. 2016, Vol. 11.

Chapter 4

Differential adipokine receptor expression on circulating leukocyte subsets in lean and obese children

Genoveva Keustermans[1*], Laila B. van der Heijden[2*], Berlinda Boer[1], Rianne Scholman[1], Roos Nuboer[3], Gerard Pasterkamp[4], Berent Prakken[1,5], Wilco de Jager[1], Eric Kalkhoven[6], Arieke J. Janse[2], Henk S. Schipper[1,5,7]

1 Laboratory for Translational Immunology, University Medical Centre Utrecht, Utrecht, The Netherlands, **2** Division of Paediatrics, Hospital Gelderse Vallei, Ede, The Netherlands, **3** Division of Paediatrics, Meander Medical Centre, Amersfoort, The Netherlands, **4** Department of Experimental Cardiology, University Medical Centre Utrecht, Utrecht, The Netherlands, **5** Division of Paediatrics, Wilhelmina Children's Hospital, University Medical Centre Utrecht, Utrecht, The Netherlands, **6** Molecular Cancer Research and Centre for Molecular Medicine, University Medical Centre Utrecht, Utrecht, The Netherlands, **7** Department of Paediatrics

*Both authors contributed equally to this work

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ABSTRACT

Background

Childhood obesity prevalence has increased worldwide and is an important risk factor for type 2 diabetes (T2D) and cardiovascular disease (CVD). The production of inflammatory adipokines by obese adipose tissue contributes to the development of T2D and CVD. While levels of circulating adipokines such as adiponectin and leptin have been established in obese children and adults, the expression of adiponectin and leptin receptors on circulating immune cells can modulate adipokine signalling, but has not been studied so far. Here, we aim to establish the expression of adiponectin and leptin receptors on circulating immune cells in obese children pre and post-lifestyle intervention compared to normal weight control children.

Methods

13 obese children before and after a 1-year lifestyle intervention were compared with an age and sex-matched normal weight control group of 15 children. Next to routine clinical and biochemical parameters, circulating adipokines were measured, and flow cytometric analysis of adiponectin receptor 1 and 2 (AdipoR1, AdipoR2) and leptin receptor expression on peripheral blood mononuclear cell subsets was performed.

Results

Obese children exhibited typical clinical and biochemical characteristics compared to controls, including a higher BMI-SD, blood pressure and circulating leptin levels, combined with a lower insulin sensitivity index (QUICKI). The 1-year lifestyle intervention resulted in stabilization of their BMI-SD. Overall, circulating leukocyte subsets showed distinct adipokine receptor expression profiles. While monocytes expressed high levels of all adipokine receptors, NK and iNKT cells predominantly expressed AdipoR2, and B-lymphocytes and $CD4^+$ and $CD8^+$ T-lymphocyte subsets expressed AdipoR2 as well as leptin receptor. Strikingly though, leukocyte subset numbers and adipokine receptor expression profiles were largely similar in obese children and controls. Obese children showed higher naïve B-cell numbers, and pre-intervention also higher numbers of immature transition B-cells and intermediate $CD14^{++}CD16^+$monocytes combined with lower total monocyte numbers, compared to controls. Furthermore, adiponectin receptor 1 expression on nonclassical $CD14^+CD16^{++}$ monocytes was consistently upregulated in obese children pre-intervention, compared to controls. However, none of the differences in leukocyte subset numbers and adipokine receptor expression profiles between obese children and controls remained significant after multiple testing correction.

Conclusions

First, the distinct adipokine receptor profiles of circulating leukocyte subsets may partly explain the differential impact of adipokines on leukocyte subsets. Second, the similarities in adipokine receptor expression profiles between obese children and normal weight controls suggest that adipokine signalling in childhood obesity is primarily modulated by circulating adipokine levels, instead of adipokine receptor expression.

1. Introduction

Obese children often remain obese in adulthood, and are at risk for metabolic syndrome and cardiovascular disease later in life (1–3). Over the last few decades, enhanced excretion of inflammatory adipose tissue derived proteins (adipokines) emerged as one of the mechanisms underlying the cardio-metabolic sequelae in obesity (4,5). The adipokine profile in childhood and adulthood obesity includes increased levels of the inflammatory adipokines chemerin and leptin, and decreased levels of the anti-inflammatory adiponectin, which together propagates systemic inflammation, insulin resistance and vascular dysfunction, as a precursor for cardio- vascular disease (4,6–11).
The impact of circulating adipokines is not merely determined by plasma levels, but also orchestrated by differential adipokine receptor expression on target organs. For example, adiponectin receptor 1 (AdipoR1) and receptor 2 (AdipoR2) are significantly homologous (67% amino acid identity), and both serve as a receptor for globular and full-length adiponectin (12). However, AdipoR1 is predominantly expressed in liver, skeletal muscle, macrophages and hypothalamus, while AdipoR2 is most abundant in liver, white adipose tissue, and the vasculature. The differential tissue distribution and downstream signalling pathways of AdipoR1 and AdipoR2 importantly contribute to the plethora of adiponectin's biological actions (10). Next to differences in tissue distribution, up/downregulation of adipokine receptor expression under specific conditions can also modulate adipokine effects. Natural Killer (NK) cells, for instance, critically depend on leptin receptor expression for their activation and function (13). While leptin receptor-deficient mice showed impaired NK cell activity, leptin receptor expression was upregulated in rats with diet-induced obesity, apparently to compensate for decreased downstream signalling (14). Taken together, both tissue distribution and disease-specific up/ downregulation of adipokine receptors can modulate adipokine effects.
Whereas studying adipose tissue, liver and vascular distribution of adipokine receptors is precluded for medical ethical reasons, circulating leukocytes are readily available and play a pivotal role in systemic inflammation in obesity (15–18). Here, we used recently available flow cytometry antibodies to study the expression of AdipoR1, AdipoR2 and leptin receptor on circulating leukocyte subsets, in addition to measuring circulating adipokine levels. Considering the pivotal role of adipokine signalling in obesity, we included obese children pre and post-life- style intervention, next to normal weight control children. This study thus aims to unravel the differential impact of circulating adipokine levels and adipokine receptor expression on adipokine signalling in childhood obesity.

2. Materials and methods

Patients

This observational cohort study included 15 lean control children and 13 obese children aged 4–18 years, all patients of the paediatric outpatient department of Meander Medical Centre in Amersfoort and Hospital Gelderse Vallei Hospital in Ede, The Netherlands. Body Mass Index standard deviation (BMI-SD) values were calculated based on results of the Fifth Dutch Growth Study (19). Obesity was defined using established international age and sex-specific BMI cut-off points (20). The obese patients were enrolled in an established 1-year multidisciplinary, multi- component, family-based treatment programme developed in the Gelderse Vallei Hospital in Ede, The Netherlands (21). Patients were included from 2010 to 2015. Anthropometric measurements, blood pressure (BP) measurements and laboratory samples were collected at base- line (lean controls, obese children), and at the end of the 1-year intervention program (obese children). The study was approved by the ethical committee of Wageningen UR (METC 12/26) and the University Medical Centre Utrecht (METC 09/217K). Written informed consent was obtained from children older than 12 years and from the parents.

Clinical variables

Waist circumference was used as a marker of central adiposity and measured with a flexible tape to the nearest 0.1 cm at umbilicus height. Blood pressure (BP) was measured in supine position with an automated blood pressure monitor (Welch Allyn VSM 300, Skaneateles Falles, NY, USA) after 5 minutes of rest during a well visit in the outpatient clinic. A minimum of two BP measurements was performed, with an interval of at least one minute between the measurements. The mean of these two measurements was collected for data analysis. Blood pressure percentile scores were obtained according to the Fourth Report on BP in children (22).

Routine laboratory measurements

Routine laboratory testing included fasting glucose, insulin levels and lipid profiles (total cholesterol, high-density lipoprotein [HDL] cholesterol, low-density lipoprotein [LDL], and tri- glycerides), as well as samples for leukocyte differentiation, and alanine-aminotranspherase (ALT). The insulin sensitivity index (QUICKI) was calculated according to international standards (23).

Flow cytometry

Whole blood samples collected in sodium heparin tubes (BD vacutainer 367876) where spun down at room temperature, 160g for 10 minutes. Plasma was subsequently removed and stored at -80°C awaiting further use. Peripheral Blood mononuclear cells (PBMC) were isolated using Ficoll-Paque density gradient centrifugation and samples were stored in foetal bovine serum (FBS) (Biowest) supplemented with 10% DMSO (Sigma-Aldrich) at -150°C. All children donated 6–12ml whole blood, with 6–10 million PBMC per 6ml whole blood sample. Upon preparation for flow cytometry, the stored PBMC samples where thawed and washed in medium comprising of RPMI1640 supplemented with L-glutamate and 25 mM HEPES (Gibco), containing 2% FBS (Biowest) and penicillin/streptomycin (100 U/mL) (Invitrogen). Cells were spun down for 10 minutes at 280g at room temperature. All samples were stained for 20 minutes in the dark at 4°C and subsequently analysed on the BD LSR Fortessa. For monocyte, NK cell, B-cell, T-helper, T-effector/memory and Treg phenotyping, 200.000 PBMCs were analysed per sample. For iNKT cell phenotyping, 750.000 PBMCs were analysed per sample. The following antibodies were used: CD3 AF700 (Biolegend, clone UCHT1), CD4 PerCP-Cy5.5 (BD, clone SK3), CD25 PE-Cy7 (BD, clone M-A251), CD45RO BV711 (Biolegend, clone UCHL1), CD127 BV421 (BD Horizon, clone HIL-7R-M21), CD8 V500 (BD, clone RPA-T8), Leptin receptor Alexaflour647 (BD, clone 52263), ADIPOR1 FITC (USBio, rabbit polyclonal antibody). ADIPOR2 PE (USBio, rabbit polyclonal antibody), CD27 APC-eFluor780 (eBio- science, clone O323), CD28 BV421 (BD Horizon, clone CD28.2), CCR6 PE-Cy7 (eBiosciences, clone R6H1), CXCR3 BV510 (Biolegend, clone GO25H7), CD16 V500 (BD Horizon, clone 3G8), CD56 PE-Cy7 (BD, clone NCAM16.2), CD1d tetramer BV421 (NIH, hCD1d-PBS-57), CD10 PE-Cy7 (BD, clone HI10A), CD19 APC-efluor 780 (eBioscience, clone HIB19), CD21 BV711 (BD, clone B-ly4), CD27 BV510 (BD Horizon, clone L128), CD38 PerCP-Cy5.5 (BD Pharmigen, clone HIT2). Leukocyte subset numbers were calculated using the differential blood count (e.g. number of $CD14^{++}CD16^{+}$ monocytes = (fraction of $CD14^{++}CD16^{+}$ monocytes / total monocytes) x differential blood count monocyte number). Gating strategy of the leukocyte subsets is shown in the S1 Fig.

Multiplex immune assay (MIA)

Plasma levels of adiponectin, chemerin and leptin were measured by a MIA using Luminex xMAP technology (xMAP, Luminex Austin TX USA) validated by the Laboratory of Translational Immunology, University Medical Centre Utrecht (24). Biorad FlexMAP3D (Biorad laboratories. Hercules USA) and xPONENT software version 4.2 (Luminex) were used for acquisition and data was analysed by 5-parametric curve fitting using Bio-Plex Manager software, version 6.1.1 (Biorad).

Statistical analysis

Differences between groups were studied with an independent-sample Student's t-test for normally distributed data, and with a Mann-Whitney U test for non-parametric comparisons. Multiple testing correction using the Benjamini and Hochberg False Discovery Rate (FDR) procedure was applied when assessing leukocyte subset numbers and comparing adipokine receptor expression of leukocyte subsets in lean versus obese children pre and post-intervention. Pearson's correlation coefficients were calculated to determine the correlation between levels of circulating adipokines and the expression of adiponectin and leptin receptors on circulating immune cells. Statistical analyses were performed with the SPSS 22 statistical package (IBM SPSS Statistics Inc, Chicago, IL, USA).

3. Results

Circulating leukocyte subset numbers

Obese children exhibited typical clinical and biochemical characteristics compared to age and sex-matched normal weight controls, with a higher BMI-SD, waist circumference and blood pressure, combined with a lower insulin sensitivity index (QUICKI) and higher plasma levels of alanine aminotransferase, chemerin and leptin (Table 1). The obese children participated in an established lifestyle intervention program (21), which resulted in stabilization of their BMI-SD and other clinical and biochemical characteristics, and enabled sampling pre and post-lifestyle intervention (Table 1).

Table 1 Patient characteristics

	Lean controls (n=15)	**Obese - pre (n=13)**	**Obese - post (n=13**
Girls (number, %)	8 (53.3)	7 (53.8)	7 (53.8)
Age (years)	11.7 ± 2.9	10.7 ± 3.9	12.0 ± 3.9
BMI	18.5 ± 2.6 *#	27.9 ± 5.2 *	28.2 ± 5.5 #
BMI-SD	0.5 ± 0.9 *#	3.3 ± 0.7 *	3.1 ± 0.7 #
Waist (cm)	64.5 ± 7.5 *#	94.4 ± 16.8 *	98.4 ± 17.0 #
Systolic blood pressure (SBP)	102.2 ± 12.7 *#	113.2 ± 12.2 *	114.8 ± 11.7 #
SBP percentile	39.9 ± 23.6 *#	65.7 ± 25.1 *	65.5 ± 28.4 #
Diastolic blood pressure (DBP)	57.7 ± 8.2 *#	67.2 ± 9.5 *	69.4 ± 10.5 #
DBP percentile	35.9 ± 19.9 *#	62.6 ± 23.9 *	65.4 ± 27.1 #
QUICKI	0.4 ± 0.0 *#	0.3 ± 0.0 *	0.3 ± 0.0 #
Alanine aminotransferase (U/l)	18.0 (14.5-21.8) *#	29.5 (22.2-53.9) *	30.3 (22.9-41.2) #
Triacylglycerol (mmol/l)	0.6 ± 0.3 *	1.0 ± 0.4 *	1.0 ± 0.7
Total cholesterol (mmol/l)	4.0 ± 0.8	4.0 ± 0.8	3.9 ± 0.9
HDL-cholesterol (mmol/l)	1.7 ± 0.9	1.2 ± 0.3	1.3 ± 0.2
LDL-cholesterol (mmol/l)	2.2 ± 0.7	2.3 ± 0.7	2.2 ± 0.8
Adiponectin (ug/ml)	18.9 (15.6-26.5)	12.9 (9.5-21.3)	13.6 (10.4-19.8)
Chemerin (ug/ml)	1.2 (1.0-1.7) *#	1.9 (1.8-2.7) *	2.3 (1.8-2.5) #
Leptin (ng/ml)	125 (16-195) *#	399 (219-692) *	411 (308-600) #

Clinical characteristics and laboratory parameters for lean controls versus obese children pre-lifestyle intervention (pre) and post-lifestyle intervention (post). Normally distributed data are shown as mean ± SD, non-parametric data as median (interquartile range). * $p<0.05$ for lean controls compared to obese-pre. # $p<0.05$ for lean controls versus obese-post.

Circulating leukocytes were analysed with multi-parameter flow cytometry, and leukocyte subsets were gated according to international standards (Fig 1, S1 Fig) (25). Overall, leukocyte subset numbers were comparable between obese children and lean controls. However, obese children pre and post-intervention showed higher naïve B-cell numbers, and obese children pre-intervention showed higher numbers of immature transition B-cells and intermediate $CD14^{++}CD16^{+}$ monocytes than lean controls (Table 2). Obese children pre-intervention additionally showed lower total monocyte numbers than lean controls. Notably, differences in leukocyte subset numbers did not survive multiple testing correction.

Table 2 Leukocyte subset numbers

	Lean controls	**Obese-pre**	**Obese-post**
Innate immunity			
Monocytes (total)	400 (300-600) *	300 (300-400) *	400 (300-500)
$CD14^{++}CD16^{-}$	352 (255-542)	244 (196-303)	330 (245-374)
$CD14^{++}CD16^{+}$	15 (11-19)*	16 (13-22)*	18 (13-39)
$CD14^{+}CD16^{++}$	37 (33-94)	39 (30-79)	40 (36-69)
Natural Killer cells ($CD16^{+}CD56^{+}$)	150 (80-211)	224 (116-321)	226 (134-287)
$CD16^{+}CD56^{++}$	8.7 (5.5-12)	8.0 (4.9-13)	9.3 (5.8-12)
$CD16^{-}CD56^{++}$	4.9 (2.8-8.0)	5.5 (3.4-8.1)	5.0 (4.4-8.9)
Bridging immunity			
Natural Killer T cells	1.2 (0.6-2.2)	1.4 (0.9-3.9)	1.2 (1.0-3.6)
Adaptive immunity			
B cells			
Naive ($CD10^{-}CD27^{-}$)	178 (132-270) *#	255 (189-454) *	316 (224-454) #
Memory ($CD10^{-}CD27^{+}$)	48 (41-73)	69 (45-113)	74 (56-117)
Immature transition ($CD10^{+}CD27^{+}$)	10 (5.4-17) *	23 (9.3-39) *	15 (10-31)
$CD4^{+}$ T helper cells			
$CD45RO^{-}$ $CXCR3^{-}$	511 (451-700)	639 (401-750)	624 (334-937)
$CD45RO^{-}$ $CXCR3^{+}$	58 (40-176)	58 (32-110) $	85 (68-162) $
$CD45RO^{+}$ $CXCR3^{-}$	140 (115-196)	151 (125-227)	207 (103-251)
$CD45RO^{+}$ $CXCR3^{+}$	111 (84-134)	67 (43-115)	84 (57-117)
CD8+ cytotoxic T cells			
$CD45RO^{-}$ $CCR7^{-}$	76 (37-138)	76 (43-178)	94 (29-161)
$CD45RO^{-}$ $CCR7^{+}$	284 (275-358)	355 (272-434)	294 (238-477)
$CD45RO^{+}$ $CCR7^{-}$	82 (44-85)	56 (38-91)	52 (42-73)
$CD45RO^{+}$ $CCR7^{+}$	15 (12-19)	13 (8.6-30)	18 (13-22)
Regulatory T cells ($CD25^{+}CD127^{-}$)	26 (14-35)	21 (16-28)	22 (13-33)

Leukocyte subset numbers (x 10^{6}) of lean controls compared to obese children pre-lifestyle intervention (pre) and post-lifestyle intervention (post). Data are presented as median (interquartile range). * $p<0.05$ for lean controls compared to obese-pre. # $p<0.05$ for lean controls versus obese-post. $ $p<0.05$ for obese-pre compared to obese-post.

Adipokine receptor expression on leukocyte subsets

Focussing on the innate immune cells, monocytes expressed high levels of adipokine receptors compared to other circulating leukocyte subsets (Fig 1). Whereas non-classical $CD14^{+}CD16^{++}$ monocytes particularly expressed high levels of AdipoR1, all monocyte subsets expressed AdipoR2, and classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocytes particularly expressed high leptin receptor levels. In contrast, Natural Killer (NK) cells and invariant Natural Killer T-cells (iNKT) predominantly showed AdipoR2 expression, next to discrete AdipoR1 expression by $CD16^{+}$ NK cells (Fig 1, Tables 3, 4 and 5).

Considering the adaptive immune cells, AdipoR1 expression appeared to be low, in general. Only part of the $CD4^{+}$ $CXCR3^{+}$ T-cells and $CD8^{+}$ T-cells and a discrete subset of regulatory T-cells showed AdipoR1 expression. In contrast, AdipoR2 was particularly expressed by B-cells, next to a subset of $CD4^{+}$ $CXCR3^{+}$ T-cells and a subset of $CD8^{+}$ $CD45RO^{+}$ (memory) T-cells. Finally, leptin receptor was predominantly expressed by B-cells, and a subset of $CD4^{+}$ $CD45RO^{-}$ (non-memory) $CXCR3^{+}$ T-cells and of $CD8^{+}$ $CD45RO^{+}$ (memory) $CCR7^{+}$ T-cells and regulatory T-cells (Fig 1, Tables 3, 4 and 5).

Taken together, circulating leukocyte subsets showed distinct adipokine receptor expression profiles. Monocytes generally expressed high levels of all adipokine receptors, while other leukocyte subsets showed a different pattern. NK and iNKT cells predominantly expressed AdipoR2, while B-lymphocytes and $CD4^{+}$ and $CD8^{+}$ T-lymphocyte subsets expressed AdipoR2 as well as leptin receptor. Notably, regulatory T-cells showed little adipokine receptor expression, but a discrete subset of regulatory T-cells expressed a combination of adipokine receptors (Figure in S2 Fig).

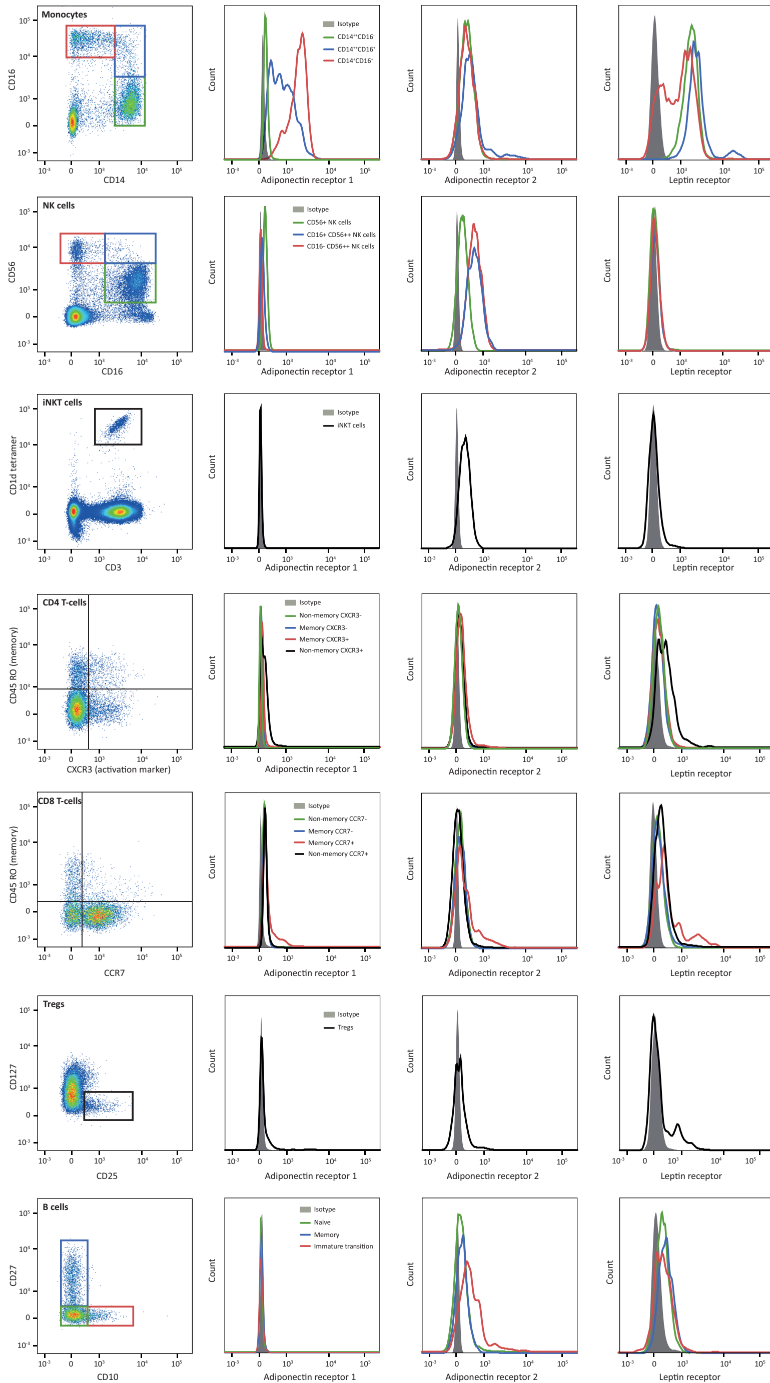

Fig 1: Adipokine receptor expression on leukocyte subsets: The first column illustrates the gating strategy for the leukocyte subsets. The other columns show representative histograms of the AdipoR1, AdipoR2 and leptin receptor expression of the different leukocyte subsets.

Table 3 Adiponectin receptor 1 expression

	Lean controls	Obese-pre	Obese-post
Innate immunity			
Monocytes (total)	21 (18-31)	31 (18-36)	25 (20-29)
$CD14^{++}CD16^{-}$	10 (8.8-16) #	8.2 (4.6-27)	7.7 (3.7-11) #
$CD14^{++}CD16^{+}$	82 (68-88)	90 (81-94)	84 (73-93)
$CD14^{+}CD16^{++}$	84 (71-92) *	94 (77-98) *	91 (85-96)
Natural Killer cells ($CD16^{+}CD56^{+}$)	82 (56-92)	86 (76-95)	88 (78-95)
$CD16^{+}CD56^{++}$	41 (29- 63)	51 (39-58)	56 (44-64)
$CD16^{-}CD56^{++}$	1.6 (0.5 -3.1)	0.9 (0.1-4.6)	0.8 (0.4-1.8)
Bridging immunity			
Natural Killer T cells	7.6 (4.1-25)	12 (4.1-18)	6.9 (5.0-15)
Adaptive immunity			
B cells			
Naive ($CD10^{-}CD27^{-}$)	1.1 (0.6-2.2) #	0.8 (0.5-2.0)	0.5 (0.4-1.0) #
Memory ($CD10^{-}CD27^{+}$)	5.9 (3.8-6.6)	5.6 (3.8-8.8)	6.4 (3.5-9.6)
Immature transition ($CD10^{+}CD27^{+}$)	0.0 (0.0-0.2) *	0.7(0.3-1.0) *$	0.1 (0.0-0.5) $
$CD4^{+}$ T helper cells			
$CD45RO^{-}$ $CXCR3^{-}$	5.2 (1.6-21)	3.3 (1.0-22)	3.6 (1.6-19)
$CD45RO^{-}$ $CXCR3^{+}$	64 (45-92)	47 (37-71)	63 (49-81)
$CD45RO^{+}$ $CXCR3^{-}$	6.1 (2.1-12)	4.7 (1.6-12)	6.6 (2.2-11)
$CD45RO^{+}$ $CXCR3^{+}$	24 (12-38)	21 (12-42)	41 (16-56)
CD8+ cytotoxic T cells			
$CD45RO^{-}$ $CCR7^{-}$	46 (41-55)	51 (42-61)	50 (42-61)
$CD45RO^{-}$ $CCR7^{+}$	60 (55-74)	54 (45-66)	53 (46-68)
$CD45RO^{+}$ $CCR7^{-}$	34(30-43)	36 (31-52)	38 (34-43)
$CD45RO^{+}$ $CCR7^{+}$	70 (66-77)	68 (52-90)	72 (67-87)
Regulatory T cells ($CD25^{+}CD127^{-}$)	3.9 (1.6-14)	4.0 (1.0-9.0)	4.8 (1.6-9.7)

Percentage expression of adiponectin receptor 1 on leukocyte subsets of lean controls compared to obese children pre-lifestyle intervention (pre) and post-lifestyle intervention (post). Data are presented as median (interquartile range). * p<0.05 for lean controls compared to obese-pre. # p<0.05 for lean controls versus obese-post. $ p<0.05 for obese-pre compared to obese-post.

Adipokine receptor expression in lean and obese children

In order to establish differences in adipokine receptor expression between obese children pre and post-intervention and normal weight controls, we studied differences in expression percentages (Tables 3, 4 and 5), as well as median fluorescence intensities (MFI) (S1–S3 Tables). In general, obese children pre and post-intervention and normal weight controls showed similar adipokine receptor expression on leukocyte subsets. In fact, none of the observed differences in adipokine receptor expression between obese children and lean controls remained significant after multiple testing correction.

As an alternative strategy to discriminate between random deviations and potentially relevant differences, we focused on differences in adipokine receptor expression that were consistent in

percentages as well as fluorescence intensities. AdipoR1 expression on non-classical $CD14^{+}CD16^{++}$ monocytes was consistently upregulated in percentages and MFI in obese children pre- intervention, compared to lean controls (Table 3 and S1 Table). The other leukocyte subsets did not show consistent differences in AdipoR1, AdipoR2 or leptin receptor expression between obese children and lean controls.

Table 4 Adiponectin receptor 2 expression

	Lean controls	**Obese-pre**	**Obese-post**
Innate immunity			
Monocytes (total)	60 (32-67)	68 (46-80)	57 (43-70)
$CD14^{++}CD16^{-}$	59 (29-66)	60 (45-80)	54 (41-67)
$CD14^{++}CD16^{+}$	79 (60-88)	80 (67-93)	75 (59-86)
$CD14^{+}CD16^{++}$	60 (44-70)	63 (46-80)	62 (44-68)
Natural Killer cells ($CD16^{+}CD56^{+}$)	67 (62-80) #	61 (53-72)	62 (53-66) #
$CD16^{+}CD56^{++}$	98 (97-100)	97 (95-99)	98 (93-99)
$CD16^{-}CD56^{++}$	99 (96-100)	97 (95-99)	98 (97-100)
Bridging immunity			
Natural Killer T cells	77 (70-89)	70 (61-74)	77 (62-82)
Adaptive immunity			
B cells			
Naive ($CD10^{-}CD27^{-}$)	38 (35-46)	36 (32-41)	35 (24-40)
Memory ($CD10^{-}CD27^{+}$)	45 (39-52)	43 (36-46)	41 (19-47)
Immature transition ($CD10^{+}CD27^{+}$)	74 (61-81)	72 (58-87)	77 (57-82)
$CD4^{+}$ T helper cells			
$CD45RO^{-}$ $CXCR3^{-}$	27 (25-32) #	25 (20-38)	25 (13-27) #
$CD45RO^{-}$ $CXCR3^{+}$	54 (46-88)	49 (40-58)	50 (43-67)
$CD45RO^{+}$ $CXCR3^{-}$	34 (31-39)	32 (28-41)	33 (14-37)
$CD45RO^{+}$ $CXCR3^{+}$	43 (36-62)	40 (33-54)	47 (33-60)
CD8+ cytotoxic T cells			
$CD45RO^{-}$ $CCR7^{-}$	29 (11-34)	31 (24-44)	29 (12-33)
$CD45RO^{-}$ $CCR7^{+}$	26 (22-48)	24 (19-44)	25 (20-38)
$CD45RO^{+}$ $CCR7^{-}$	31 (13-41)	36 (30-44)	30 (7.6-35)
$CD45RO^{+}$ $CCR7^{+}$	58 (52-76)	55 (35-80)	58 (52-77)
Regulatory T cells ($CD25^{+}CD127^{-}$)	23 (21-27)	20 (15-27)	22 (19-28)

Percentage expression of adiponectin receptor 2 on leukocyte subsets of lean controls compared to obese children pre-lifestyle intervention (pre) and post-lifestyle intervention (post). Data are presented as median (interquartile range). * $p<0.05$ for lean controls compared to obese-pre. # $p<0.05$ for lean controls versus obese-post. $ $p<0.05$ for obese-pre compared to obese-post.

Table 5 Leptin receptor expression

	Lean controls	Obese-pre	Obese-post
Innate immunity			
Monocytes (total)	96 (95-98)	97 (92-98)	97 (94-98)
CD14^{++}CD16^{-}	99 (99-100)	100 (99-100)	100 (99-100)
CD14^{++}CD16^{+}	100 (99-100)	100 (99-100)	100 (99-100)
CD14^{+}CD16^{++}	70 (61-81)	75 (65-84)	77 (77-81)
Natural Killer cells (CD16^{+}CD56^{+})	8.0 (5.3-12)	8.8 (3.3-18)	6.1 (4.4-10)
CD16^{+}CD56^{++}	7.2 (5.3-9.6)	5.7 (3.0-14)	6.2 (3.0-9.7)
CD16^{-}CD56^{++}	6.6 (5.9-7.7)	5.3 (3.1-7.1)	6.4 (2.9-7.8)
Bridging immunity			
Natural Killer T cells	11 (5.8-19)	13 (5.2-32)	9.8 (8.7-18)
Adaptive immunity			
B cells			
Naive (CD10^{-}CD27^{-})	51 (44-56)	51 (46-58)	47 (46-55)
Memory (CD10^{-}CD27^{+})	60 (57-65)	63 (54-65)	57 (50-62)
Immature transition (CD10^{+}CD27^{+})	46 (39-54)	50 (35-57)	46 (39-51)
CD4^{+} T helper cells			
CD45RO^{-} CXCR3^{-}	31 (28-40)	29 (24-41)	28 (24-36)
CD45RO^{-} CXCR3^{+}	69 (59-93)	62 (50-73)	71 (64-80)
CD45RO^{+} CXCR3^{-}	26 (22-29)	25 (21-26)	24 (21-29)
CD45RO^{+} CXCR3^{+}	42 (29-50)	41 (28-49) $	55 (44-66) $
CD8+ cytotoxic T cells			
CD45RO^{-} CCR7^{-}	22 (16-26)	22 (17-34)	25 (19-29)
CD45RO^{-} CCR7^{+}	41 (31-52)	39 (31-52)	38 (32-48)
CD45RO^{+} CCR7^{-}	21 (19-32)	24 (19-32)	28 (24-31)
CD45RO^{+} CCR7^{+}	65 (56-72)	67 (48-87)	69 (61-80)
Regulatory T cells (CD25^{+}CD127^{-})	10 (7.8-17)	12 (5.9-15)	13 (11-17)

Percentage expression of leptin receptor on leukocyte subsets of lean controls compared to obese children pre-lifestyle intervention (pre) and post-lifestyle intervention (post). Data are presented as median (interquartile range). * p<0.05 for lean controls compared to obese-pre. # p<0.05 for lean controls versus obese-post. $ p<0.05 for obese-pre compared to obese-post.

Circulating adipokines and adipokine receptor expression

Finally, we wondered whether high circulating adipokine levels were associated with alterations in adipokine receptor expression, which could modulate adipokine signalling. First, we studied the relationship between circulating adipokine levels and adipokine receptor expression on monocytes. Neither did we observe a correlation between circulating adiponectin levels and AdipoR1/AdipoR2 expression (Fig 2A and 2B), nor a correlation between circulating leptin levels and leptin receptor expression (Fig 2C, Fig B in S3 Fig). Second, screening other leukocyte subsets did not yield a correlation between circulating adipokine levels and adipokine receptor expression either (Fig A in S3 Fig).

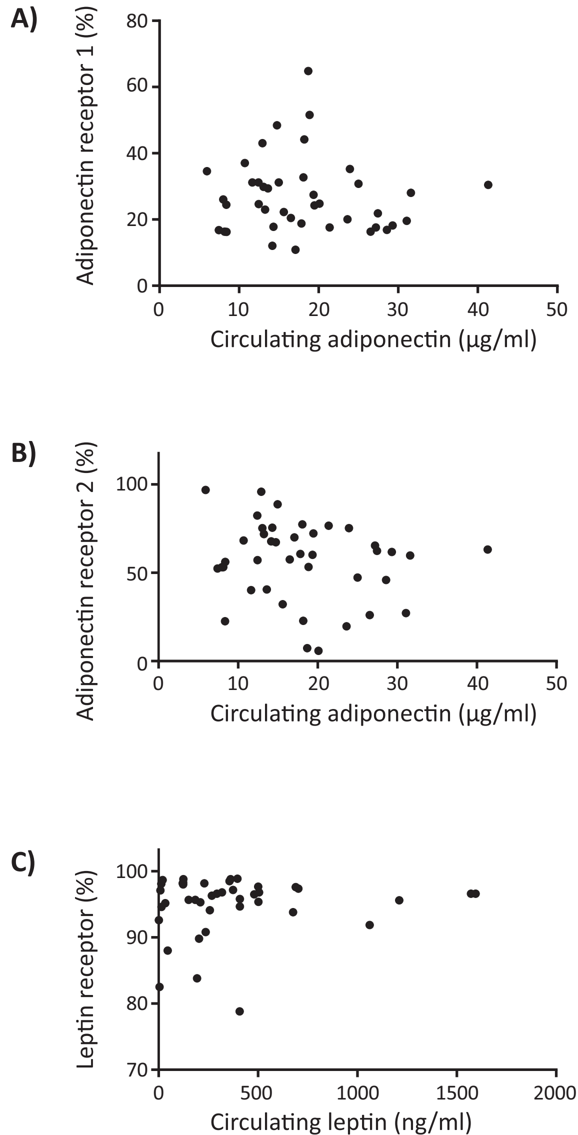

Fig 2: Circulating adipokine levels and monocyte adipokine receptor expression: (A) Circulating adiponectin levels versus percentage of AdipoR1 expressing monocytes (Pearson's correlation coefficient -0.079, p = 0.623). (B) Circulating adiponectin levels versus percentage of AdipoR2 expressing monocytes (Pearson's correlation coefficient -0.178, p = 0.265). (C) Circulating leptin levels versus percentage of leptin receptor expressing monocytes (Pearson's correlation coefficient 0.107, p = 0.505).

4. Discussion

Over the last decades, enhanced circulating leptin levels and decreased adiponectin levels emerged as one of the mechanisms underlying the cardio-metabolic sequelae in obesity (4,5,11). This study aimed to establish the expression of adiponectin and leptin receptors on circulating immune cell subsets in obese children and normal weight controls, since adipokine receptor expression on circulating

leukocytes can modulate adipokine signalling and obesity- induced systemic inflammation (15,16). Here, we will discuss our two main findings.

First, circulating leukocyte subsets showed distinct adipokine receptor expression profiles, which may partly explain the differential impact of adipokines on leukocyte subsets. Whereas enhanced AdipoR1/R2 expression on the myeloid cell lineage has for example been associated with anti-inflammatory (M2) macrophage polarization and suppression of foam cell formation (26,27), the decreased AdipoR1 expression on classical $CD14^{++}CD16^{-}$ monocytes may be involved in their inflammatory fate and pivotal role in the development of cardiovascular disease (28,29). Another example is the discrete subpopulation of Tregs expressing a combination of leptin receptor and adiponectin receptors (S2 Fig). Considering the role of peroxisome-proliferator-activated receptor γ (PPARγ) induced adiponectin signalling in adipose tissue differentiation of Tregs (30,31), and the pivotal role of leptin in T-cell differentiation (32–34), it is tempting to speculate that circulating leptin and adiponectin receptor positive Tregs represent recirculating adipose tissue Tregs. Taken together, our data provide an exciting starting point for future studies to the role of adipokine receptors in leukocyte differentiation and function.

Second, we observed largely similar leukocyte subset numbers and adipokine receptor expression profiles in obese children and controls. The high naive $CD10^{-}CD27^{-}$ B-cell numbers in obese children pre and post-lifestyle intervention appear to be an exception to that. Interestingly, our findings correspond with recent studies observing high naive B-cell numbers in obese adults (35,36). The high naïve B-cell numbers may be explained by leptin-induced B- cell hyperstimulation, which can impair B-cell function (35,36). Indeed, B-cell responses to vaccination can be impaired in obesity (35,37). With respect to the similar adipokine receptor expression profiles of obese children and lean controls, our findings suggest that in childhood obesity, adipokine signalling in circulating leukocytes is primarily modulated by circulating adipokine levels, instead of adipokine receptor expression.

Our study has a few limitations that have to be taken into account. Storage or freeze-thawing of the peripheral blood mononuclear cells may have neutralized differences in adipokine receptor expression between obese children and controls. Notably, differences in adipokine receptor expression between leukocyte subsets were preserved, which argues against significant storage or freeze-thawing effects. Next, while adipokine receptor expression in liver, skeletal muscle, adipose tissue and other tissues plays in important role in adipokine signalling as well, medical ethical reasons precluded tissue collection. Importantly, our observations in circulating leukocyte subsets do not extend to other tissues. Finally, our study may have been underpowered to identify subtle differences in adipokine receptor expression due to relatively small patient numbers. In obese adults, lymphocyte AdipoR1 and AdipoR2 mRNA expression was reduced compared to anorexic adults (38). Likewise, reduced monocyte AdipoR1 and AdipoR2 protein expression was observed in obese adults with coronary artery disease, compared to obese adults without cardiovascular disease (39). However, the results in these studies may have been distorted due to the fact that multiple testing corrections were not applied and nor differentiation was made between functionally distinct leukocyte subsets.

In conclusion, our results cannot exclude subtle differences in adipokine receptor expression, but suggest that adipokine signalling in circulating leukocytes in childhood obesity is primarily modulated by altered adipokine levels.

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Author Contributions

Conceptualization
Genoveva Keustermans, Laila B. van der Heijden, Berent Prakken, Wilco de Jager, Eric Kalkhoven, Henk S. Schipper.

Data curation
Genoveva Keustermans, Laila B. van der Heijden, Berlinda Boer, Rianne Schol- man, Roos Nuboer, Arieke J. Janse, Henk S. Schipper.

Formal analysis
Genoveva Keustermans, Laila B. van der Heijden, Berlinda Boer, Henk S. Schipper.

Funding acquisition
Gerard Pasterkamp.

Investigation
Genoveva Keustermans, Laila B. van der Heijden, Berlinda Boer, Rianne Scholman, Henk S. Schipper.

Methodology
Genoveva Keustermans, Laila B. van der Heijden, Rianne Scholman, Roos Nuboer, Wilco de Jager, Arieke J. Janse, Henk S. Schipper.

Resources
Berent Prakken, Eric Kalkhoven.

Software
Genoveva Keustermans, Laila B. van der Heijden, Rianne Scholman, Henk S. Schipper.

Supervision
Gerard Pasterkamp, Berent Prakken, Wilco de Jager, Eric Kalkhoven, Arieke J. Janse, Henk S. Schipper.

Visualization
Genoveva Keustermans, Laila B. van der Heijden.

Writing – original draft
Genoveva Keustermans, Laila B. van der Heijden, Henk S. Schipper.

Writing – review & editing
Genoveva Keustermans, Laila B. van der Heijden, Gerard Pasterkamp, Henk S. Schipper.

References

1. Twig G, Yaniv G, Levine H, Leiba A, Goldberger N, Derazne E, et al. *Body-Mass Index in 2.3 Mil- lion Adolescents and Cardiovascular Death in Adulthood.* N Engl J Med. 2016, Vol. 374, pp. 2430–2440.

2. Baker J.L, Olsen L.W, Sorensen T.I. *Childhood body-mass index and the risk of coronary heart disease in adulthood.* N Engl J Med. 2007, Vol. 357, pp. 2329–2337.

3. Lloyd L.J, Langley-Evans S.C, McMullen S. *Childhood obesity and risk of the adult metabolic syndrome: a systematic review*. Int J Obes (Lond). 2012, Vol. 36, pp.1–11.

4. Ouchi N, Parker J.L, Lugus J.J, Walsh K. *Adipokines in inflammation and metabolic disease.* Nat Rev Immunol. 2011, Vol. 11, pp. 85–97.

5. Freitas Lima L.C, Braga V.A, do Socorro de Franca Silva M, Cruz J.C, Sousa Santos S.H, de Oliveira Monteiro M.M, et al. *Adipokines, diabetes and atherosclerosis: an inflammatory association.* Front Physiol. 2015, Vol. 6, pp. 304.

6. Schipper H.S, Nuboer R, Prop S, van den Ham H.J, de Boer F.K, Kesmir C, et al. *Systemic inflammation in childhood obesity: circulating inflammatory mediators and activated CD14(++) monocytes.* Diabetologia. 2012, Vol. 55, pp. 2800–2810.

7. Bonomini M, Pandolfi A. Chem*erin in renal dysfunction and cardiovascular disease.* Vascul Pharmacol. 2016, Vol. 77, pp. 28–34.

8. Rourke J.L, Dranse H.J, Sinal C.J. *Towards an integrative approach to understanding the role of chemerin in human health and disease*. Obes Rev. 2013, Vol. 14, pp. 245–262.

9. Abella V, Scotece M, Conde J, Pino J, Gonzalez-Gay MA, Gomez-Reino J.J, et al*. Leptin in the interplay of inflammation, metabolism and immune system disorders*. Nat Rev Rheumatol. 2017, Vol. 13, pp. 100– 109.

10. Yamauchi T, Kadowaki T. *Adiponectin receptor as a key player in healthy longevity and obesity-related diseases.* Cell Metab. 2013, Vol. 17, pp. 185–196.

11. Ohashi K, Shibata R, Murohara T, Ouchi N. *Role of anti-inflammatory adipokines in obesity- related diseases.* Trends Endocrinol Metab. 2014, Vol. 25, pp. 348–355.

12. Yamauchi T, Iwabu M, Okada-Iwabu M, Kadowaki T. *Adiponectin receptors: a review of their structure, function and how they work.* Best Pract Res Clin Endocrinol Metab. 2014, Vol. 28, pp. 15–23.

13. Tian Z, Sun R, Wei H, Gao B. *Impaired natural killer (NK) cell activity in leptin receptor deficient mice: leptin as a critical regulator in NK cell development and activation*. Biochem Biophys Res Com- mun. 2002, Vol. 298, pp. 297–302.

14. Nave H, Mueller G, Siegmund B, Jacobs R, Stroh T, Schueler U, et al. *Resistance of Janus kinase-2 dependent leptin signaling in natural killer (NK) cells: a novel mechanism of NK cell dysfunction in diet-induced obesity.* Endocrinology. 2008, Vol. 149, pp. 3370–3378.

15. Procaccini C, De Rosa V, Galgani M, Carbone F, La Rocca C, Formisano L, et al. *Role of adipo- kines*

signaling in the modulation of T cells function. Front Immunol. 2013, Vol. 4, pp. 332.

16. Sanchez-Margalet V, Martin-Romero C, Santos-Alvarez J, Goberna R, Najib S, Gonzalez-Yanes C. *Role of leptin as an immunomodulator of blood mononuclear cells: mechanisms of action*. Clin Exp Immunol. 2003, Vol. 133, pp. 11–19.

17. Pang T.T, Narendran P. *The distribution of adiponectin receptors on human peripheral blood mononuclear cells.* Ann N Y Acad Sci. 2008, Vol. 1150, pp. 143–145.

18. Tsiotra P.C, Pappa V, Raptis S.A, Tsigos C. *Expression of the long and short leptin receptor iso-forms in peripheral blood mononuclear cells: implications for leptin's actions.* Metabolism. 2000, Vol. 49, pp. 1537– 1541.

19. Schonbeck Y, Talma H, van Dommelen P, Bakker B, Buitendijk SE, Hirasing RA, et al. *Increase in prevalence of overweight in Dutch children and adolescents: a comparison of nationwide growth studies in 1980, 1997 and 2009.* PLoS One. 2011, Vol. 6, pp. e27608.

20. Cole T.J, Bellizzi M.C, Flegal K.M, Dietz W.H. *Establishing a standard definition for child overweight and obesity worldwide: international survey*. BMJ. 2000, Vol. 320, pp. 1240–1243.

21. van Hoek E, Bouwman L.I, Koelen M.A, Lutt M.A, Feskens E.J, Janse A.J. *Development of a Dutch intervention for obese young children.* Health Promot Int. 2016.

22. *National High Blood Pressure Education Program Working Group on High Blood Pressure in C, Adoles- cents (2004) The fourth report on the diagnosis, evaluation, and treatment of high blood pressure in chil- dren and adolescents.* Pediatrics. Vol. 114, pp. 555–576.

23. Katz A, Nambi S.S, Mather K, Baron A.D, Follmann D.A, Sullivan G, et al. *Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans.* J Clin Endocrinol Metab. 2000, Vol. 85, pp. 2402–2410.

24. Schipper H.S, de Jager W, van Dijk M.E, Meerding J, Zelissen P.M, Adan R.A, et al. *A multiplex immunoassay for human adipokine profiling*. Clin Chem. 2010, Vol. 56, pp. 1320–1328.

25. Maecker H.T, McCoy J.P, Nussenblatt R. *Standardizing immunophenotyping for the Human Immunology Project.* Nat Rev Immunol. 2012, Vol. 12, pp. 191–200.

26. Tian L, Luo N, Zhu X, Chung B.H, Garvey W.T, Fu Y. *Adiponectin-AdipoR1/2-APPL1 signaling axis suppresses human foam cell formation: differential ability of AdipoR1 and AdipoR2 to regulate inflammatory cytokine responses.* Atherosclerosis. 2012, Vol. 221, pp. 66–75.

27. van Stijn C.M, Kim J, Lusis A.J, Barish G.D, Tangirala R.K. *Macrophage polarization phenotype reg-ulates adiponectin receptor expression and adiponectin anti-inflammatory response.* FASEB J. 2015, Vol. 29, pp. 636–649.

28. Berg K.E, Ljungcrantz I, Andersson L, Bryngelsson C, Hedblad B, Fredrikson G.N, et al. *Elevated CD14++CD16- monocytes predict cardiovascular events.* Circ Cardiovasc Genet. 2012, Vol. 5, pp. 122–131.

29. Weber C, Shantsila E, Hristov M, Caligiuri G, Guzik T, Heine G.H, et al. *Role and analysis of monocyte subsets in cardiovascular disease. Joint consensus document of the European Society of Cardiology (ESC) Working Groups "Atherosclerosis & Vascular Biology" and "Thrombosis".* Thromb

Haemost. 2016, Vol. 116, pp. 626–637.

30. Cipolletta D. *Adipose tissue-resident regulatory T cells: phenotypic specialization, functions and therapeutic potential.* Immunology. 2014, Vol. 142, pp. 517–525.

31. Cipolletta D, Feuerer M, Li A, Kamei N, Lee J, Shoelson S.E, et al. *PPAR-gamma is a major driver of the accumulation and phenotype of adipose tissue Treg cells*. Nature. 2012, Vol. 486, pp. 549–553.

32. Saucillo D.C, Gerriets V.A, Sheng J, Rathmell J.C, Maciver N.J. *Leptin metabolically licenses T cells for activation to link nutrition and immunity.* J Immunol. 2014, Vol. 192, pp. 136–144.

33. Reis B.S, Lee K, Fanok M.H, Mascaraque C, Amoury M, Cohn L.B, et al. *Leptin receptor signaling in T cells is required for Th17 differentiation.* J Immunol. 2015, Vol. 194, pp. 5253–5260.

34. Naylor C, Petri W.A Jr. *Leptin Regulation of Immune Responses*. Trends Mol Med. 2016, Vol. 22, pp. 88–98.

35. Frasca D, Ferracci F, Diaz A, Romero M, Lechner S, Blomberg B.B. *Obesity decreases B cell responses in young and elderly individuals.* Obesity (Silver Spring). 2016, Vol. 24, pp. 615–625.

36. Kosaraju R, Guesdon W, Crouch M.J, Teague H.L, Sullivan E.M, Karlsson E.A, et al. *B Cell Activity Is Impaired in Human and Mouse Obesity and Is Responsive to an Essential Fatty Acid upon Murine Influenza Infection*. J Immunol. 2017.

37. Fan W, Chen X.F, Shen C, Guo Z.R, Dong C. *Hepatitis B vaccine response in obesity: A meta-analysis.* Vaccine. 2016, Vol. 34, pp. 4835–4841.

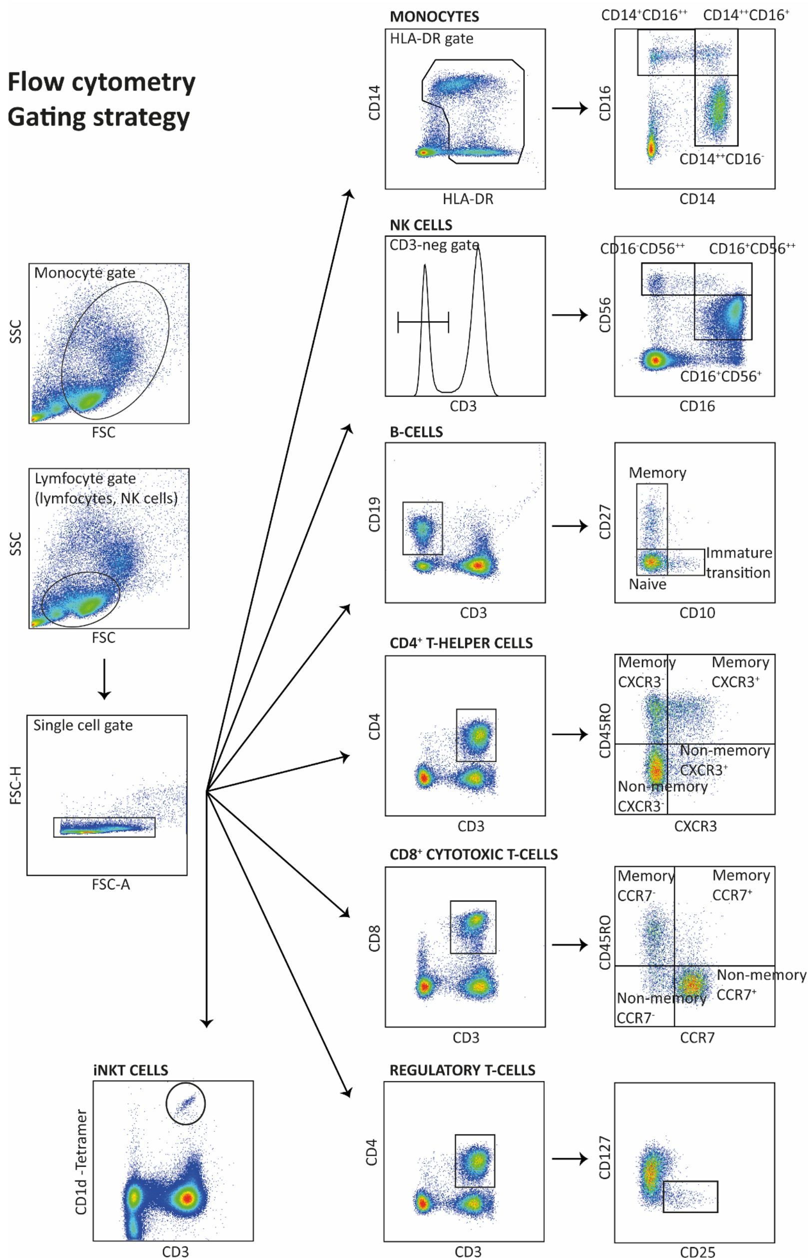
38. Alberti L, Gilardini L, Girola A, Moro M, Cavagnini F, Invitti C. *Adiponectin receptors gene expression in lymphocytes of obese and anorexic patients.* Diabetes Obes Metab. 2007, Vol. 9, pp. 344–349.

39. Kollias A, Tsiotra P.C, Ikonomidis I, Maratou E, Mitrou P, Kyriazi E, et al. *Adiponectin levels and expression of adiponectin receptors in isolated monocytes from overweight patients with coronary artery disease.* Cardiovasc Diabetol. 2011, Vol. 10, pp. 14.

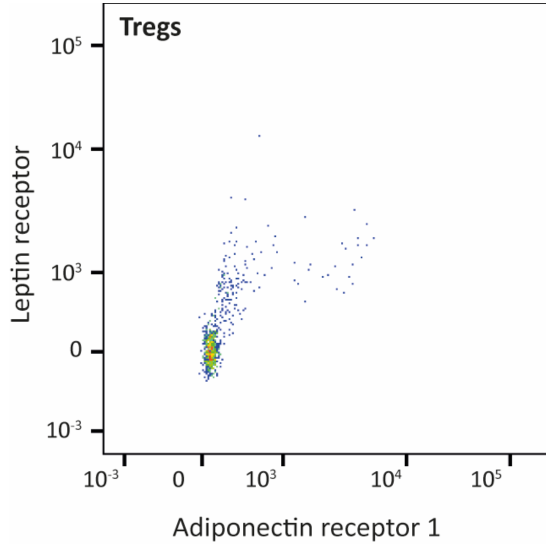
Supporting information

S1 Fig. Gating strategy of the leukocyte subsets.

First, forward scatter (FSC) and sideward scatter (SSC) profiles were used to roughly distinguish monocytes and lymphocytes/NK cells. Second, doublet cells were excluded using FSC-Area (FSC-A) and FSC-Height (FSC-H) gating. Finally, leukocyte subsets were gated on their marker expression.

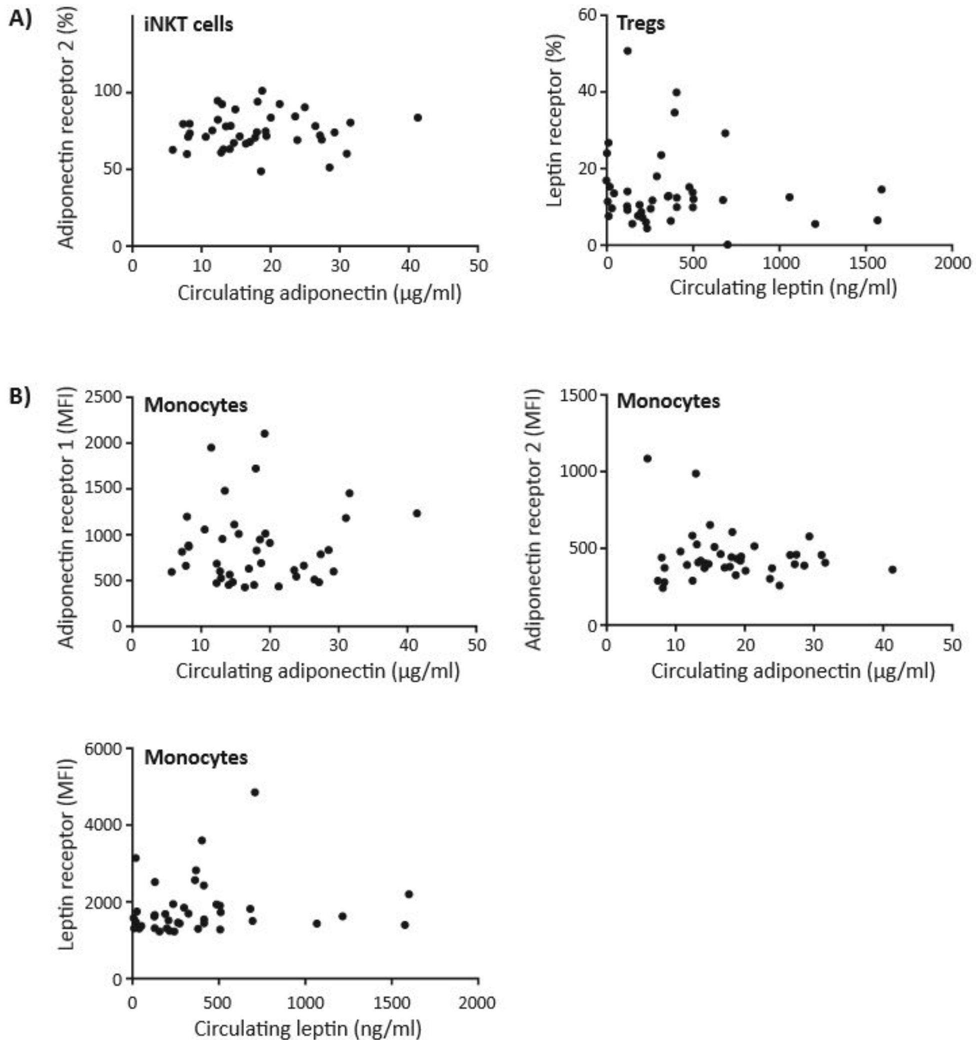


S2 Fig. Combined adiponectin receptor 1 and leptin receptor expression on regulatory T cells (Tregs). Focussing on the $CD25^{hi}CD127^{low}CD4^{+}$ T-cells (regulatory T-cells), the adipo- nectin receptor 1-positive subset also expresses the leptin receptor.



S3 Fig. Circulating adipokine levels and adipokine receptor expression.

*(*A) Circulating adiponectin levels versus percentage of adiponectin receptor 2 expressing iNKT cells (Pearson's correlation coefficient 0.048, p = 0.764), and circulating leptin levels versus percentage of leptin receptor expressing regulatory T-cells (Pearson's correlation coefficient -0.130, p = 0.416). (B) Circulating adiponectin levels versus median fluorescence intensity (MFI) of adiponectin receptor 1 and 2 expression (Pearson's correlation coefficient AdipoR1 0.052, p = 0.746; Adi- poR2–0.172, p = 0.284), and circulating leptin levels versus median fluorescence intensity (MFI) of leptin receptor expression (Pearson's correlation coefficient 0.126, p = 0.431).



S1 Table Adiponectin receptor 1 expression

	Lean controls	Obese-pre	Obese-post
Innate immunity			
Monocytes (total)	583 (465-769) *#	931 (582-1306) *	866 (656-1104) #
$CD14^{++}CD16^{-}$	405 (354-522)	403 (342-479)	433 (377-461)
$CD14^{++}CD16^{+}$	737 (606-1036)	935 (738-1164)	835 (745-1063)
$CD14^{+}CD16^{++}$	812 (549-1456) *	1317 (1014-2379) *	1232 (968-1885)
Natural Killer cells ($CD16^{+}CD56^{+}$)	335 (262-453)	399 (321-510)	406 (336-517)
$CD16^{+}CD56^{++}$	297 (261-396)	322 (280-366)	328 (293-390)
$CD16^{-}CD56^{++}$	225 (220-270)	236 (207-266)	215 (211-265)
Bridging immunity			
Natural Killer T cells	236 (221-395)	317 (239-333)	273 (241-366)
Adaptive immunity			
B cells			
Naive ($CD10^{-}CD27^{-}$)	249 (230-279)	242 (225-264)	236 (231-264)
Memory ($CD10^{-}CD27^{+}$)	228 (219-234) #	224 (218-232)	217 (215-225) #
Immature transition ($CD10^{+}CD27^{+}$)	224 (197-251) #	217 (202-240)	249 (224-496) #
$CD4^{+}$ T helper cells			
$CD45RO^{-}$ $CXCR3^{-}$	255 (242-348)	278 (251-381)	258 (245-304)
$CD45RO^{-}$ $CXCR3^{+}$	297 (285-383)	339 (320-457) $	308 (299-339) $
$CD45RO^{+}$ $CXCR3^{-}$	259 (241-332)	245 (234-370)	249 (239-314)
$CD45RO^{+}$ $CXCR3^{+}$	279 (263-347)	299 (262-382)	286 (262-315)
CD8+ cytotoxic T cells			
$CD45RO^{-}$ $CCR7^{-}$	230 (228-233) #	244 (225-251)	236 (228-252) #
$CD45RO^{-}$ $CCR7^{+}$	246 (230-306)	241 (224-356)	241 (231-231)
$CD45RO^{+}$ $CCR7^{-}$	225 (224-228)	229 (222-244)	229 (225-233)
$CD45RO^{+}$ $CCR7^{+}$	279 (273-365)	354 (255-426)	310 (275-375)
Regulatory T cells ($CD25^{+}CD127^{-}$)	282 (240-314)	248 (210-277)	257 (224-271)

Median Fluorescence Intensity (MFI) of adiponectin receptor 1 on leukocyte subsets of lean controls compared to obese children pre-lifestyle intervention (pre) and post-lifestyle intervention (post). Data are presented as median (interquartile range). * p<0.05 for lean controls compared to obese-pre. # p<0.05 for lean controls versus obese-post. $ p<0.05 for obese-pre compared to obese-post.

S2 Table **Adiponectin receptor 2 expression**

	Lean controls	Obese-pre	Obese-post
Innate immunity			
Monocytes (total)	460 (401-518)	410 (376-535)	392 (326-434)
$CD14^{++}CD16^{-}$	455 (394-515) #	405 (369-515)	378 (321-419) #
$CD14^{++}CD16^{+}$	641 (499-752)	507 (433-1100)	491 (414-648
$CD14^{+}CD16^{++}$	487 (417-553)	445 (381-561)	421 (372-482)
Natural Killer cells ($CD16^{+}CD56^{+}$)	370 (358-417)	351 (296-437)	352 (291-377)
$CD16^{+}CD56^{++}$	690 (617-858)	690 (579-753)	631 (548-726)
$CD16^{-}CD56^{++}$	752 (695-941)	705 (613-778)	668 (594-771)
Bridging immunity			
Natural Killer T cells	378 (376-602)	399 (354-418)	392 (353-419)
Adaptive immunity			
B cells			
Naive ($CD10^{-}CD27^{-}$)	306 (286-318) #	294 (281-310)	290 (268-299) #
Memory ($CD10^{-}CD27^{+}$)	308 (289-317)	301 (282-313)	305 (271-312)
Immature transition ($CD10^{+}CD27^{+}$)	444 (389-474)	454 (395-504)	423 (357-454)
$CD4^{+}$ T helper cells			
$CD45RO^{-}$ $CXCR3^{-}$	294 (282-345)	297 (273-346)	283 (278-324)
$CD45RO^{-}$ $CXCR3^{+}$	348 (328-409)	345 (314-467)	341 (308-365)
$CD45RO^{+}$ $CXCR3^{-}$	308 (293-347)	304 (280-360)	298 (282-356)
$CD45RO^{+}$ $CXCR3^{+}$	333 (305-448)	332 (292-397)	331 (291-391)
CD8+ cytotoxic T cells			
$CD45RO^{-}$ $CCR7^{-}$	293 (278-304)	291 (279 319)	291 (286-317,)
$CD45RO^{-}$ $CCR7^{+}$	331 (285-901)	321 (281-530	314 (290-698)
$CD45RO^{+}$ $CCR7^{-}$	290 (275-314)	295 (280 -318)	291 (286-308)
$CD45RO^{+}$ $CCR7^{+}$	535 (471-1086)	600 (393-769)	833 (505-964)
Regulatory T cells ($CD25^{+}CD127^{-}$)	297 (286-617)	297 (263-419)	320 (280-437)

Median Fluorescence Intensity (MFI) of adiponectin receptor 2 on leukocyte subsets of lean controls compared to obese children pre-lifestyle intervention (pre) and post-lifestyle intervention (post). Data are presented as median (interquartile range). * p<0.05 for lean controls compared to obese-pre. # p<0.05 for lean controls versus obese-post. $ p<0.05 for obese-pre compared to obese-post.

S3 Table Leptin receptor expression

	Lean controls	Obese-pre	Obese-post
Innate immunity			
Monocytes (total)	1451 (1317-1699)	1670 (1439-2326)	1705 (1454-1932)
$CD14^{++}CD16^{-}$	1467 (1302-1699)	1677 (1405-2451)	1532 (1393-1872)
$CD14^{++}CD16^{+}$	2236 (1715-2433)	2524 (1827-4133)	2227 (2008-2893)
$CD14^{+}CD16^{++}$	1188 (1141-1529)	1402 (1139-1999)	1467 (1467-1971)
Natural Killer cells ($CD16^{+}CD56^{+}$)	387 (370-416)	384 (362-426)	388 (367-416)
$CD16^{+}CD56^{++}$	380 (350-409)	365 (342-381)	374 (356-403)
$CD16^{-}CD56^{++}$	376 (356-403)	377 (365-401)	378 (350-424)
Bridging immunity			
Natural Killer T cells	483 (403-774)	577 (462-774) $	440 (415-603) $
Adaptive immunity			
B cells			
Naive ($CD10^{-}CD27^{-}$)	465 (445-487)	467 (449-479)	460 (434-480)
Memory ($CD10^{-}CD27^{+}$)	521 (502-546)	527 (492-560)	507 (481-523)
Immature transition ($CD10^{+}CD27^{+}$)	470 (447-500)	477 (451-529)	468 (449-516)
$CD4^{+}$ T helper cells			
$CD45RO^{-}$ $CXCR3^{-}$	417 (409-487)	410 (405-504)	419 (407-430)
$CD45RO^{-}$ $CXCR3^{+}$	597 (562-737)	646 (562-846)	582 (571-648)
$CD45RO^{+}$ $CXCR3^{-}$	421 (406-441)	426 (412-529)	424 (409-451)
$CD45RO^{+}$ $CXCR3^{+}$	504 (444-727)	527 (437-632)	577 (502-607)
CD8+ cytotoxic T cells			
$CD45RO^{-}$ $CCR7^{-}$	410 (398-424)	402 (388 -464)	414 (403-445)
$CD45RO^{-}$ $CCR7^{+}$	463 (424-624)	457 (415-691)	443 (432-537)
$CD45RO^{+}$ $CCR7^{-}$	412 (395-470)	429 (400-481)	436 (417-464)
$CD45RO^{+}$ $CCR7^{+}$	676 (583-753)	886 (545-1063)	737 (631-1006)
Regulatory T cells ($CD25^{+}CD127^{-}$)	410 (401-494)	426 (410-489)	451 (429 -566)

Median Fluorescence Intensity (MFI) of leptin receptor on leukocyte subsets of lean controls compared to obese children pre-lifestyle intervention (pre) and post-lifestyle intervention (post). Data are presented as median (interquartile range). * $p<0.05$ for lean controls compared to obese-pre. # $p<0.05$ for lean controls versus obese-post. $ $p<0.05$ for obese-pre compared to obese-post.

Chapter 5

Monocyte gene expression in childhood obesity is associated with obesity and complexity of atherosclerosis in adults

G. C. Keustermans[1*], D. Kofink[2*], A. Eikendal[3,4], W. de Jager [1], J. Meerding[1], R. Nuboer[5], J. Waltenberger[6], A. O. Kraaijeveld[2], J. W. Jukema[7], J. W. Sels[8], J. Garssen[9,10], B. J. Prakken[1,11], F. W. Asselbergs [2,12,13], E. Kalkhoven[14], I. E. Hoefer[3], G. Pasterkamp[3] & H. S. Schipper [1,11,15]

1 Laboratory for Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands. **2** Department of Cardiology, Division of Heart and Lungs, University Medical Center Utrecht, Utrecht, The Netherlands. **3** Department of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands. **4** Department of Internal medicine, Gastroenterology and Pulmonology, Red Cross Hospital, Beverwijk, The Netherlands. **5** Department of Pediatrics, Meander Medical Center, Amersfoort, The Netherlands. **6** Department of Cardiovascular Medicine, University Hospital Muenster, Muenster, Germany. **7** Department of Cardiology, Leiden University Medical Center, Leiden, The Netherlands. **8** Departments of Cardiology and Intensive Care, Maastricht University Medical Center, Maastricht, The Netherlands. **9** Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, The Netherlands. **10** Department of Immunology, Nutricia Research, Utrecht, The Netherlands. **11** Division of Pediatrics, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands. **12** Durrer Center for Cardiogenetic Research, ICIN- Netherlands Heart Institute, Utrecht, The Netherlands. **13** Institute of Cardiovascular Science, Faculty of Population Health Sciences, University College London, London, United Kingdom. **14** Molecular Cancer Research and Center for Molecular Medicine, University Medical Center Utrecht, Utrecht, The Netherlands. **15** Department of Pediatric Cardiology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands.

*Both authors contributed equally to this work

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ABSTRACT

Childhood obesity coincides with increased numbers of circulating classical CD14++CD16- and intermediate CD14++CD16+ monocytes. Monocytes are key players in the development and exacerbation of atherosclerosis, which prompts the question as to whether the monocytosis in childhood obesity contributes to atherogenesis over the years. Here, we dissected the monocyte gene expression profile in childhood obesity using an Illumina microarray platform on sorted monocytes of 35 obese children and 16 lean controls. Obese children displayed a distinctive monocyte gene expression profile compared to lean controls. Upon validation with quantitative PCR, we studied the association of the top 5 differentially regulated monocyte genes in childhood obesity with obesity and complexity of coronary atherosclerosis (SYNTAX score) in a cohort of 351 adults at risk for ischemic cardiovascular disease. The downregulation of monocyte IMPDH2 and TMEM134 in childhood obesity was also observed in obese adults. Moreover, downregulation of monocyte TMEM134 was associated with a higher SYNTAX atherosclerosis score in adults. In conclusion, childhood obesity entails monocyte gene expression alterations associated with obesity and enhanced complexity of coronary atherosclerosis in adults.

1. Introduction

The childhood obesity epidemic has alarming cardiovascular consequences, and thereby limits the worldwide increase in life expectancy (1,2). Obesity early in life may contribute to the development of cardiovascular disease in several ways. First, childhood obesity tends to result in adulthood obesity, which is an important risk factor for cardiovascular disease, especially when it concerns visceral adiposity (3,4). Second, childhood and adulthood obesity share independent risk factors for cardiovascular disease, such as a high blood pressure (5). Furthermore, obesity-induced insulin resistance and hyperglycaemia lead to defective insulin signalling in vascular wall lesional cells, which promotes atherosclerosis at the level of the arterial wall (6). Finally, obesity is associated with low-grade systemic inflammation, which promotes atherogenesis (7,8).

At a cellular level, monocytes appear to be a pivotal link between obesity and cardiovascular disease. Obesity is accompanied by leukocytosis, particularly of the myeloid lineage (7,9). Recent studies indicate that adipose tissue derived inflammatory factors such as IL-1β stimulate bone marrow myeloid progenitors, leading to monocytosis in obesity (10). Next to increased numbers, monocytes show an activated and inflammatory phenotype in obesity. In humans, monocytes fall into three phenotypical categories: classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$ and non-classical $CD14^{+}CD16^{++}$ monocytes (11). Previously, we have shown that childhood obesity is accompanied by increased numbers and an activated phenotype of the classical $CD14^{++}CD16^{-}$ monocyte subset (7). These monocytes are equivalent to $GR1^{+}Ly6c^{high}$ monocytes in mice, that differentiate into inflammatory macrophages and foam cells in various atherosclerosis models (12,13). The increased inflammatory monocyte numbers in childhood obesity may thus contribute to atherogenesis over the years.

The aim of this study was to obtain in-depth understanding of the monocyte gene expression profile in childhood obesity as compared to normal weight controls using micro-array analyses of sorted monocytes. Furthermore, monocyte gene expression profiles were compared with an established cohort of 351 adults at risk for ischemic cardiovascular disease, to study whether monocyte gene expression profiles in childhood obesity overlap with an atherogenic monocyte phenotype in adults. The adult cohort encompassed several clinical parameters, but we focused on the relation between monocyte gene expression and the SYNTAX atherosclerosis score because it is an established angiographic grading system for evaluating the complexity of coronary atherosclerotic lesions, widely used as a readout for atherosclerotic burden (14-18).

2. Methods

Paediatric cohort

Peripheral blood mononuclear cells (PBMC) were studied of 51 children aged 6–16 years (35 obese, 16 lean controls). The cells were derived from a previously published cross-sectional study at the Paediatric Outpatient Department of the Meander Medical Centre in Amersfoort, the Netherlands, consisting of 60 obese children and 30 age- and sex-matched lean controls (7). Because PBMC were available for 35 obese children and 16 lean controls, these children were included in the current study. Importantly, the availability of stored PBMC depended on the amount of blood a patient donated upon inclusion, which varied randomly. Therefore, we believe patients in the current study are a random selection of the previous study.

BMI-SD was calculated using the outcomes of the Fifth Dutch Growth Study (2008–2010). Childhood obesity was defined as BMI-SD >2.5, which can be extrapolated toward the international definition of obesity as BMI >30 kg/m2 for adults (30,31). Blood pressure was measured using an automated oscillometric method (Dinamap; GE Healthcare, Amersham, UK). Lipid profiles where obtained using standardized laboratory procedures. Written informed consent was obtained from all

children and their parents. The study was approved by the Institutional Medical Ethical Review Board of the University Medical Centre Utrecht, The Netherlands. All experiments with human biological materials were performed in accordance with the relevant guidelines and regulations.

Adult cohort

CTMM Circulating Cells is a multi-centre cohort of four Dutch medical centres that enrolled patients with stable or unstable angina pectoris undergoing coronary angiography, with the aim of identifying cellular biomarkers for the prediction of adverse cardiovascular events. Patients were recruited between March 2009 and September 2011. Details of the study design have been described elsewhere (32). All participants provided written informed consent. The study was approved by the Institutional Medical Ethical Review Board of the University Medical Centre Utrecht, The Netherlands. Data from 351 patients were included in the final analysis after removal of samples with outlying median intensity (Supplemental Table 8). Gene expression profiles were quantile-normalized followed by log2 transformation. The complexity of coronary atherosclerosis was assessed with coronary angiography using the SYNTAX score system. Two independent observers quantified SYNTAX scores, using SYNTAX score calculator version 2.11. e SYNTAX score is a tool for evaluating the complexity of coronary artery disease, taking into account the number of atherosclerotic lesions, their location and their functional impact (14). SYNTAX scores were available from 196 of the 351 patients.

Monocytes

In both cohorts, peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque density gradient centrifugation. In the paediatric cohort, flow cytometric phenotyping was performed in earlier studies (7). Subsequent to isolation, samples were stored in freeze medium (FCS with 10% DMSO, Sigma-Aldrich) until further use. In order to isolate monocytes, stored samples where thawed and washed in medium comprising of RPMI1640 supplemented with l-glutamate and 25 mM HEPES (Gibco), containing 2% FCS and penicillin/streptomycin (100 U/mL) (Invitrogen). Cells where spun down for 10 min, 1600 rpm at room temperature. PBMCs where then resuspended in MACS buffer - 2%FBS (Biowest), 2%EDTA (VWR chemicals) in PBS (Gibco) - and counted using the trypan blue exclusion method (Gibco). Anti-human CD14 magnetic particles where subsequently used to isolate monocytes using the company protocol (BD IMag). The CD14 positive cells were then re-suspended in 500ul of TRIZOL (Life Technologies) and stored at −80 °C.

Microarray and data processing

RNA was isolated from the trizol-lysed samples by AROS Applied Biotechnology. Samples of the 35 obese children and 16 healthy control children and the 351 adults underwent the same isolation procedures, and were similarly processed. In short, samples were labelled using the Illumina TotalPrep RNA Ampli cation Kit and 100 ng of total RNA. The IVT product was QC-checked on gel and quantitated using the NanoDrop (Thermo Scientifc). 750 ng of cDNA was used for the standard Illumina protocol before samples were hybridized on the arrays (Illumina humanHT-12 v3). Arrays were scanned using a Bead Array Reader (Illumina). After inspection of the sample median intensities, samples with a median intensity of <50 were removed. Subsequently, the expression data was quantile-normalised and log2 transformed using the lumi R package (33).

qPCR Validation

To validate the 67 differentially expressed genes, qPCR primers were designed for the top 20 hits. Of these primer pairs 17 functioned optimally and were deemed applicable for the validation process (Supplementary Table 6). High quality RNA of 27 obese children and 11 healthy controls was available for the qPCR validation studies. qPCR analysis was performed using SYBR Select Master Mix reagents

(Thermo Fischer Scientific) and run using the QuantStudio Flex system (Thermo Fischer Scientific). Data was normalized for housekeeping gene expression of GUSB, 36B4 and B2M, in accordance with international standards (34).

Statistics

First, demographic characteristics of the study population were presented as numbers and percentages for categorical variables and as means with standard deviation (SD) or medians with interquartile ranges (Q1, Q3) for normal and non-normally distributed continuous variables, respectively. Subsequently, monocyte gene expression profiles of lean and obese children were compared and significant differences between both groups were assessed using Mann Whitney U tests for continuous variables and X^2 test for binary variables.

Second, monocyte gene expression profiles were compared using the Limma package in R. In short, the Limma package uses empirical Baysian methods for the analysis of gene expression microarray data and is specifically designed for analysing smaller datasets (33). For this analysis, the genes functioned as outcome variables (dependent variables) and obesity status as determinant (independent variable). Age and sex were included in the model as covariates. In addition, to adjust for multiple testing, Benjamini Hochberg (BH) correction was applied. To illustrate the results of the microarray analysis, a heat map was generated using the heatmap.2 function in R. Hierarchical clustering was performed using complete linkage.

Third, linear regression was used to study the relation between obesity status and the gene expression (dependent variable) For this analysis, two models were constructed; a crude model (Model 1) and a model in which age and sex were included as covariates (Model 2) (Supplemental Table 2).
Fourth, the relation between the 5 qPCR validated genes and clinical variables was studied in the whole paediatric cohort (*n* = 51) as well as the obese subgroup (*n* = 35) and the adult cohort (*n* = 351) using linear regression analysis (Supplemental Tables 3 and 4). For the analysis performed in the whole paediatric cohort age, sex and BMI-SD were included as covariates whereas for the analysis in the obese subgroup age and sex were included as covariates. $P_{2\text{-sided}} < 0.05$ was considered statistically significant.

Data availability

The paediatric datasets generated and analysed during the current study are available from the corresponding author on reasonable request. Restrictions apply to data of the adult CTMM Circulating Cells dataset, which were used under license for the current study, and are not publicly available. Data are available from the authors upon reasonable request and after permission of the CTMM Circulating Cells consortium.

3. Results

Monocytes in childhood obesity show a distinctive gene expression profile.

Obese children exhibited typical clinical and biochemical characteristics with a significantly higher Body Mass Index standard deviation for age and sex (BMI-SD) compared to lean controls (3.4 versus 0.4, p < 0.001), a higher systolic blood pressure (BP) (123 mmHg, versus 110 mmHg, p = 0.005), lower Quantitative insulin sensitivity index (QUICKI) (0.3 versus 0.4, p < 0.001) and lower High-density lipoprotein (HDL) cholesterol level (1.2 mmol/L versus 1.5 mmol/L, p = 0.004) (Table 1). Furthermore, the obese subgroup showed a higher total monocyte number (0.6×10^9/ml versus 0.4×10^9/ml, p < 0.001), reflecting a higher classical $CD14^{++}CD16^{-}$ monocyte number (52.0×10^7/ml vs. 36.2×10^7/ml, p = 0.001) and a higher intermediate $CD14^{++}CD16^{+}$ monocyte number (4.6×10^7/ml versus 3.3 ×

10^7/ml, $p < 0.001$). Notably, the obese population showed a higher age compared to the lean controls (13.9 versus 10.5 years), and a lower percentage of boys (31% versus 44%). In order to avoid confounding, all subsequent analyses were corrected for age and sex.
Following microarray multiple testing correction, 67 genes were significantly and differently expressed between the obese and lean participants (Supplemental Tables 1 and 2). An unbiased clustering approach revealed a clear separation in monocyte gene expression profiles between lean and obese individuals (Fig. 1). The microarray data thus highlighted a distinctive monocyte gene expression profile in obese children.

Table 1 Characteristics of the pediatric study population

	Lean children (n=16)	**Obese children (n=35)**
Age (years)	10.5 (8.4, 12.9) *	13.9 (10.8, 14.9) *
Boys (number, %)	7 (44)	11 (31)
BMI-SD	0.4 (-0.7, 0.9) §	3.4 (3.1, 3.7) §
QUICKI	0.4 (0.3, 0.4) §	0.3 (0.3, 0.3) §
Systolic blood pressure (SBP, mmHg)	110 (98, 120) §	123 (113, 129) §
HDL-cholesterol (mmol/L)	1.5 (1.3, 1.7) §	1.2 (1.0, 1.4) §
LDL-cholesterol (mmol/L)	2.2 (2.2, 2.4)	2.3 (2.0, 3.0)
Triglycerides (mmol/L)	0.7 (0.5, 0.9)	0.9 (0.7, 1.3)
Total monocyte number (x 10^9)	0.4 (0.4, 0.5) §	0.6 (0.5, 0.7) §
$CD14^{++}CD16^{-}$ monocyte number (x 10^7)	36.2 (34.3, 43.2) §	52.0 (43.3, 64.7) §
$CD14^{++}CD16^{+}$ monocyte number (x 10^7)	1.8 (1.2, 2.3) §	4.6 (3.0, 7.0) §

Characteristics of the paediatric study population: Clinical characteristics and laboratory parameters for lean controls versus obese children. Data is shown as median (interquartile range). * $p<0.05$, § $p<0.01$. BMI-SD: standard deviation of body mass index corrected for age and sex, HDL: high-density lipoprotein, LDL: low-density lipoprotein, QUICKI: quantitative insulin sensitivity index.

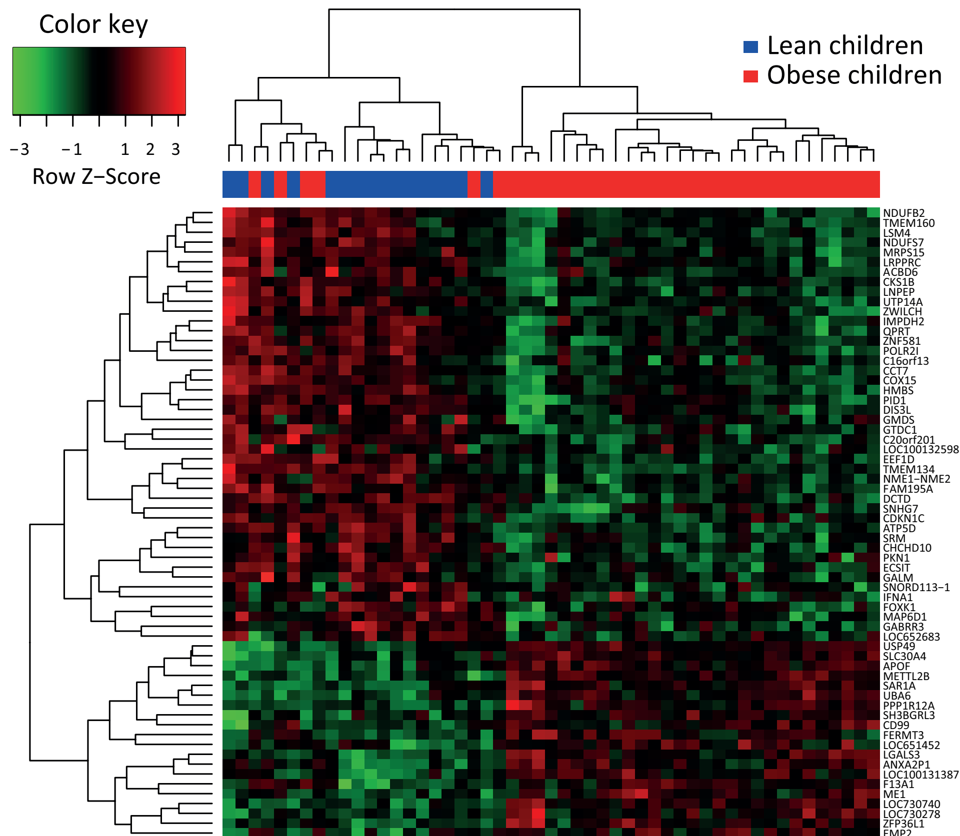

Figure 1: Heat map and cluster analysis of monocyte gene expression. e heat map depicts the gene cluster structure as a hierarchical tree with distinct branches and uses row z-score to depict data that deviates above or below the population mean.

Quantitative PCR validation.

Quantitative PCR (qPCR) was used to confirm the gene expression results, focusing on the top 20 microarray hits. qPCR analyses confirmed the observed downregulation of the monocyte genes Hydroxymethylbilane synthase (HMBS) ($p = 0.01$), Leucine Rich Pentatricopeptide Repeat Containing (LRPPRC) ($p = 0.005$), Transmembrane protein 134 (TMEM134) ($p = 0.028$) and Zwilch Kinetochore Protein (ZWILCH) ($p = 0.005$) in childhood obesity compared to lean controls (Fig. 2, Supplemental Table 7). Furthermore, Inosine Monophosphate Dehydrogenase 2 (IMPDH2) showed a trend towards downregulation in obese monocytes ($p = 0.06$). Because of its significant downregulation in monocytes from obese adults, IMPDH2 was included in subsequent analyses as well. Together, these 5 downregulated genes formed the starting point for subsequent studies. First, the association between the 5 downregulated monocyte genes and a selection of clinical variables in childhood was explored (Supplemental Tables 3 and 4). Second, the association with obesity and atherosclerosis was studied in an adult cohort at risk for ischemic cardiovascular disease.

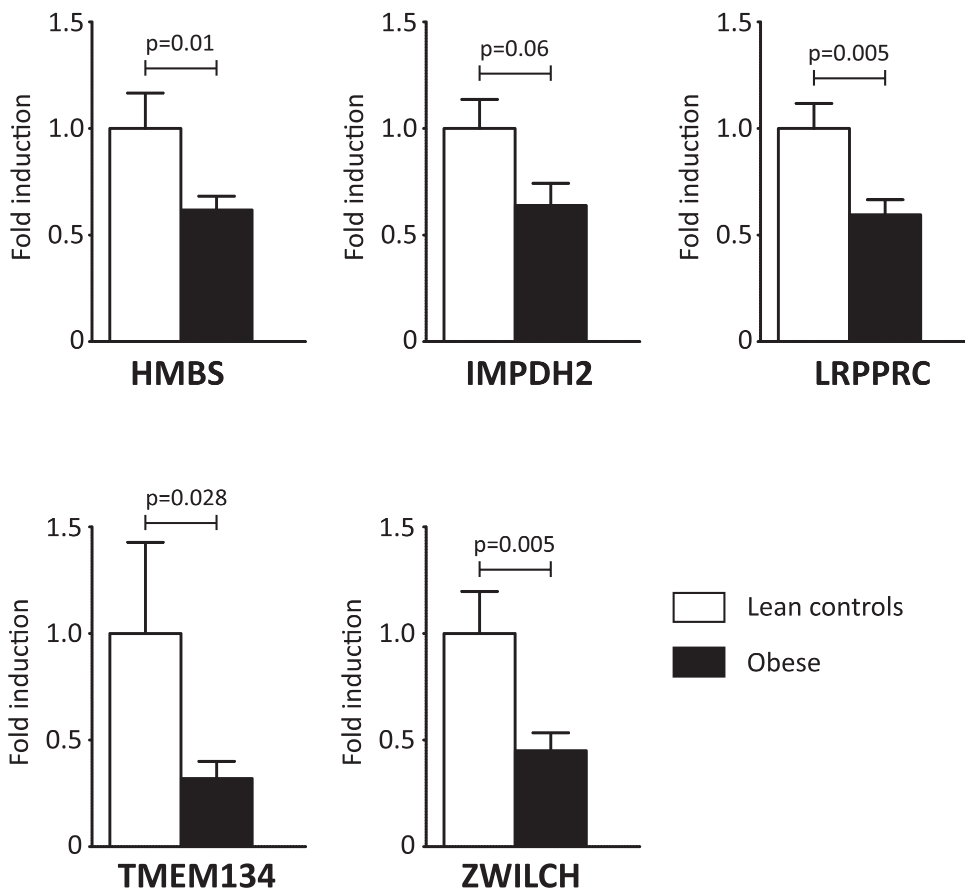

Figure 2: Quantitative PCR con rms downregulation of 5 monocyte genes in childhood obesity. Graphs show fold induction of the gene of interest, normalized for housekeeping gene expression. Error bars represent SEM.

Pathway analysis.

Focusing on the functional relevance of the observed gene expression profile, the functional enrichment of the differentially expressed genes in biological processes was assessed using ToppFun, a GO Term enrichment tool. Sixty-four out of 67 genes could be identified by ToppFun and were mapped to pathways involved in biological processes, using a minimum pathway size of 10 genes. These genes were particularly involved in oxidative phosphorylation and distinct metabolic processes (Supplemental Table 5, Supplemental Fig. 1).

Monocyte gene expression and adult obesity and cardiovascular risk.

To investigate whether adults at risk show a monocyte gene expression profile similar to obese children, the 5 validated monocyte genes were studied in a cohort of 351 adults at risk for ischemic cardiovascular disease. Clinical characteristics of the adult cohort are provided in Supplemental Table 8.

Downregulation of monocyte IMPDH2 ($\beta = -0.496$, $p = 0.004$) and TMEM134 ($\beta = -0.314$, $p = 0.043$) was associated with obesity in adults (BMI >30 kg/m^2), paralleling our findings in children. These

relationships remained significant after adjustment for age and sex (Table 2). Next, we tested whether monocyte gene expression was associated with the established SYNTAX (Synergy between percutaneous coronary intervention with Taxus and Cardiac Surgery) coronary atherosclerosis score. The SYNTAX score uses coronary angiography findings to quantify the complexity of coronary atherosclerosis, based on the number of atherosclerotic lesions, their location and their functional impact (14). The Syntax score was originally developed to help clinicians select the most appropriate revascularization strategy, but is increasingly being used as a risk stratification tool for adverse ischemic events (14,19). The SYNTAX score was available for 196 of the 351 adults. Monocyte TMEM134 downregulation was associated with higher SYNTAX scores ($\beta = -0.247$, $p = 0.041$) (Table 3). While adjustment of age and sex did not alter this association ($\beta = -0.251$, $p = 0.033$), addition of BMI to the model attenuated the association between TMEM134 expression and SYNTAX scores. In summary, monocyte TMEM134 downregulation is observed in childhood obesity, associated with obesity in adults at risk, and correlates with a higher SYNTAX score in adults at risk in an obesity-dependent fashion.

Table 2 Monocyte gene expression and obesity in the adult cohort

			Model 1	***Model 2***
Gene ID	**Transcript**	**Array ID**	**β (95% CI)**	**β (95% CI)**
HMBS	ILMN_16358	6060278	-0.213 (-0.505, 0.078)	-0.233 (-0.541, 0.076)
HMBS	ILMN_16358	7320021	-0.194 (-0.496, 0.108)	-0.227 (-0.549, 0.095)
IMPDH2	ILMN_3439	4590026	-0.424 (-0.753, -0.095)*	-0.496 (-0.837, -0.156)§
LRPPRC	ILMN_23753	6380064	-0.007 (-0.287, 0.272)	-0.013 (-0.304, 0.277)
TMEM134	ILMN_176754	5690711	-0.061 (-0.318, 0.195)	-0.068 (-0.340, 0.203)
TMEM134	ILMN_183533	670671	-0.311 (-0.610, -0.011)*	-0.314 (-0.620, -0.009)*
ZWILCH	ILMN_166966	7000743	-0.167 (-0.467, 0.133)	-0.168 (-0.485, 0.148)
ZWILCH	ILMN_3475	4850221	-0.247 (-0.556, 0.062)	-0.260 (-0.581, 0.062)

Monocyte gene expression and obesity in the adult cohort : The association of the 5 monocyte genes with obesity in the adult cohort was studied using a logistic regression model. Adult obesity was defined as a BMI >30kg/m^2, according to international standards and compared to a normal weight BMI <25kg/m^2. Logistic regression coefficients β with 95% confidence intervals (95% CI) for the monocyte genes are shown, both unadjusted (model 1) and adjusted for age and sex (model 2). *p<0.05, § p<0.01.

Table 3 Monocyte gene expression and SYNTAX score in the adult cohort

			Model 1	***Model 2***	***Model 3***
Gene ID	**Transcript**	**Array ID**	**β (95% CI)**	**β (95% CI)**	**β (95% CI)**
HMBS	ILMN_16358	6060278	-0.005 (-0.243, 0.232)	0.002 (-0.232, 0.237)	-0.013 (-0.252, 0.227)
HMBS	ILMN_16358	7320021	-0.043 (-0.281, 0.195)	-0.013 (-0.247, 0.221)	-0.030 (-0.268, 0.208)
IMPDH2	ILMN_3439	4590026	-0.133 (-0.370, 0.104)	-0.116 (-0.347, 0.115)	-0.090 (-0.331, 0.150)
LRPPRC	ILMN_23753	6380064	-0.187 (-0.424, 0.049)	-0.188 (-0.419, 0.043)	-0.201 (-0.437, 0.036)
TMEM134	ILMN_176754	5690711	-0.222 (-0.458, 0.014)	-0.211 (-0.443, 0.021)	-0.193 (-0.43, 0.044)
TMEM134	ILMN_183533	670671	-0.247 (-0.483, -0.012)*	-0.251 (-0.481, -0.022)*	-0.227 (-0.464, 0.011)
ZWILCH	ILMN_166966	7000743	-0.102 (-0.339, 0.136)	-0.171 (-0.404, 0.062)	-0.168 (-0.408, 0.071)
ZWILCH	ILMN_3475	4850221	0.030 (-0.207, 0.268)	0.010 (-0.223, 0.242)	0.048 (-0.197, 0.293)

Monocyte gene expression and SYNTAX score in the adult cohort: The association of the 5 validated genes with severity of coronary artery atherosclerosis (SYNTAX score, square-root transformed) was studied using linear regression. Linear regression coefficients β with 95% confidence intervals (95% CI) for the monocyte genes are shown. Model 1: unadjusted, model 2: adjusted for age and sex, model 3: adjusted for age, sex and BMI. *p<0.05.

4. Discussion

Monocytes are key players in the development and exacerbation of atherosclerosis, both via their role as macrophage foam cell precursors and their role in systemic inflammation (20). In human studies, increased numbers of classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocytes predict cardiovascular events independent of age, sex and classical cardiovascular risk factors (21,22). Interestingly, childhood obesity also coincides with increased circulating numbers of classical and intermediate monocytes (7), which prompts the question as to whether the monocytosis in childhood obesity contributes to atherogenesis over the years. The atherogenic role of monocytes in childhood obesity is difficult to study in human models, since longitudinal data are lacking. To the best of our knowledge, this is the first monocyte gene expression study in childhood obesity, and the first endeavour to crosscheck gene expression profiles in adults at risk for ischemic cardiovascular disease. Our study showed a distinctive monocyte gene expression profile in childhood obesity, and downregulation of monocyte IMPDH2 and TMEM134 was also associated with obesity in the adult cohort at risk. Finally, downregulated TMEM134 coincided with a higher SYNTAX score in adults at risk, reflecting an enhanced atherosclerotic burden (14,19).

Our results stress the relevance of the monocytosis in childhood obesity and raise several interesting questions. First, pathway analysis of differentially regulated monocyte genes in childhood obesity revealed an over-representation of oxidative phosphorylation, oxidative stress, and intracellular metabolism pathways, which apparently reflects reprogramming to aerobic glycolysis. While resting immune cells primarily need ATP to meet cellular demands, and use glucose-pyruvate conversion (glycolysis) and oxidative phosphorylation to fulfil these needs, many immune cells in inflammatory microenvironments undergo metabolic reprogramming to aerobic glycolysis in order to engage in cellular growth and proliferation (23). Interestingly, an upregulation of aerobic glycolysis also coincides with the development of 'trained immunity' (24). Upon repetitive stimulation with microbial moieties and/or metabolites, monocytes undergo epigenetic reprogramming towards aerobic glycolysis, which enhances the response of the trained monocytes in case of re-stimulation (23,25). Recent literature suggests that the development of trained immunity contributes to the development of systemic inflammation and atherosclerosis, and represents an intriguing target for therapeutic intervention (25).

Second, the role of TMEM134 in monocytes gains traction. In human monocyte studies, the expression of TMEM134 was decreased in classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocytes, in contrast to non-classical $CD14^{+}CD16^{++}$ monocytes (26). Hence TMEM134 downregulation in childhood obesity and adults with cardiovascular risk may reflect obesity-induced $CD14^{++}CD16^{-}$ and $CD14^{++}CD16^{+}$ monocytosis. Whether the highly conserved 21.5 kDa transmembrane protein TMEM134 plays an active role in monocyte differentiation remains to be elucidated. Notably, the existing studies indicate that TMEM134 affects the prototypical inflammatory nuclear factor-κB (NF-κB) signalling pathway. TMEM 134 was identified as a binding protein of latent membrane protein 1 (LMP1) and Hepatitis E Virus Open Reading Frame 2 (ORF2), and affected downstream NF-κB signalling via these binding partners (27,28). Importantly, modulation of downstream NF-κB signalling is considered one of the hallmarks of innate immune programming in chronic inflammation (29). Therefore, it is tempting to speculate that the observed downregulation of TMEM134 in childhood obesity monocytes is connected to the development of trained immunity, as discussed previously.

Finally, limitations of the current study have to be taken into account. Since our paediatric study population was relatively small, the associations reported in our study are of subtle strength. Second, environmental factors such as freeze-thawing of the monocytes may have influenced gene expression profiles. Though paediatric and adult samples were treated similarly, minor processing differences could impact gene expression profiles. Third, we chose to focus on the 5 qPCR-validated monocyte genes. Thereby, we may have disregarded important monocyte genes that were not included in the qPCR validation. Finally, CD14-positive magnetic bead sorting skewed the analysed

monocyte compartments towards classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocytes (Supplemental Fig. 2), and partly disregarded the non-classical $CD14^{+}CD16^{++}$ monocyte subset, which is considered less important for atherosclerosis development (21,22).

In conclusion, childhood obesity entails monocyte gene expression alterations associated with obesity and enhanced complexity of coronary atherosclerosis in adults. Especially the role of TMEM134 in monocytes gains traction, as downregulation of monocyte TMEM134 was associated with obesity in children and adults, and coincided with a higher SYNTAX atherosclerosis score in adults at risk for ischemic cardiovascular disease.

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Author Contributions

B.J.P., G.P., E.K., J.G. and H.S.S. designed the study. R.N., J.W., A.O.K., J.W.J. and J.W.S. recruited participants. G.C.K. and J.M. did the experimental work. G.C.K., D.K., A.E. and W.d.J. collected data and participated in the analysis of the data, supervised by H.S.S. D.K. and A.E. performed statistical analyses. G.C.K., D.K., A.E. and H.S.S. drafted the manuscript, and all authors revised it critically for intellectual content. All authors have approved the final version.

Additional Information

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Competing Interests
J. Garssen is employed at Nutricia Research, The Netherlands. The other authors declare no competing financial interests.

References

1. Twig, G. *et al*. *Body-Mass Index in 2.3 Million Adolescents and Cardiovascular Death in Adulthood.* The New Engl J of Med. 2016, Vol. 374, pp. 2430–2440.

2. Baker J. L., Olsen L. W, Sorensen, T. I. *Childhood body-mass index and the risk of coronary heart disease in adulthood.* The New Engl J of Med. 2007, Vol. 357, pp. 2329–2337.

3. Arner, P. *et al*. *Dynamics of human adipose lipid turnover in health and metabolic disease*. Nature. 2011, Vol. 478, pp. 110–113.

4. Long M. T, Fox, C. S. *The Framingham Heart Study–67 years of discovery in metabolic disease.* Nat Rev Endocrinol. 2016, Vol. 12, pp. 177–183.

5. Hall J. E, do Carmo J. M, da Silva A. A, Wang Z, Hall M. E. *Obesity-induced hypertension: interaction of neurohumoral and renal mechanisms.* Circulation research. 2015, Vol. 116, pp. 991–1006.

6. Bornfeldt K. E, Tabas I. *Insulin resistance, hyperglycemia, and atherosclerosis.* Cell metabolism. 2011, Vol. 14, pp. 575–585.

7. Schipper, H. S. *et al*. *Systemic inflammation in childhood obesity: circulating inflammatory mediators and activated CD14(++) monocytes.* Diabetologia. 2012, Vol. 55, pp. 2800–2810.

8. Ouchi N, Parker J. L, Lugus J. J, Walsh K. *Adipokines in inflammation and metabolic disease*. Nat Rev Immunol. 2011, Vol. 11, pp. 85–97.

9. Schmidt M. I. *et al*. *Markers of inflammation and prediction of diabetes mellitus in adults (Atherosclerosis Risk in Communities study): a cohort study.* Lancet. 1999, Vol. 353, pp. 1649–1652.

10. Nagareddy P. R. *et al*. *Adipose tissue macrophages promote myelopoiesis and monocytosis in obesity.* Cell metabolism. 2014, Vol. 19, pp. 821–835.

11. Ziegler-Heitbrock L. *Monocyte subsets in man and other species.* Cellular immunology. 2014, Vol. 289, pp. 135–139.

12. Woollard K. J, Geissmann, F. *Monocytes in atherosclerosis: subsets and functions*. Nat Rev Cardiol. 2010, Vol. 7, pp. 77–86.

13. Cros J. *et al*. *Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors.* Immunity. 2010, Vol. 33, pp. 375–386.

14. Sianos G. *et al*. *e SYNTAX Score: an angiographic tool grading the complexity of coronary artery disease.* EuroIntervention. 2005, Vol. 1, pp. 219–227.

15. Karadeniz M. *et al*. *High Sensitive CRP Level Is Associated With Intermediate and High Syntax Score in Patients With Acute Coronary Syndrome*. Int Heart J. 2015, Vol. 56, pp. 377–380.

16. Isik T. *et al*. *Relation of red cell distribution width with the presence, severity, and complexity of coronary artery disease*. Coron Artery Dis. 2012, Vol. 23, pp. 51–56.

17. Altun B. *et al*. *The relationship between high-sensitive troponin T, neutrophil lymphocyte ratio and SYNTAX Score*. Scand J Clin Lab Invest. 2014, Vol. 74, pp. 108–115.

18. Gijsberts C. M. *et al*. *Routinely analyzed leukocyte characteristics improve prediction of mortality after coronary angiography*. Eur J Prev Cardiol. 2016, Vol. 23, pp. 1211–1220.

19. Yadav, M. *et al*. *Prediction of coronary risk by SYNTAX and derived scores: synergy between percutaneous coronary intervention with taxus and cardiac surgery.* J American College of Cardiol. 2013, Vol. 62, pp. 1219–1230.

20. Hilgendorf I, Swirski F. K, Robbins C. S. *Monocyte fate in atherosclerosis. Artherioscl thromb vasc boil.* 2015, Vol. 35, pp. 272–27.

21. Rogacev K. S. *et al*. *CD14++ CD16+ monocytes and cardiovascular outcome in patients with chronic kidney disease*. Euro heart J. 2011, Vol. 32, pp. 84–92.

22. Berg K. E. *et al*. *Elevated CD14++ CD16− monocytes predict cardiovascular events*. Circ Cardiovasc Gene. 2012, Vol. 5, pp. 122–131.

23. Loftus R. M, Finlay D. K. *Immunometabolism: Cellular Metabolism Turns Immune Regulator*. The J Biol Chem. 2016, Vol. 291, pp. 1–10.

24. Netea M. G, Quintin J., van der Meer J. W. *Trained immunity: a memory for innate host defense*. Cell host & microbe**.** 2011, Vol. 9, pp. 355–361.

25. Norata G. D. *et al*. *The Cellular and Molecular Basis of Translational Immunometabolism*. Immunity 2015, Vol. 43, pp. 421–434.

26. Schmidl C. *et al*. *Transcription and enhancer pro ling in human monocyte subsets*. Blood**.** 2015, Vol. 123, pp. e90–99.

27. Talaty P, Emery A, Holthusen K, Everly D. N. Jr. *Identification of transmembrane protein 134 as a novel LMP1-binding protein by using bimolecular fluorescence complementation and an enhanced retroviral mutagen.* Journal of virology. 2012, Vol. 86, pp. 11345–11355.

28. Tian Y. *et al*. *Systematic identification of hepatitis E virus ORF2 interactome reveals that TMEM134 engages in ORF2-mediated NF-kappaB pathway.* Virus Res. 2016.

29. Morris M. C, Gilliam E. A, Li L. *Innate immune programing by endotoxin and its pathological consequences.* Frontiers in immunology. 2014, Vol. 5, pp. 680.

30. Schonbeck Y. *et al*. *Increase in prevalence of overweight in Dutch children and adolescents: a comparison of nationwide growth studies in 1980, 1997 and 2009*. PLoS One**.** 2011, Vol. 6, pp. e27608.

31. Cole T. J, Bellizzi M. C, Flegal K. M, Dietz W. H. *Establishing a standard de nition for child overweight and obesity worldwide: international survey*. BMJ. 2000, Vol. 320, pp. 1240–1243.

32. Hoefer I. E. *et al*. *Circulating cells as predictors of secondary manifestations of cardiovascular disease: design of the CIRCULATING CELLS study*. Clin Res Cardiol**.** 2013, Vol. 102, pp. 847–856.

33. Du P, Kibbe W. A, Lin S. M. *lumi: a pipeline for processing Illumina microarray.* Bioinformatics. 2008, Vol. 24, pp. 1547–1548.

34. Vandesompele J. *et al*. *Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.* Genome Biol. 2002, Vol. 3, pp. RESEARCH0034.

Supporting information

Supplemental table 1 Significantly different genes between obese and lean children in total study population (n = 51)

	Log Fold change	Crude p-value	FDR-adjusted p-value	Beta
ACBD6	-0.16	<0.0001	0.03	3.35
ANXA2P1	0.25	<0.0001	0.04	1.75
APOF	0.23	<0.0001	0.03	2.07
ATP5D	-0.16	<0.0001	0.03	2.31
C16orf13	-0.17	<0.0001	0.05	1.33
C20orf201	-0.15	<0.0001	0.04	1.98
CCT7	-0.29	<0.0001	0.03	2.47
CD99	0.29	<0.0001	0.04	1.85
CDKN1C	-0.41	<0.0001	0.05	1.32
CHCHD10	-0.17	<0.0001	0.04	1.86
CKS1B	-0.30	<0.0001	0.03	2.46
COX15	-0.32	<0.0001	0.04	1.57
DCTD	-0.14	<0.0001	0.03	2.88
DIS3L	-0.32	<0.0001	0.04	1.92
ECSIT	-0.21	<0.0001	0.03	3.69
EEF1D	-0.17	<0.0001	0.03	3.04
EMP2	0.14	<0.0001	0.03	2.36
F13A1	0.62	<0.0001	0.03	3.70
FAM195A	-0.35	<0.0001	0.03	2.42
FERMT3	0.11	<0.0001	0.03	3.29
FOXK1	-0.19	<0.0001	0.04	1.89
GABRR3	-0.09	<0.0001	0.03	2.57
GALM	-0.17	<0.0001	0.04	1.47
GMDS	-0.20	<0.0001	0.04	1.57
GTDC1	-0.10	<0.0001	0.04	1.73
HMBS	-0.27	<0.0001	0.03	2.76
IFNA1	-0.09	<0.0001	0.03	2.07
IMPDH2	-0.28	<0.0001	0.02	4.98
LGALS3	0.31	<0.0001	0.03	2.80
LNPEP	-0.12	0.0001	0.05	1.22
LOC100130604	0.36	<0.0001	0.03	2.18
LOC100131387	0.16	<0.0001	0.03	2.19
LOC100132598	-0.08	0.0001	0.05	1.19
LOC651452	0.07	<0.0001	0.03	2.38
LOC652683	-0.10	<0.0001	0.03	2.10
LOC653778	0.40	<0.0001	0.04	1.84
LOC654121	0.20	<0.0001	0.03	2.65
LOC730278	0.31	<0.0001	0.03	2.51
LOC730740	0.16	<0.0001	0.03	3.24
LRPPRC	-0.21	<0.0001	0.03	3.15
LSM4	-0.23	0.0001	0.05	1.20

MAP6D1	-0.33	<0.0001	0.03	2.78
ME1	0.27	<0.0001	0.02	4.37
METTL2B	0.14	<0.0001	0.03	2.86
MRPS15	-0.16	<0.0001	0.05	1.30
NDUFB2	-0.22	<0.0001	0.04	1.63
NDUFS7	-0.23	<0.0001	0.05	1.28
NME1-NME2	-0.23	<0.0001	0.03	3.42
PID1	-0.44	<0.0001	0.03	2.07
PKN1	-0.15	<0.0001	0.03	2.07
POLR2I	-0.20	0.0001	0.05	1.22
PPP1R12A	0.25	<0.0001	0.04	1.48
QPRT	-0.32	<0.0001	0.00	8.56
SAR1A	0.32	<0.0001	0.04	1.75
SH3BGRL3	0.26	<0.0001	0.03	3.24
SLC30A4	0.19	<0.0001	0.03	2.48
SNHG7	-0.39	<0.0001	0.02	4.61
SNORD113-1	-0.08	<0.0001	0.04	1.73
SRM	-0.17	<0.0001	0.04	1.42
TMEM134	-0.19	<0.0001	0.02	4.54
TMEM160	-0.28	<0.0001	0.04	1.77
UBA6	0.36	<0.0001	0.04	1.53
USP49	0.55	<0.0001	0.04	1.56
UTP14A	-0.16	<0.0001	0.05	1.37
ZFP36L1	0.40	<0.0001	0.04	1.96
ZNF581	-0.17	<0.0001	0.04	1.51
ZWILCH	-0.12	<0.0001	0.03	2.62

Supplemental table 2 Association between genes and obesity status in total study population (n = 51)

	Model 1*† β (95% CI)	p value	Model 2*† β (95% CI)	p value
ACBD6	-0.16 (-0.22, -0.10)	<0.0001	-0.16 (-0.22, -0.09)	<0.0001
ANXA2P1	0.26 (0.16, 0.37)	<0.0001	0.25 (0.13, 0.36)	<0.0001
APOF	0.22 (0.12, 0.32)	<0.0001	0.23 (0.12, 0.33)	<0.0001
ATP5D	-0.17 (-0.23, -0.10)	<0.0001	-0.16 (-0.23, -0.09)	<0.0001
C16orf13	-0.15 (-0.23, -0.07)	0.0004	-0.17 (-0.26, -0.09)	<0.0001
C20orf201	-0.16 (-0.22, -0.09)	<0.0001	-0.15 (-0.22, -0.08)	0.0001
CCT7	-0.28 (-0.40, -0.16)	<0.0001	-0.29 (-0.42, -0.16)	<0.0001
CD99	0.26 (0.13, 0.39)	0.0002	0.29 (0.16, 0.43)	<0.0001
CDKN1C	-0.40 (-0.59, -0.22)	<0.0001	-0.41 (-0.61, -0.21)	0.0001
CHCHD10	-0.19 (-0.26, -0.11)	<0.0001	-0.17 (-0.25, -0.09)	<0.0001
CKS1B	-0.28 (-0.41, -0.16)	<0.0001	-0.30 (-0.43, -0.16)	<0.0001
COX15	-0.29 (-0.43, -0.15)	<0.0001	-0.32 (-0.47, -0.17)	0.0001
DCTD	-0.16 (-0.22, -0.11)	<0.0001	-0.14 (-0.20, -0.08)	<0.0001
DIS3L	-0.31 (-0.45, -0.18)	<0.0001	-0.32 (-0.46, -0.17)	<0.0001
ECSIT	-0.21 (-0.29, -0.13)	<0.0001	-0.21 (-0.30, -0.13)	<0.0001
EEF1D	-0.15 (-0.22, -0.08)	<0.0001	-0.17 (-0.24, -0.10)	<0.0001
EMP2	0.12 (0.06, 0.18)	0.0002	0.14 (0.08, 0.20)	<0.0001
F13A1	0.64 (0.41, 0.88)	<0.0001	0.62 (-0.37, 0.88)	<0.0001
FAM195A	-0.34 (-0.48, -0.19)	<0.0001	-0.35 (-0.51, -0.20)	<0.0001
FERMT3	0.11 (0.07, 0.15)	<0.0001	0.11 (0.07, 0.15)	<0.0001
FOXK1	-0.19 (-0.27, -0.11)	<0.0001	-0.19 (-0.28, -0.10)	<0.0001
GABRR3	-0.08 (-0.12, -0.05)	<0.0001	-0.09 (-0.13, -0.05)	<0.0001
GALM	-0.18 (-0.26, -0.11)	<0.0001	-0.17 (-0.26, -0.09)	0.0001
GMDS	-0.18 (-0.27, -0.09)	0.0001	-0.20 (-0.29, -0.10)	<0.0001
GTDC1	-0.10 (-0.14, -0.06)	<0.0001	-0.10 (-0.15, -0.06)	<0.0001
HMBS	-0.25 (-0.36, -0.15)	<0.0001	-0.27 (-0.38, -0.15)	<0.0001
IFNA1	-0.08 (-0.11, -0.04)	0.0003	-0.09 (-0.13, -0.05)	<0.0001
IMPDH2	-0.29 (-0.39, -0.19)	<0.0001	-0.28 (-0.39, -0.18)	<0.0001
LGALS3	0.35 (0.22, 0.48)	<0.0001	0.31 (0.18, 0.44)	<0.0001
LNPEP	-0.13 (-0.18, -0.07)	<0.0001	-0.12 (-0.18, -0.06)	0.0001
LOC100130604	0.38 (0.23, 0.53)	<0.0001	0.36 (0.20, 0.53)	<0.0001
LOC100131387	0.17 (0.11, 0.24)	<0.0001	0.16 (0.09, 0.23)	<0.0001
LOC100132598	-0.08 (-0.11, -0.04)	<0.0001	-0.08 (-0.12, -0.04)	<0.0001
LOC651452	0.06 (0.04, 0.09)	<0.0001	0.07 (0.04, 0.10)	<0.0001
LOC652683	-0.09 (-0.13, -0.05)	<0.0001	-0.10 (-0.14, -0.05)	<0.0001
LOC653778	0.39 (0.21, 0.56)	<0.0001	0.40 (0.21, 0.59)	<0.0001
LOC654121	0.20 (0.13, 0.28)	<0.0001	0.20 (-0.11, 0.28)	<0.0001
LOC730278	0.32 (0.19, 0.45)	<0.0001	0.31 (0.18, 0.45)	<0.0001
LOC730740	0.17 (0.11, 0.23)	<0.0001	0.16 (0.09, 0.23)	<0.0001
LRPPRC	-0.21 (-0.30, -0.13)	<0.0001	-0.21 (-0.30, -0.12)	<0.0001
LSM4	-0.22 (-0.33, -0.12)	<0.0001	-0.23 (-0.34, -0.12)	0.0001
MAP6D1	-0.36 (-0.49, -0.23)	<0.0001	-0.33 (-0.48, -0.19)	<0.0001
ME1	0.27 (0.17, 0.38)	<0.0001	0.27 (0.17, 0.38)	<0.0001

METTL2B	0.13 (0.08, 0.19)	<0.0001	0.14 (0.08, 0.21)	<0.0001
MRPS15	-0.17 (-0.24, -0.10)	<0.0001	-0.16 (-0.24, -0.08)	0.0001
NDUFB2	-0.22 (-0.32, -0.13)	<0.0001	-0.22 (-0.33, -0.12)	<0.0001
NDUFS7	-0.24 (-0.34, -0.14)	<0.0001	-0.23 (-0.34, -0.12)	0.0001
NME1-NME2	-0.23 (-0.31, -0.14)	<0.0001	-0.23 (-0.32, -0.13)	<0.0001
PID1	-0.43 (-0.62, -0.25)	<0.0001	-0.44 (-0.65, -0.24)	<0.0001
PKN1	-0.14 (-0.20, -0.08)	<0.0001	-0.15 (-0.22, -0.08)	<0.0001
POLR2I	-0.21 (-0.30, -0.12)	<0.0001	-0.20 (-0.30, -0.11)	0.0001
PPP1R12A	0.27 (0.16, 0.38)	<0.0001	0.25 (0.13, 0.37)	0.0001
QPRT	-0.31 (-0.40, -0.22)	<0.0001	-0.32 (-0.42, -0.22)	<0.0001
SAR1A	0.33 (0.19, 0.46)	<0.0001	0.32 (0.17, 0.47)	<0.0001
SH3BGRL3	0.25 (0.14, 0.36)	<0.0001	0.26 (0.15, 0.38)	<0.0001
SLC30A4	0.16 (0.09, 0.24)	<0.0001	0.19 (0.11, 0.27)	<0.0001
SNHG7	-0.37 (-0.51, -0.24)	<0.0001	-0.39 (-0.54, -0.24)	<0.0001
SNORD113-1	-0.07 (-0.10, -0.03)	0.0003	-0.08 (-0.12, -0.05)	<0.0001
SRM	-0.18 (-0.25, -0.10)	<0.0001	-0.17 (-0.25, -0.09)	<0.0001
TMEM134	-0.19 (-0.26, -0.13)	<0.0001	-0.19 (-0.26, -0.12)	<0.0001
TMEM160	-0.28 (-0.40, -0.16)	<0.0001	-0.28 (-0.42, -0.15)	<0.0001
UBA6	0.33 (0.17, 0.49)	0.0002	0.36 (0.19, 0.54)	0.0001
USP49	0.55 (0.31, 0.79)	<0.0001	0.55 (0.29, 0.81)	0.0001
UTP14A	-0.14 (-0.21, -0.07)	0.0002	-0.16 (-0.23, -0.08)	0.0001
ZFP36L1	0.44 (0.27, 0.62)	<0.0001	0.40 (0.22, 0.59)	<0.0001
ZNF581	-0.18 (-0.25, -0.10)	<0.0001	-0.17 (-0.25, -0.09)	<0.0001
ZWILCH	-0.11 (-0.16, -0.06)	<0.0001	-0.12 (-0.17, -0.07)	<0.0001

* Model 1: crude, Model 2: adjusted for age and sex (reference category: lean)
† Values are linear regression coefficients (β) with 95% confidence intervals (CI)

Supplemental table 3 Monocyte gene expression and cardiometabolic parameters in the total pediatric population

		Genes HMBS	IMPDH2	LRPPRC	TMEM134	ZWILCH
Cardiovascular risk factors						
Systolic BP	β	-2.52	11.46	-0.66	-3.13	-26.43
	95% CI	-18.87, 13.84	-6.47, 29.38	-21.39, 20.07	-29.94, 23.67	-63.45, 10.59
QUICKI	β	-0.01	0.03	-0.06	0.06	-0.02
	95% CI	-0.06, 0.05	-0.02, 0.09	-0.13, 0.001	-0.02, 0.14	-0.14, 0.10
Monocytes						
Total monocyte nr	β	-0.06	-0.25	-0.15	-0.18	-0.51
	95% CI	-0.33, 0.20	-0.54, 0.04	-0.49, 0.18	-0.61, 0.24	-1.11, 0.09
$CD14^{++}CD16^{-}$ nr	β	-5.08	-25.98	-12.28	-25.81	-54.99
	95% CI	-32.18, 22.01	-55.11, 3.15	-46.14, 21.58	-68.19, 16.56	-114.69, 4.71
$CD14^{++}CD16^{+}$ nr	β	-0.26	-2.16	-5.55	2.48	-7.97
	95% CI	-6.86, 6.34	-9.47, 5.15	-13.66, 2.56	-7.98, 12.93	-22.85, 6.91
$CD14^{++}CD16^{-}$ CD11b MFI	β	-2442	-3071	-10184 *	-5021.0	-19312 *
	95% CI	-10380, 5495	-12069, 5927	-18305, -2062	-18839, 8797	-36097, -2527
$CD14^{++}CD16^{+}$ CD11b MFI	β	362	-1705	-6765	2012	-8736
	95% CI	-7767, 8491	-1094, 7507	-15456, 1925	-12154, 16178	-26844, 9373
Adipokines						
Adiponectin	β	13.56	10.41	10.33	20.66	36.01 *
	95% CI	-1.79, 28.90	-7.67, 28.49	-9.17, 29.83	-3.96, 45.29	1.43, 70.60
Chemerin	β	-0.84 *	-0.25	-0.27	-1.24 *	-0.14
	95% CI	-1.60, -0.09	-1.13, 0.63	-1.26, 0.76	-2.45, -0.03	-1.96, 1.68
Leptin	β	-7.05	106.93	27.60	116.45	311.12
	95% CI	-204.94, 190.85	-110.29, 324.16	-220.06, 275.26	-195.93, 428.84	-130.88, 753.11
TNF-R2	β	0.11	1.01 *	0.48	1.02	1.86
	95% CI	-0.77, 0.99	0.08, 1.94	-0.62, 1.57	-0.34, 2.39	-0.07, 3.79

The association of the 5 validated genes with a selection of cardiometabolic parameters was studied using a linear regression model. Linear regression coefficients (β) with 95% confidence intervals (95% CI) for the monocyte genes are shown, adjusted for age, sex and BMI-SD. *$p<0.05$, § $p<0.01$. MFI: median fluorescence intensity, nr: number, QUICKI: quantitative insulin sensitivity index, Systolic BP: systolic blood pressure.

Supplemental table 4 Monocyte gene expression and cardiometabolic parameters in the obese pediatric subgroup

		Genes HMBS	IMPDH2	LRPPRC	TMEM134	ZWILCH
Cardiovascular risk factors						
Systolic BP	β	-1.41	20.72 *	11.22	-5.82	-25.87
	95% CI	-19.59, 16.78	1.25, 40.19	-15.87, 38.30	-38.63, 26.99	-74.00, 22.26
QUICKI	β	-0.03	-0.03	-0.08 *	-0.01	-0.14 *
	95% CI	-0.09, 0.02	-0.09, 0.04	-0.16, -0.001	-0.12, 0.09	-0.28, -0.001
Monocytes						
Total monocyte nr	β	0.02	-0.17	-0.05	0.02	-0.35
	95% CI	-0.37, 0.41	-0.61, 0.28	-0.64, 0.54	-0.69, 0.72	-1.39, 0.70
$CD14^{++}CD16^{-}$ nr	β	4.15	1.09	-3.80	12.66	-6.53
	95% CI	-5.81, 14.11	-10.44, 12.62	-18.78, 11.18	-4.75, 30.07	-33.05, 19.98
$CD14^{++}CD16^{+}$ nr	β	6.98	-15.09	1.94	-4.53	-36.60
	95% CI	-33.07, 47.02	-60.64, 30.46	-57.94, 61.82	-76.39, 67.34	-141.66, 68.47
$CD14^{++}CD16^{-}$ CD11b MFI	β	3389	-8455	-15385 *	10387	-24070
	95% CI	-9927, 16705	-12017, 14902	-29859, -912	-18329, 39102	-62508, 14369
$CD14^{++}CD16^{+}$ CD11b MFI	β	2323	-839	-19214 §	17823	-23782
	95% CI	-11681, 16326	-14954, 13275	-33658, -4771	-11611, 47257	-64228, 16665
Adipokines						
Adiponectin	β	16.47	7.72	-0.55	16.56	23.05
	95% CI	-0.54, 33.48	-13.85, 29.29	-27.07, 26.01	-16.04, 49.17	-24.31, 70.40
Chemerin	β	-1.09 *	-0.17	-0.28	-2.68 §	-1.17
	95% CI	-2.14, -0.03	-1.46, 1.12	-1.95, 1.40	-4.45, -0.92	-4.13, 1.78
Leptin	β	580	238	144	377	759
	95% CI	-265, 380	-120, 596	-333, 621	-186, 939	-46, 1563
TNF-R2	β	0.16	1.28 *	0.58	1.17	1.19
	95% CI	-0.99, 1.30	0.07, 2.49	-1.10, 2.26	-0.83, 3.17	-1.80, 4.17

The association of the 5 validated genes with a selection of cardiometabolic parameters was studied using a linear regression model. Linear regression coefficients (β) with 95% confidence intervals (95% CI) for the monocyte genes are shown, adjusted for age and sex. *p<0.05, § p<0.01. MFI: median fluorescence intensity, nr: number, QUICKI: quantitative insulin sensitivity index, Systolic BP: systolic blood pressure.

Supplemental table 5 Pathway analysis

Name	Gene ID	Hits in query list	Number of genes in pathway	Raw p-value	P-value (FDR-adjusted)	Hits in Query List
oxidative phosphorylation	GO:0006119	5	108	1,58E-05	4,24E-03	ATP5D,CHCHD10,NDUFS7,COX15,NDUFB2
purine ribonucleoside monophosphate metabolic process	GO:0009167	7	279	1,63E-05	4,24E-03	ATP5D,CHCHD10,NDUFS7,IMPDH,COX15,NDUFB2,PID1
purine nucleoside monophosphate metabolic process	GO:0009126	7	280	1,67E-05	4,24E-03	ATP5D,CHCHD10,NDUFS7,IMPDH,COX15,NDUFB2,PID1
ribonucleoside monophosphate metabolic process	GO:0009161	7	291	2,14E-05	4,24E-03	ATP5D,CHCHD10,NDUFS7,IMPDH,COX15,NDUFB2,PID1
nucleoside metabolic process	GO:0009116	8	414	2,56E-05	4,24E-03	ATP5D,CHCHD10,NDUFS7,IMPDH,COX15,DCTD,NDUFB2,PID1
nucleoside monophosphate metabolic process	GO:0009123	7	304	2,83E-05	4,24E-03	ATP5D,CHCHD10,NDUFS7,IMPDH,COX15,NDUFB2,PID1
nucleotide metabolic process	GO:0009117	10	694	2,88E-05	4,24E-03	ATP5D,CHCHD10,NDUFS7,IMPDH,QPRT,COX15,DCTD,NDUFB2,ME1,PID1
nucleoside phosphate metabolic process	GO:0006753	10	704	3,26E-05	4,24E-03	ATP5D,CHCHD10,NDUFS7,IMPDH,QPRT,COX15,DCTD,NDUFB2,ME1,PID1
glycosyl compound metabolic process	GO:1901657	8	436	3,70E-05	4,24E-03	ATP5D,CHCHD10,NDUFS7,IMPDH,COX15,DCTD,NDUFB2,PID1
nucleoside biosynthetic process	GO:0009163	5	131	4,02E-05	4,24E-03	ATP5D,CHCHD10,IMPDH2,DCTD,PID1
nucleotide biosynthetic process	GO:0009165	7	322	4,08E-05	4,24E-03	ATP5D,CHCHD10,IMPDH2,QPRT,DCTD,ME1,PID1
glycosyl compound biosynthetic process	GO:1901659	5	133	4,32E-05	4,24E-03	ATP5D,CHCHD10,IMPDH2,DCTD,PID1
nucleoside phosphate biosynthetic process	GO:1901293	7	325	4,33E-05	4,24E-03	ATP5D,CHCHD10,IMPDH2,QPRT,DCTD,ME1,PID1
organonitrogen compound biosynthetic process	GO:1901566	14	1445	5,17E-05	4,69E-03	ATP5D,LRPPRC,EEF1D,CHCHD10,IMPDH2,ZFP36L1,QPRT,MRPS15,SRM,HMBS,COX15,DCTD,ME1,PID1
purine nucleoside monophosphate biosynthetic process	GO:0009127	4	74	6,50E-05	4,96E-03	ATP5D,CHCHD10,IMPDH2,PID1
purine ribonucleoside monophosphate biosynthetic process	GO:0009168	4	74	6,50E-05	4,96E-03	ATP5D,CHCHD10,IMPDH2,PID1
nucleobase-containing small molecule metabolic process	GO:0055086	10	766	6,63E-05	4,96E-03	ATP5D,CHCHD10,NDUFS7,IMPDH2,QPRT,COX15,DCTD,NDUFB2,ME1,PID1

ATP metabolic process	GO:0046034	6	242	7,35E-05	5,19E-03	ATP5D,CHCHD10,NDUFS7,COX15,NDUFB2,PID1
purine ribonucleoside metabolic process	GO:0046128	7	362	8,53E-05	5,71E-03	ATP5D,CHCHD10,NDUFS7,IMPDH2,COX15,NDUFB2,PID1
purine nucleoside metabolic process	GO:0042278	7	365	8,98E-05	5,71E-03	ATP5D,CHCHD10,NDUFS7,IMPDH2,COX15,NDUFB2,PID1
purine ribonucleoside triphosphate metabolic process	GO:0009205	6	263	1,16E-04	6,76E-03	ATP5D,CHCHD10,NDUFS7,COX15,NDUFB2,PID1
ribonucleoside monophosphate biosynthetic process	GO:0009156	4	86	1,17E-04	6,76E-03	ATP5D,CHCHD10,IMPDH2,PID1
ribonucleoside triphosphate metabolic process	GO:0009199	6	270	1,34E-04	7,12E-03	ATP5D,CHCHD10,NDUFS7,COX15,NDUFB2,PID1
purine nucleoside triphosphate metabolic process	GO:0009144	6	271	1,37E-04	7,12E-03	ATP5D,CHCHD10,NDUFS7,COX15,NDUFB2,PID1
ribonucleoside metabolic process	GO:0009119	7	392	1,40E-04	7,12E-03	ATP5D,CHCHD10,NDUFS7,IMPDH2,COX15,NDUFB2,PID1
nucleoside monophosphate biosynthetic process	GO:0009124	4	94	1,65E-04	8,07E-03	ATP5D,CHCHD10,IMPDH2,PID1
nucleoside triphosphate metabolic process	GO:0009141	6	295	2,17E-04	9,91E-03	ATP5D,CHCHD10,NDUFS7,COX15,NDUFB2,PID1
purine nucleoside biosynthetic process	GO:0042451	4	102	2,26E-04	9,91E-03	ATP5D,CHCHD10,IMPDH2,PID1
purine ribonucleoside biosynthetic process	GO:0046129	4	102	2,26E-04	9,91E-03	ATP5D,CHCHD10,IMPDH2,PID1
ATP biosynthetic process	GO:0006754	3	48	3,74E-04	1,59E-02	ATP5D,CHCHD10,PID1
ribonucleoside biosynthetic process	GO:0042455	4	121	4,33E-04	1,78E-02	ATP5D,CHCHD10,IMPDH2,PID1
mitochondrial respiratory chain complex I assembly	GO:0032981	3	56	5,90E-04	2,21E-02	NDUFS7,ECSIT,NDUFB2
NADH dehydrogenase complex assembly	GO:0010257	3	56	5,90E-04	2,21E-02	NDUFS7,ECSIT,NDUFB2
mitochondrial respiratory chain complex I biogenesis	GO:0097031	3	56	5,90E-04	2,21E-02	NDUFS7,ECSIT,NDUFB2
purine ribonucleoside triphosphate biosynthetic process	GO:0009206	3	59	6,88E-04	2,50E-02	ATP5D,CHCHD10,PID1
purine nucleoside triphosphate biosynthetic process	GO:0009145	3	60	7,22E-04	2,55E-02	ATP5D,CHCHD10,PID1
ribonucleoside triphosphate biosynthetic process	GO:0009201	3	65	9,12E-04	3,11E-02	ATP5D,CHCHD10,PID1
purine ribonucleotide metabolic process	GO:0009150	7	537	9,28E-04	3,11E-02	ATP5D,CHCHD10,NDUFS7,IMPDH2,COX15,NDUFB2,PID1
generation of precursor metabolites and energy	GO:0006091	6	395	1,01E-03	3,30E-02	ATP5D,CHCHD10,NDUFS7,COX15,NDUFB2,PID1
ribonucleotide metabolic process	GO:0009259	7	552	1,09E-03	3,46E-02	ATP5D,CHCHD10,NDUFS7,IMPDH2,COX15,NDUFB2,PID1

purine nucleotide metabolic process	GO:0006163	7	556	1,14E-03	3,52E-02	ATP5D,CHCHD10,NDUFS7,IMPDH2,COX15,NDUFB2,PID1
ribose phosphate metabolic process	GO:0019693	7	567	1,27E-03	3,85E-02	ATP5D,CHCHD10,NDUFS7,IMPDH2,COX15,NDUFB2,PID1
mitochondrial respiratory chain complex assembly	GO:0033108	3	77	1,49E-03	4,28E-02	NDUFS7,ECSIT,NDUFB2
nucleoside triphosphate biosynthetic process	GO:0009142	3	77	1,49E-03	4,28E-02	ATP5D,CHCHD10,PID1
nicotinamide nucleotide biosynthetic process	GO:0019359	2	20	1,55E-03	4,28E-02	QPRT,ME1
pyridine nucleotide biosynthetic process	GO:0019363	2	20	1,55E-03	4,28E-02	QPRT,ME1
mitochondrion organization	GO:0007005	8	770	1,70E-03	4,60E-02	LRPPRC,CHCHD10,NDUFS7,MRPS15,LSM4,ECSIT,NDUFB2,PID1
mitochondrial ATP synthesis coupled electron transport	GO:0042775	3	82	1,79E-03	4,73E-02	NDUFS7,COX15,NDUFB2
purine-containing compound metabolic process	GO:0072521	7	605	1,84E-03	4,73E-02	ATP5D,CHCHD10,NDUFS7,IMPDH2,COX15,NDUFB2,PID1
organophosphate metabolic process	GO:0019637	10	1167	1,86E-03	4,73E-02	ATP5D,CHCHD10,NDUFS7,IMPDH2,QPRT,COX15,DCTD,NDUFB,ME1,PID1
cofactor biosynthetic process	GO:0051188	4	181	1,94E-03	4,81E-02	QPRT,HMBS,COX15,ME1
ATP synthesis coupled electron transport	GO:0042773	3	85	1,98E-03	4,81E-02	NDUFS7,COX15,NDUFB2
organophosphate biosynthetic process	GO:0090407	7	614	2,00E-03	4,81E-02	ATP5D,CHCHD10,IMPDH2,QPRT,DCTD,ME1,PID1
heme biosynthetic process	GO:0006783	2	23	2,05E-03	4,82E-02	HMBS,COX15
organelle inner membrane	GO:0019866	8	564	1,69E-04	3,30E-02	ATP5D,LRPPRC,NDUFS7,MRPS15,COX15,ECSIT,NDUFB2,LGALS3

Results of GO Term enrichment analysis. The table lists GO term pathways (> 10 genes) involved in biological processes, the number of overlapping genes in the query gene list and each pathway, the size of each pathway, the nominal p value derived from a hypergeometric test and the corresponding FDR adjusted p-value.

Supplemental table 6 qPCR Primer sequences

Gene	Forward	Reverse
36B4	CGGGAAGGCTGTGGTGCTG	GTGAACACAAAGCCCACATTCC
GUSB	CACCAGGGACCATCCAATACC	GCAGTCCAGCGTAGTTGAAAAA
B2M	CCAGCAGAGAATGGAAAGTC	GATGCTGCTTACATGTCTCG
ACBD6	TTGGTGGGCCAGTTATTAGTTC	CCAGTGAAGTAGAGCCCTACC
CHCHD10	CTACCATGGTCTGAGCTCCC	CATTTGTGTCTTGGGTTATCTGTG
CKS1B	CGACGACGAGGAGTTTGAGTATC	TTAGGGACCAGCTTGGCTATGT
COX15	TGGAGTAACTAGGTTGACAGAG	TGGAAATTGCTGGTATCTTTGG
ECSIT	ACAGGAACCTCCATCTCTCAG	GGACTGTTCAGAGCTATGGGC
FERMT3	AGCAGATCAATCGCAAGCAG	ATCCCGTACTTGTCCAGTGT
F13A1	GTGAAGATGATGCTGTGTATCTG	ATGCCATCTTCAAACTGACC
HMBS	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC
IFNA1	GCCTCGCCCTTTGCTTTACT	CTGTGGGTCTCAGGGAGATCA
IMPDH2	GGGCATCATCTCCTCCAGGG	TGCTGCGCTGCAGAATTTCA
LRPPRC	GAGAGATGCCGGAATTGAGC	CTCGGACTTCTCCACCTTCT
NME-1	AAGGAGATCGGCTTGTGGTTT	CTGAGCACAGCTCGTGTAATC
NME-2	CATTGACCTGAAAGACCGAC	ATGATGTTCCTGCCAACCTG
QPRT	GGGCAGCCTTTCTTCGATG	GGAGCCCATACTTCTCCACCA
TMEM134	CAGTTCAGCATTGATGATGCC	TTCTCCAGGTTCTGGTAGCG
USP49	CTCATCCCCTTCTCCCAGAG	TTCCAGGGATAGGTCCCAAA
ZWILCH	TTGGCTGATGGTTTGAGGAC	TGGTATGAAATCACACTACTGCTC

Supplemental table 7 qPCR validation data

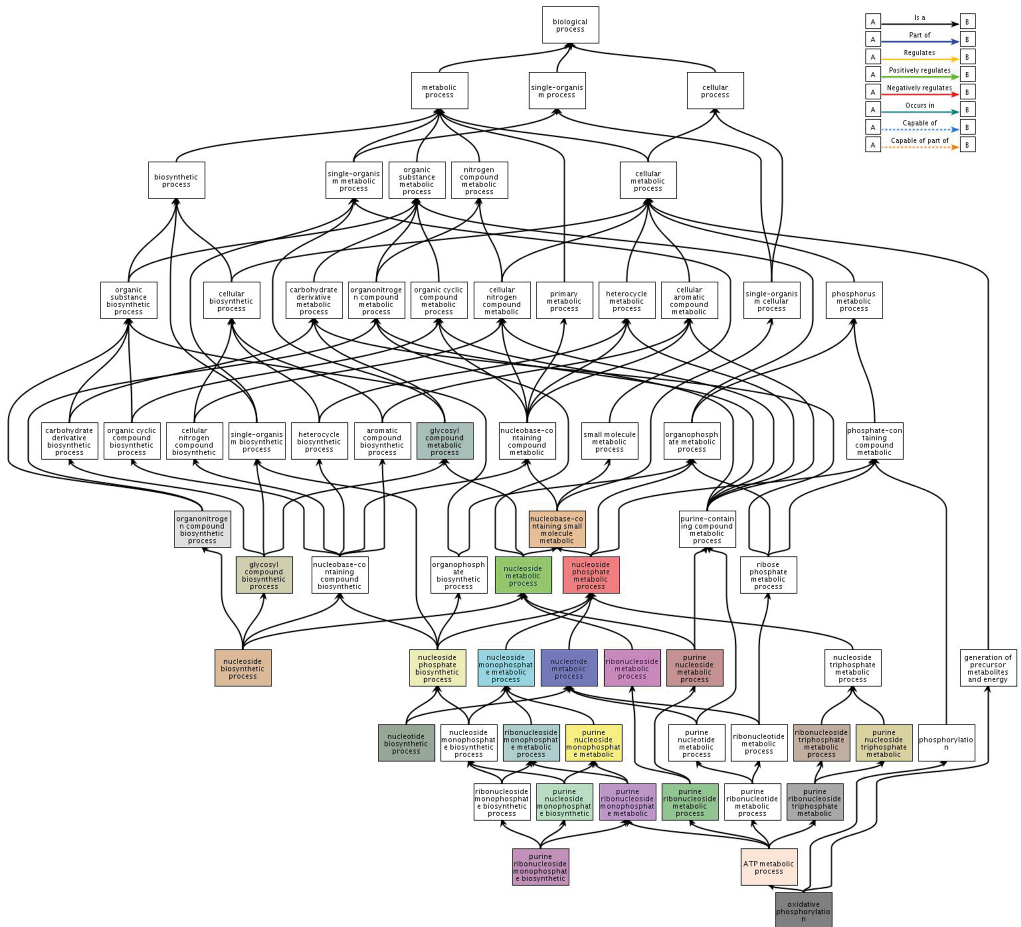
Gene	Fold Induction obese group (95% CI difference in fold induction)	P-value
ACBD6	0.59 (-0.18 to 1.0)	0.17
CHCHD10	1.13 (-0.63 to 0.37)	0.60
CKS1B	0.85 (-0.19 to 0.48)	0.38
COX15	1.13 (-0.97 to 0.72)	0.76
ECSIT	1.35 (-0.87 to 0.16)	0.17
FERMT3	1.04 (-0.44 to 0.36)	0.85
F13A1	1.28 (-0.88 to 0.32)	0.35
HMBS	0.62 (0.087 to 0.68)	0.01
IFNA1	1.23 (-1.04 to 0.59)	0.58
IMPDH2	0.64 (-0.014 to 0.74)	0.06
LRPPRC	0.60 (0.14 to 0.68)	0.005
NME-1	0.71 (-0.071 to 0.65)	0.11
NME-2	0.85 (-0.58 to 0.89)	0.68
QPRT	0.73 (-0.39 to 0.94)	0.41
TMEM134	0.32 (0.079 to 1.28)	0.028
USP49	0.84 (-0.28 to 0.60)	0.47
ZWILCH	0.45 (0.18 to 0.92)	0.005

Supplemental table 8 Clinical characteristics of the adult cohort at risk

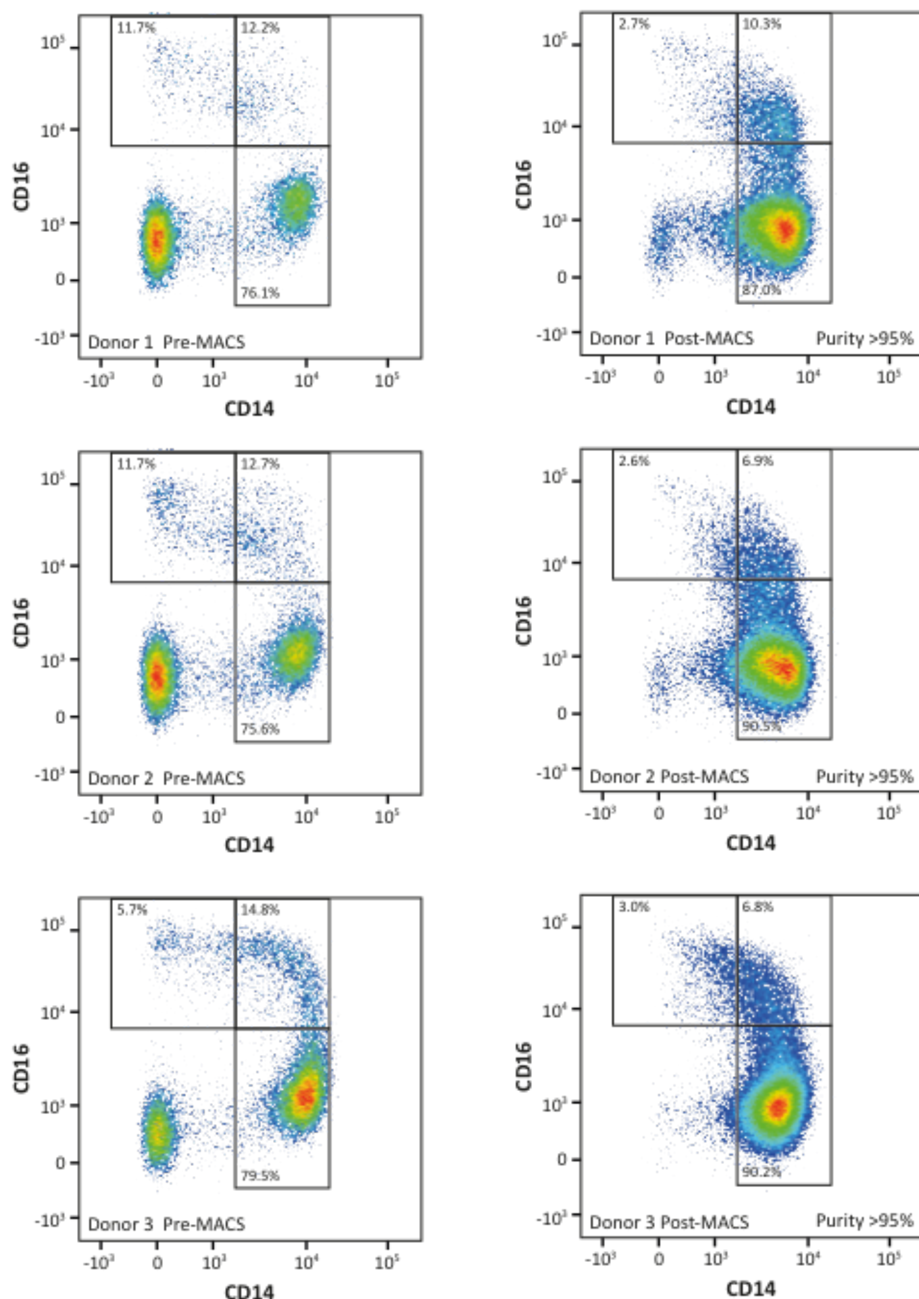
Variables	
Age, years	62.4 (10.1)
Male sex	262 (74.6)
BMI, kg/m2	27.4 (4.3)
Obese (BMI>30)	80 (23.3)
Hypertension	229 (65.2)
Hypercholesterolemia	230 (65.5)
Diabetes mellitus	75 (21.4)
Current smoker	71 (20.2)
Positive family history	151 (43.0)
prior MI	111 (31.6)
prior PCI	130 (37.0)
prior CABG	32 (9.1)
SYNTAX score*	13 (6-22)

Baseline characteristics of the adults with cardiovascular risk (n=351). Discrete variables are given as absolute count (%), continuous variables as mean (SD) or as median (IQR). *median (IQR).

Supplemental Figure 1 Nodal Map



Supplemental figure 2 Magnetic bead sorting



The monocyte subsets of 3 random patients before and after magnetic bead sorting are shown. Purity of the monocyte fraction after magnetic bead sorting was >95%.

Chapter 6

General Discussion

Discussion

Over the last few decades chronic inflammation has come to the forefront as a key player in creating long-term detrimental morbidities within the human populace. This thesis has endeavoured to understand and dissect the role of various key players in chronic inflammation by using a 3-tier approach: cytokines, extracellular vesicles (EVs) and immune cells. In this chapter, each tier will be addressed individually. By understanding the role and postulating the relevance of each tier, this discussion attempts to clarify old and provide new insights into the chronic inflammatory state.

1 Cytokines

1.1 Summary of findings

In **chapter 2** of this thesis we reviewed existing methodologies of sample isolation, storage and measurement when determining cytokine concentrations in a given study. Firstly, sample handling and storage influence cytokine recovery and stability as exemplified by the fact cytokines are most stable in EDTA and sodium heparin tubes. Secondly, temperature and time delay to processing also influence cytokine measurements. The duration of contact between serum or plasma and blood cells can increase artefacts in cytokine measurements (44). Thirdly, when looking at long term stability and repetitive measurements, the levels of most cytokines are stable within a two-year period with a decrease of 10–20% occurring for each year after that (44). Repetitive freeze thawing of samples also greatly influences sample measurement with cytokine levels remaining stable, drastically decreasing or increasing as a result of this process. Lastly, many techniques may be applied when determining cytokine levels however, multiplex immunoassays in particular provide an ideal method by which to measure multiple cytokines in parallel (44). Note, control samples should undergo identical handling, storage and use in order to truly compare samples. Thus, for all current and future work that will look at determining and manipulating cytokine involvement in chronic inflammation it is important that the afore mentioned factors are carefully monitored and taken into consideration when attempting to extrapolate data to the biological situation.

In **chapter 3** of this thesis cytokines were used as read out in determining EV functionality within the context of JIA. As mentioned above cytokines play a key role in JIA pathology, pro-inflammatory cytokines cause increased immune cell infiltration and activation inducing phenotypic changes as well as further cytokine or EV release (2, 3, 4). Our data indicated that PBMCs stimulated by EVs produced significantly higher levels of IL-6 (**chapter 3**). As previously described, IL-6 plays a central role in chronic inflammation (45). Our data could thus provide hints towards a novel way in which cytokine production may be influenced within the context of JIA. Administration of biologicals within the joint could thus be considered a mechanism of curbing inflammation however, the content of EVs has to be ascertained first i.e.: if IL-6 is carried within JIA derived EVs, administration of biologicals may not induce the desired effect as the cytokine will be protected within the membrane. If IL-6 production is induced by vesicle content then, inhibiting vesicle fusion or binding with immune cells could be considered a mechanism by which to reduce inflammation.

In **chapters 4** and **5** we elaborated on the role of adipokines within the context of obesity induced chronic inflammation. In congruence with previous data patients demonstrated increased levels of leptin and decreased levels of adiponectin (6, 7). In order to decipher the specific mechanism by which these adipokines may contribute to these disease states, in **chapter 4** we analysed adipokine receptor expression on various immune subsets and compared these to adipokine levels. Circulating leukocyte subsets exhibited distinct adipokine receptor expression profiles. In order to determine if adipokine effects where modulated by high adipokine levels and receptor expression, the relationship between these two factors was ascertained. When looking at monocytes no correlation was observed between circulating adiponectin or leptin levels and the corresponding adipokine receptor expression similarly, other leukocyte subsets showed the same phenomenon. Our data also identified analogous adipokine receptor expression profiles between obese children and healthy controls, thus suggesting that, rather

than receptor expression, adipokine levels modulate adipokine induced signalling in circulating leukocytes (46). This data suggests that future treatment of obesity associated inflammation should look at neutralizing elevated circulating pro-inflammatory adipokines or enhancing anti-inflammatory adipokine levels rather than altering adipokine receptor activity (eg. blockade or receptor upregulation).

1.2 The broader context

Of the cytokines measured and analysed in this thesis, this section will look particularly at the role of IL-6 and leptin within chronic pediatric inflammation.

IL-6 and IL-6 type cytokines signal through cytokine specific receptors that form a complex with at least one gp130 subunit (47). This signalling results in the activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) and MAPK (mitogen- activated protein kinase) pathways (47). IL-6 has both a membrane bound and soluble receptor. This means that cells that do not express IL-6R can still react to IL-6 through soluble receptor complex formation (47), allowing it to influence various immune cells and tissues. IL-6 is known to function in a pleiotropic manner where under normal conditions the cytokine initiates the acute phase response and promotes wound healing (47) (45) however, chronic IL-6 production has detrimental outcomes. Polymorphisms in IL-6 promotor regions or the IL-6R gene locus have been shown to be associated with systemic JIA (48). CVD studies have furthermore shown short term IL-6 stimulation has a cardio-protective function for acute damage while an excess IL-6R production or chronic signalling has a causal effect on CVD (49). This data thus suggests targeting IL-6 and IL-6R signalling as method of treating chronic inflammatory diseases. For example, when looking at our data, EVs have the ability to induce PBMC IL-6 production. This stimulation could be the result of inducing IL-6 production or releasing vesicle contained IL-6. Thus, although local IL-6 blockade within the joint of JIA patients can be proposed as a therapeutic treatment, this may not affect membrane bound vesicular contents and may consequently not influence EV mediated IL-6 induction. When looking to treat CVD or other systemic chronic inflammatory diseases with anti-IL-6 therapies the pleotropic function of this molecule must be taken into consideration. Clinical studies with IL-6 targeting therapies have indicated that drug administration can cause increased risk to opportunistic infections (50). Individual patient cytokine levels must thus be measured from blood and treatment administration must be tailored to allow the removal of excess IL-6 while permitting a basal IL-6 concentrations to remain.

Similar to IL-6, leptin signals through the activation of the JAK/STAT signal transduction pathway (51) (52) and exhibits pleotropic effects. As mentioned before Leptin causes increased production of IL-6, TNF and IFNγ (51). From our studies, we were able to identify elevated levels of leptin in obese children however, this did not coincide with a difference in leptin receptor expression profiles between obese children and controls. Our data proposes an adipokine effect due to levels of adipokines in circulation. This in combination with leukocyte specific or, as previously shown, organ specific receptor expression could highlight a mechanism by which leptin can influence various chronic inflammatory diseases. This effect could be the result of the fact that, depending on the disease, different immune cell profiles are found. Considering that we show differential adipokine receptor expression on different leukocyte subsets, leptin binding to the leukocyte subset predominantly present in a disease type could induce a disease specific effect. An additional point of interest is the fact chronic inflammation, as a result of obesity, is commonly accompanied by cardiovascular disease and type 2 diabetes. Augmented leptin concentrations could increase leptin receptor activation on cardiomyocytes or pancreatic cells and could thus cause these obesity specific manifestations. This is supported by the fact improved glucose tolerance is observed in pancreas specific leptin receptor knock out mice (53). Additionally, experiments have shown that stimulation of human cardiomyocytes with leptin leads to significantly increased cell size (53). There is however, disparate data on the hypertrophic and anti-hypertrophic effects of leptin (14, 15). Nonetheless, what can be hypothesised is the possible direct interaction of leptin with cardiomyocytes and subsequent induction of pro-inflammatory cytokine production that

may result in increased monocyte recruitment and differentiation in the heart and thus, through this mechanism, contribute to observed deleterious cardiovascular outcomes. Leptin deficient individuals exhibit an aberrant immune shift from a Th1 to Th2 profile accompanied by increased susceptibility to infectious diseases (54). When considering the use of leptin therapy in chronic inflammatory diseases, this particular characteristic of leptin activity could be applied. In the context of JIA, polyarticular and extended oligoarticular patients exhibit a Th1 skewed response within synovial fluid derived immune cells (55). It could thus be postulated that local blockade of leptin activity within these individuals could help skew the immune response away from the Th1 profile. Thus, like IL-6, administration or blockade of leptin could be considered a treatment method for inflammatory diseases however, the neurobiological, physiological and metabolic effects of this cytokine must be taken into careful consideration. This complexity has been exemplified in research involving type 2 diabetes. Leptin administration in non-obese type 2 diabetes individuals resulted in improved insulin resistance however, obese type 2 diabetes subjects do not respond to this therapy (18, 19).

1.3 Strengths and weaknesses

Although measuring cytokines provides a window into the severity of the inflammatory state, several factors may influence measured data (**Chapter 2**). The sensitivity in sample handling, storage and measurement may make it difficult to extrapolate measurements to the biological situation and may confound data comparison. Furthermore, cytokine levels in circulation may not always reflect the local situation as is evidenced by extremely elevated cytokine levels in the joints of JIA patients in comparison to peripheral blood measurements. Additionally, little is understood about cytokine network dynamics. Previous data has shown that cytokine interactions can greatly influence the immune response (56) thus, in the presence of multiple equilibria, external stimuli (e.g. antibodies) that affect these interactions can cause a shift in the inflammatory state (56). Lastly, redundancy in cytokine functionality (20, 21) is not always taken into consideration when interpreting data. If these pitfalls in measuring cytokines are, however, kept in mind when performing experiments and analysing data these molecules can be applied optimally. Cytokine and adipokine profiling has in fact already proved itself to be extremely useful in the diagnosis and monitoring of various chronic diseases (2, 22). The fact these molecules correlate with disease progression or regression, and the ease, speed and relative accuracy by which they can be measured using high throughput multiplex technology cements their position as key components to be used when monitoring the immune system and disease.

1.4 Future directions

Cytokine and cytokine activity can be used as both disease related biomarkers as well as therapeutic interventions. Some studies have already looked into the administration of monoclonal IL-6 receptor Tocilizumab in chronic inflammatory disease models (23, 24, 25). Data suggests Tocilizumab improves glycaemic parameters in rheumatoid arthritis patients (57) however, in contrast, weight gain and dyslipidaemia occurred in some clinical studies with tocilizumab (24, 25, 26). This data exemplifies the fact that several factors must be carefully investigated before cytokine based therapy can be used to its full potential. Firstly, as demonstrated by our data in **chapter 2**, the scientific community must agree on a standardized method by which measure and store cytokines. Using a universal technique will allow data from different settings to be truly compared. Secondly, the pleotropic nature of cytokine activity should be thoroughly investigated. This goes hand in hand with understanding cytokine network dynamics. By dissecting these two factors specific information can be garnered on the administration of cytokines, antibodies or cytokine receptors. These molecular interactions, particularly the strength and duration of them, are key in understanding the nature of cytokines in the context of chronic inflammatory diseases and in designing cytokine based therapy that results in an optimal and desired effect.

2 Extracellular vesicles

2.1 Summary of findings

In **chapter 3** of this thesis we delved into the role of EVs in chronic inflammation. Using JIA as our disease model, 3 key points were drawn from this study. Firstly, using both a sucrose dependant and independent technique we were able to isolate EVs from as little as 1 ml of biological fluid. Secondly, we showed a difference in EV population distribution between peripheral blood and synovial fluid derived vesicles. Lastly, functionality testing revealed that EVs where able to induce significant IL-6 production from healthy control PBMCs regardless of the EV source.

2.2 The broader context

EVs can cause both immune activation and suppression. Work on RA patients revealed EV counts are associated with diseases severity and RA synovial fluid derived vesicles are able to influence synoviocyte activity. Additionally, the presence of EVs in human atherosclerotic lesions has also been described (58). Data suggests that EVs are involved in lesion formation by influencing monocytes and endothelial cells (58), and in lesion progression by stimulating foam cell formation (58) and are associated with plaque stability. EVs can thus be postulated to play an important role within chronic inflammatory diseases. These particles allow the packaged transport of immune regulating information to target cells. The fact JIA patients, similar to RA patients, have copious amounts of vesicles within their synovial fluid could be indicative of a role for these particles in disease pathology. Although our data shows no difference in IL-6 induction by EVs derived from JIA patients and healthy adult controls, this pilot study supports the functional activity of these particles within the context of paediatric immune regulation.

2.3 Strengths and weaknesses

Vesicle isolation techniques are one of the main weaknesses in determining the function of these particles in chronic inflammation. Methods currently focus on bulk sample isolation and analysis which does not permit separating the EVs into various subsets. EV subsets are derived from various immune cells and immune cell compartments. With separation and identification methodology currently in its infancy, these specific populations cannot be differentiated. EV composition and dynamics are greatly influenced by the cell and compartment from which they are produced. Therefore, the inability to separate EV subsets means their immune stimulating or inhibitory effects can as of yet not fully be understood. Bulk isolation methods also result in the co-isolation of debris. Although sucrose gradient isolation is currently on the forefront of "clean" vesicle isolation, this method is not applicable to the small sample volumes of (paediatric) patient material. In line with EV isolation methods, the equipment, time and expertise required to process and produce these particles means they cannot yet be implemented in large patient studies or clinical situations that require fast results. Vesicle quantification can also be considered a confounding factor when working with these particles. Technology has come a long way however, as exemplified by FACS technology, this is mainly based on extrapolating the number of vesicles measured within a scattering volume at a single point in time towards a concentration per unit volume (59). This method contains inbuilt errors that include particles being lost below detection thresholds as well as the fact vesicle size differences influence concentration measurements (59). Most of the downfalls of working with vesicles exist in the methods used to isolate and identify them. Once a protocol can be designed that allows high resolution individual vesicle analysis a new door can be opened into using these particles in immune regulation.

2.4 Future directions

Currently, much work is done on vesicle isolation. Future techniques should support high resolution single particle analysis, this will enable vesicle content and dynamics to truly be understood. By ascertaining whether vesicle populations act in a direct or indirect manner with immune cells, and subsequently choosing the most robust and efficient method of interaction, individual particles can be directed to target cells to induce a desired immune-regulatory effect. In keeping with this,

understanding and separating vesicle populations will allow the activity of these various groups to be determined. It has already been shown that different disease conditions have different vesicle numbers. Thus, if EVs can be separated and characterized, not only will cellular-EV dynamics within a disease context be determined, researchers can 1) emulate EV membrane structures and use these as therapeutic delivery systems to target cells or 2) introduce therapies that interact specifically with pathogenic EV populations and inhibit their activity.

3 Immune cells

3.1 Summary of findings

In **chapter 4** and **5** of this thesis we looked to analyse and dissect key immune cells involved in chronic inflammation.

Chapter 4 analysed adipokine receptor expression on various immune cell subsets. When looking at innate immune cells, monocytes expressed higher levels of adipokine receptors compared to other leukocyte subsets. When looking closer at the monocyte subset, non-classical $CD14^{+}CD16^{++}$ monocytes exhibited high levels of AdipoR1, all monocyte subsets expressed AdipoR2, and classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocytes showed high leptin receptor levels. When looking at adaptive immune cell populations, B-lymphocytes, $CD4^{+}$ and $CD8^{+}$ T-lymphocyte subsets expressed AdipoR2 as well as leptin receptor. In all, obese children pre- and post-intervention and normal weight controls exhibited similar adipokine receptor expression on leukocyte subsets. This data thus reveals that immune cell subsets may differentially react to adipokine stimulation due to varying receptor expression.

Chapter 5 took a closer look at the monocyte subset in the context of obesity. As seen in literature, the obese group exhibited higher total monocyte numbers with higher classical $CD14^{++}CD16^{-}$ monocyte numbers and higher intermediate $CD14^{++}CD16^{+}$ monocyte numbers in particular. Gene expression analysis revealed that 67 genes were significantly and differentially expressed between obese and lean children. 64 of these 67 genes were mapped to pathways associated with oxidative phosphorylation and metabolic processes. 5 of the 67 genes were confirmed using qPCR. Comparison to clinical data revealed an association between these genes and adipokine levels. Lastly, these 5 genes were used to analyze if an adult cohort with obesity and cardiovascular risk exhibited a comparable expression profile to obese children. Downregulation of 2 of the 5 genes (IMPDH2 and TMEM134) was associated with obesity in adults. TMEM134 downregulation was furthermore associated with a higher SYNTAX score in the adult cohort however, addition of BMI to the analysis model diminished this association. As a whole, TMEM134 downregulation was observed in pediatric obesity and was correlated to a higher SYNTAX score in adults at risk of cardiovascular disease in an obesity dependent manner.

3.2 The broader context

Although in **Chapter 4** we investigated a variety of immune cells involved in chronic inflammation, this section will particularly focus on monocytes. When looking at adipokine receptor expression monocytes robustly expressed these proteins. Previous work has also shown that AdipoR1/R2 expression on myeloid cells has been associated with anti-inflammatory macrophage polarization and suppression of foam cell formation (60). As elaborated upon in *section 1.2* above, the immune cell specific adipokine receptor expression patterns we demonstrated could represent a new mechanism by which adipokines can influence chronic inflammatory diseases. Namely, due the fact these diseases exhibit different immune cell phenotypes. For example, it has previously been demonstrated that human leptin causes monocyte proliferation and activation in a dose-dependent manner *in vitro* (61). Therefore, the high leptin receptor expression on classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocytes observed in obesity in combination with elevated blood leptin levels within

this populace could alter the inflammatory state of these cells and thus play a role in obesity associated disease development.

As mentioned before, childhood obesity is associated with increased numbers of circulating classical $CD14^{++}CD16^{-}$ and intermediate $CD14^{++}CD16^{+}$ monocytes. Monocytes also play a key role in the development and propagation of atherosclerosis. Although much work has focused on determining cytokine production, receptor expression and immune cell activity in both adult and pediatric obesity derived circulating monocytes, one facet remains untouched. That is, the influence of metabolic pathways on immune cell activity and function. Our genetic data in **Chapter 5**, although indicative, is only the tip of the iceberg. Our investigations revealed that genes significantly and differentially regulated within the obese pediatric cohort were associated with oxidative phosphorylation and metabolic processes. It is commonly known that the adaptive immune response results in the development of immunological memory. Recently, however, innate immune memory (trained immunity) has been brought to the forefront (62). Innate immune memory has been demonstrated in human monocytes and macrophages in response to repetitive inflammatory stimuli. These stimuli can come in the form of pathogens however, environmental changes are able to induce similar effects. In the context of obesity, nutrient excess can cause intracellular metabolic changes that result in functional and epigenetic reprogramming in immune cells (32, 33). Modification of immune cell energy production could cause phenotypic (within cell) and environmental (released cellular products) changes that could propagate the pro-inflammatory environment and thereby bolster chronic inflammation (33, 34). As previously mentioned, type 2 diabetes is associated with obesity. We thus ask if changes in immune cell metabolic processes could be influenced by hyperglycemia and insulin resistance. Previous research has shown that transient hyperglycemia induces persistent epigenetic changes in the promoter of the NF- B p65 sub- unit in cultured human aortic endothelial cells (63). These changes cause increased expression of the pro-artherogenic genes MCP1 and VCAM1 (63). It has furthermore been shown that chronic exposure to a moderately high level of hyperglycaemia has persistent effects on diabetic complications even during periods of improved glycemia in a process termed "metabolic memory" (32, 35). The fact that transient hyperglycaemia affects artherogenesis and monocyte associated genes in combination with the monocytosis, glucose intolerance and cardiovascular incidences observed in obese individuals could thus indicate a new mechanism by which cardiovascular disease is propagated within an obese cohort. The fact our data indicates that genes altered in both the paediatric *and* adult cohort at risk are associated with metabolic processes could provide the first window into how epigenetic changes as a result of hyperglycaemia could persist and subsequently place the obese paediatric cohort at risk of detrimental cardiovascular outcomes in later life even after the necessary intervention has occurred.

3.3 Strength and weaknesses

Technologies of cellular isolation and manipulation are continuously being improved and pioneered. The ability to isolate single cells and specific populations using FACS sorting technology means that distinct disease related populations can be studied and understood (64). Our work illustrates, however, that deciphering the role of immune cells in chronic inflammation and atherogenisis is difficult to do using only FACS technology. Cellular markers do not solely represent immune cell function as factors such as immune cell metabolism, cytokine production and various cellular processes also contribute to immune cell activity. Extensive immune cell phenotyping is combination with functional assays is therefore necessary to truly ascertain the role of immune cells within the context of chronic inflammation.

3.4 Future directions

Monocyte activation, gene expression and cell metabolism are 3 key factors that need to be defined in chronic inflammatory diseases. Understanding the intracellular metabolism of immune cells and combining this with extensive phenotyping of monocyte subsets may provide novel therapeutic

angles. This has already been demonstrated in cases of post-sepsis immunoparalysis. Although these monocytes were thoroughly phenotyped, analysis of cellular metabolism reveled extensive defects (37, 38). Reversal of these defects using recombinant interferon-γ therapy caused partial restoration of immune function to these cells (31, 37). Furthermore, early persistent hyperglycaemia has extended effects on diabetic complications during periods of improved glycemia (32, 35). Therefore, for obesity associated diseases such as diabetes or cardiovascular disease inhibiting metabolic pathways that propagate pathology, or stimulating pathways that alter short-term disease activity and induce long term epigenetic changes could be a method of ensuring that children do not have to deal with the detrimental outcomes of their maladies for the rest of their lives. It should be noted however that in chronic inflammation, many factors may influence the metabolic status of immune cells. These factors may include hypoxia, hyperlipidemia, fatty acid abundance and systemic metabolism which have all been shown to contribute to alterations in immune metabolism (62). Therefore, although modulating immune cell metabolism represents a novel therapeutic process, knowledge over various fields including immunology, endocrinology and lipidology must be combined.

4 Conclusion

In all, combining old knowledge, optimizing technologies, progressing current work and implementing the future steps postulated in this thesis could open new gate ways into understanding and manipulating the chronic inflammatory state. Of the many and important immunological facets described in this thesis taking a robust step towards uncovering the roll of immuno-metabolism in the context of chronic inflammatory diseases could provide a promising and effective new treatment methodology. As described above epigenetic changes in immune cell metabolism can alter cellular phenotype and activity on both the short and long term. These changes have an over-arching effect that may even override typical cellular phenotypic (cell markers) and activity (cytokines) markers as illustrated by metabolic memory. As mentioned above, however, cellular metabolism can in itself be influenced by a great many factors. It is therefore important that a multi-disciplinary approached be applied in future investigations. This informed manner of treating inflammation is particularly pertinent when dealing with the paediatric population as treatments applied in children should maintain quality of life and most importantly, ensure disease free longevity in this population.

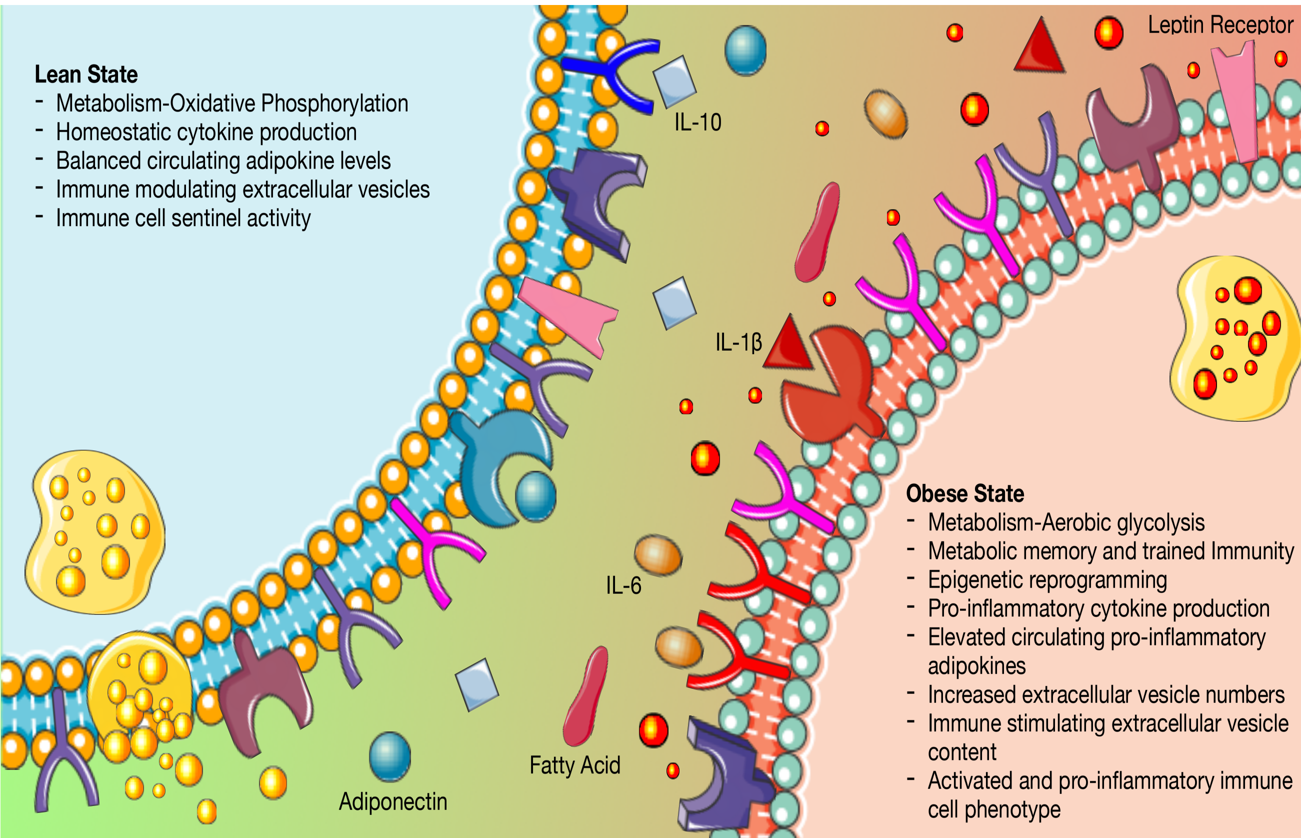


Fig 2: A summary of the findings within this thesis. Here a comparison between the lean and obese state is used as an illustration of chronic inflammation. It can be seen that several factors contribute to disease propagation. This orchestra of occurrence includes alterations in immune cell metabolism, elevated pro-inflammatory cytokine production, changes in immune cell phenotype and receptor expression, altered immune cell activity, epigenetic reprogramming and increased extracellular vesicle release.

References

1. Keustermans G.C, Hoeks S.B, Meerding J.M, Prakken B.J, de Jager W. *Cytokine assays: an assessment of the preparation and treatment of blood and tissue samples.* Methods. 2013, Vol. 61, pp. 10-17.

2. de Jager W, Hoppenreijs E.P.A.H , Wulffraat N.M, Wedderburn L.R, Kuis W, Prakken B.J. *Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study* Ann Rheum. 2007, Vol. 66, pp. 589–598.

3. Prakken B, Albani S, Martini A. *Juvenile idiopathic arthritis.* Lancet. 2011, Vol. 377, pp. 2138-2149.

4. Buzas E.I, Gyorgy B, Nagy G, A Falus, Gay S. *Emerging role of extracellular vesicles in inflammatory diseases.* Nat Rev Rheumatol,. 2014, Vol. 10, pp. 356-364.

5. Gabay C. *Interleukin-6 and chronic inflammation.* Arthritis Res Ther. 2006, Vol. 8, p. S3.

6. Ouchi N, Parker J.L, Lugus J.J, Walsh K. *Adipokines in inflammation and metabolic disease.* Nat Rev Immunol. 2011, Vol. 11, pp. 85-97.

7. Jung U.J, Choi M.S. *Obesity and Its Metabolic Complications: The Role of Adipokines and the Relationship between Obesity, Inflammation, Insulin Resistance, Dyslipidemia and Nonalcoholic Fatty Liver Disease.* Int J Mol Sci. 2014, Vol. 15, pp. 6184–6223.

8. Keustermans G.C.E, van der Heijden L.B , Boer B, Scholman R, Nuboer R, Pasterkamp G, Prakken B, de Jager W, Kalkhoven E, Janse A.J, Schipper H.S. *Differential adipokine receptor expression on circulating leukocyte subsets in lean and obese children.* PLOS one. 2017, pp. 1-15.

9. Heinrich P.C, Behrmann I, Haan S, Hermanns H.M, Muller-Newen G, Schaper F. *Principles of interleukin (IL)-6-type cytokine signalling and its regulation.* Biochem. J. 2003, Vol. 374, pp. 1–20 .

10. Pignatti P, Vivarelli M, Meazza C, Rizzolo M.G, Martini A, De Benedetti F. *Abnormal regulation of interleukin 6 in systemic juvenile idiopathic arthritis.* J Rheumatol. 2001, Vol. 28, pp. 1670-1676.

11. Fontes J.A, Rose N.R, Čiháková D. *The varying faces of IL-6: From cardiac protection to cardiac failure.* Cytokine. 2015, Vol. 74, pp. 62-68.

12. Rose-John S, Winthrop K, Calabrese L. *The role of IL-6 in host defence against infections: immunobiology and clinical implications.* Nat Rev Rheumatol. 2017, Vol. 13, pp. 399-409.

13. G, Frühbeck. *Intracellular signalling pathways activated by leptin.* Biochem J. 2006, Vol. 393, pp. 7–20.

14. Friedman J. *The long road to leptin.* J Clin Invest. 2016 , Vol. 126, pp. 4727-4734.

15. Hall M.E, Harmancey R, Stec D.E. *Lean heart: Role of leptin in cardiac hypertrophy and metabolism.* World J Cardiol. 2015, Vol. 7, pp. 511–524.

16. Díaz B.B, Rodríguez I.M , González D.A, del Cristo Rodríguez Pérez M, de León A.C. *An Overview of Leptin and the Th1/Th2 Balance.* Open J Immunol. 2014, Vol. 4, pp. 42-50.

17. Mahendra A, Misra R, Aggarwal A. *Th1 and Th17 Predominance in the Enthesitis-related Arthritis Form of Juvenile Idiopathic Arthritis*. J Rheumatol. 2009, Vol. 36, pp. 1730-1736.

18. Coppari R, Bjørbæk C. *Leptin revisited: its mechanism of action and potential for treating diabetes.* Nature Reviews Drug Discovery. 2012, Vol. 11, pp. 692–708 .

19. Kwon H, Pessin J.E. *Adipokines Mediate Inflammation and Insulin Resistance.* Front Endocrinol . 2013, Vol. 4, p. 71.

20. Seymour R.M, Henderson B. *Pro-inflammatory–anti-inflammatory cytokine dynamics mediated by cytokine-receptor dynamics in monocytes.* IMA J Mathematics Applied in Med and Biol. 2001, Vol. 18, pp. 159–192.

21. Ozaki K, Leonard W.J. *Cytokine and Cytokine Receptor Pleiotropy and Redundancy.* JBC. 2002, Vol. 277, pp. 29355–29358.

22. Kaminiarczyk-Pyzalka D, Adamczak K, Mikos H, Klimecka I, Moczko J, Niedziela M. *Serum TNF-α levels and indicators of disease activity in children with oligoarticular juvenile idiopathic arthritis (oJIA) in the first year of the disease.* Clin Lab. 2014, Vol. 60, pp. 799-807.

23. Harris J.G, Kessler E.A, Verbsky J.W. *Update on the Treatment of Juvenile Idiopathic Arthritis.* Curr Allergy Asthma Rep. 2013, Vol. 13, pp. 337–346.

24. Y, Okuda. *Review of tocilizumab in the treatment of rheumatoid arthritis.* Biologics. 2008, Vol. 2, pp. 75–82.

25. Tanaka T, Kishimoto T. *Targeting Interleukin-6: All the Way to Treat Autoimmune and Inflammatory Diseases.* Int J Biol Sci. 2012, Vol. 8, pp. 1227-1236.

26. Nuti F, Civitelli F, Cucchiara S. *Long-Term Safety of Immunomodulators in Pediatric Inflammatory Diseases.* Pediatric Drugs. 2014, Vol. 16, pp. 343–352.

27. Boulanger C.M, Loyer X, Rautou P.E, Amabile N. *Extracellular vesicles in coronary artery disease.* Nat Rev Cardiol. 2017, Vol. 14, pp. 259–272.

28. van der Vlist E.J, Nolte-'t Hoen E.N.M, Stoorvogel W, Arkesteijn G.J.A. Wauben M.H.M. *Fluorescent labeling of nano-sized vesicles released by cells and subsequent quantitative and qualitative analysis by high-resolution flow cytometry.* Nat prot. 2012, Vol. 7.

29. Tian L, Luo N, Zhu X, Chung B.H, Garvey W.T, Fu Y. *Adiponectin-AdipoR1/2-APPL1 signaling axis suppresses human foam cell formation: differential ability of AdipoR1 and AdipoR2 to regulate inflammatory cytokine responses.* Atherosclerosis. 2012, Vol. 221, pp. 66-75.

30. Santos-Alvarez J, Goberna R, Sánchez-Margalet V. *Human leptin stimulates proliferation and activation of human circulating monocytes.* Cell Immunol. 1999, Vol. 194, pp. 6-11.

31. Arts R.J.W, Joosten L.A.B, Netea M.G. *Immunometabolic circuits in trained immunity.* Seminars in Immunol. 2016, Vol. 28, pp. 425–430.

32. El-Osta A, Brasacchio D, Yao D, Pocai A, Jones P.L, Roeder R.G, Cooper M.E, Brownlee M. *Transient high glucose causes persistent epigenetic changes and altered gene expression during subsequent normoglycemia.* J Exp Med. 2008, Vol. 205 , pp. 2409-2417.

33. Norata G.D, Caligiuri G, Chavakis T, Matarese G, Netea M.G, Nicoletti A, O'Neill L.A.G, Marelli-Berg F.M. *The Cellular and Molecular Basis of Translational Immunometabolism.* Immunity. 2015 , Vol. 3, pp. 421-434.

34. Loftus R.M, Finlay D.K. *Immunometabolism: Cellular Metabolism Turns Immune Regulator.* J Biological Chem. 2016, Vol. 291, pp. 1–10.

35. Brasacchio D, Okabe J, Tikellis C, Balcerczyk A, George P, Baker E.K, Calkin A.C, Brownlee M, Cooper M.E, El-Ost A. *Hyperglycemia Induces a Dynamic Cooperativity of Histone Methylase and Demethylase Enzymes Associated With Gene-Activating Epigenetic Marks That Coexist on the Lysine Tail.* Diabetes. 2009 , Vol. 58, pp. 1229-1236.

36. Hu P, Zhang W, Xin H, Deng G. *Single Cell Isolation and Analysis.* Front Cell Dev Biol. 2016, Vol. 4, p. 116.

37. Arts R.J, Gresnigt M.S, Joosten L.A, Netea M.G. *Cellular metabolism of myeloid cells in sepsis.* J Leukoc Biol. 2017 , Vol. 101, pp. 151-164.

38. Cheng S.C, Scicluna B.P, Arts R.J, Gresnigt M.S, Lachmandas E, Giamarellos-Bourboulis E.J, Kox M, Manjeri G.R, Wagenaars J.A, Cremer O.L, Leentjens J, Van der Meer A.J, van de Veerdonk F.L, Bonten M.J, Schultz M.J, Willems P.H, Pickkers P, Joosten L.A, van der Poll T, Netea M.G. *Broad defects in the energy metabolism of leukocytes underlie immunoparalysis in sepsis.* Nat Immunol. 2016, Vol. 17, pp. 406-413.

Chapter 7

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Nederlandse Samenvatting

Inflammatie (ontsteking) is de belangrijkste respons van onze lichaam om pathogene stimuli te bestrijden. Verschillende triggers kunnen ontstekingen teweegbrengen, variërend van extrinsieke factoren zoals pathogenen tot intrinsieke activators zoals cel beschadiging. De functie van deze immuunrespons is het elimineren van een schadelijke factor, i.e. het verwijderen van beschadigde cellen en weefsel en het induceren van herstel door betrokkenheid van bloedvaten, en daarnaast het rekruteren van immuun cellen en een overvloed aan eiwit-bemiddelaars. Gebrekkige ontsteking kan leiden tot progressieve weefselbeschadiging door pathogenen, terwijl chronische ontsteking resulteert in chronische inflammatoire systemische ziekten (CISD) waaronder atherosclerose, reumatoïde artritis en kanker. Acute ontsteking is een primaire reactie van het lichaam op schadelijke prikkels, maar als deze niet wordt opgelost, kan de acute ontsteking een lange termijn/chronische fase ingaan: chronische ontsteking. In dit proefschrift verwijzen alle vermelding van ontsteking naar de chronische categorie.

Veel factoren spelen een rol bij het bewerkstelligen van chronische ontstekingen, in dit proefschrift hebben we ons gefocust op drie niveaus: cytokines, extracellulaire blaasjes (vesikels) en immuun cellen. Gevallen van obesitas en juveniele idiopathische artritis (JIA) zijn bovendien gebruikt om chronische ontsteking in de pediatrische populatie te illustreren en te analyseren.

Het eerste niveau (**hoofdstuk 2**) richt zich op cytokines als de kleinste factoren die betrokken zijn bij ontstekingen. Cytokines worden geacht de ontstekingsreactie te orkestreren door de interactie en communicatie tussen cellen te beïnvloeden. Chemokinen, zijn een subgroep van cytokines die chemotaxis induceren en adipokines zijn cytokines en andere immunologisch actieve eiwitten die worden uitgescheiden door adipocyten en/ of vetweefsel (AT) resident immuun cellen. In het bijzonder, belicht Hoofdstuk 2 de methodologische stappen die nodig zijn bij het implementeren van cytokines als biomarkers voor chronische ontstekingen. Door te begrijpen hoe bepaalde factoren cytokine niveaus kunnen manipuleren en te testen met de nodige controles en methodologie, kan een objectief beeld van cytokine niveaus binnen een ziekteproces worden bereikt. We raden aan dat het overhandigen en verwerken van monsters op een gestandaardiseerde manier gebeurt. De bloedbemonsteringsbuizen binnen een onderzoek moeten hetzelfde blijven. Bovendien raden wij aan om direct na afname het monster op ijs of op 4 ° C te houden, alvorens het monster te scheiden. Het proces van monster-scheiding moet vervolgens zo snel mogelijk worden uitgevoerd en de producten van deze scheiding moeten vervolgens worden bewaard bij -80 ° C voor langdurige opslag. Bij het uitvoeren van cytokine-analyses, afhankelijk van de herkomst van het monster, moet matrix interferentie worden uitgevoerd door het verwijderen van belemmerende stoffen of door reagentia te kiezen die resulteren in de laagste interferentie tijdens de test. Door dit algemene overzicht te volgen, zullen de gegevens die in een onderzoek zijn geproduceerd, de cytokine niveaus bij patiënten duidelijk weergeven en maximale datamining mogelijk maken.

Het tweede niveau (**hoofdstuk 3**) onderzoekt de rol van extracellulaire blaasjes (EVs) bij chronische ontstekingen. EVs zijn kleine membraangebonden deeltjes die door alle menselijke celtypen worden afgescheiden. De EV-populatie is een heterogene mix van deeltjes met verschillende groottes. Het kan worden voortgebracht in de endosomale compartimenten van cellen of wordt in de omgeving gestort na het ontluiken en de splijting van het plasmamembraan. EVs kunnen immuun stimulatie en -onderdrukking beïnvloeden, daarnaast is het aangetoond dat ze de pathogenese van ontstekings-, auto-immuun- en infectieziekten bevorderen. In het kader van onze experimenten konden we aantonen dat EVs uit monsters van pediatrische patiënten met chronische ontsteking met succes kunnen worden geïsoleerd en geïmplementeerd in functionele testen. Onze gegevens wijzen erop dat EVs kunnen worden geïsoleerd van kleine hoeveelheden biologische vloeistof (synoviale vloeistof en plasma uit perifeer bloed). Bovendien kunnen EVs, ongeacht de bron-vloeistof, JIA-geassocieerde cytokineproductie in beperkte mate wijzigen in vergelijking met niet-gestimuleerde omstandigheden. Hoewel deze en andere gegevens de mogelijke rol van EVs bij chronische

ontstekingen ondersteunen, blijft het, vanwege suboptimale isolatie- en identificatie methoden, moeilijk om de specifieke functies van EV-populaties echt te specificeren en vast te stellen.

In het laatste niveau van dit proefschrift richten we ons op cellulair niveau door ons te concentreren op de rol van immuun cellen bij pediatrische chronische ontstekingen. Monocyten, macrofagen en T-lymfocyten zijn de primaire immuun cellen die betrokken zijn bij chronische ontsteking. In **hoofdstuk 4** en **5** wordt chronische ontsteking geïllustreerd aan de hand van obesitas bij kinderen.

In **hoofdstuk 4** analyseren we adipokine receptor-expressie op verschillende cellulaire subsets in combinatie met circulerende adipokine niveaus. We konden inzicht verschaffen in een eerder onontgonnen deel van pediatrische obesitas. Zwaarlijvige kinderen vertoonden kenmerkende klinische en biochemische kenmerken vergeleken met een controlegroep. Subgroepen van circulerende leukocyten vertoonden verschillende adipokine-receptor expressie-profielen. Opvallend was dat de aantallen leukocyt subsets en adipokine receptor expressie-profielen grotendeels vergelijkbaar waren bij kinderen met obesitas en de controlegroep. Zwaarlijvige kinderen vertoonden hogere naïeve B-celaantallen en, pre-interventie ook hogere aantallen onvolwassen transitite (immature transition) B-cellen en intermediaire CD14 + + CD16 + monocyten gecombineerd met lagere totale monocytenaantallen, vergeleken met de controlegroep. Bovendien werd de adiponectine receptor 1-expressie op niet-klassieke CD14 + CD16 ++ -monocyten consequent upgereguleerd in de pre-interventie bij obese kinderen, in vergelijking met de controles. Echter, de verschillen in leukocyten subset en adipokine receptor expressie tussen obese kinderen en de controlegroep bleken niet statistisch significant na meerdere test correcties. Veelal kunnen de verschillende adipokine receptor-profielen van circulerende leukocyten subsets het onderscheidende effect van adipokines op leukocyten subsets enigszins verklaren. Ten tweede suggereren de overeenkomsten in adipokine receptor expressie-profielen van beide groepen dat adipokine signalering bij kinderen met obesitas primair wordt gemoduleerd door circulerende adipokine niveaus, in plaats van adipokine receptor-expressie.

In **hoofdstuk 5** onderzoeken we bij een pediatrische cohort met obesitas de invloed van obesitas op de genexpressie van monocyten en de daaropvolgende associatie van deze genen met circulerende adipokines en klinische variabelen. Daarnaast wordt het gevonden genexpressie profiel vergeleken met een volwassene cohort met coronaire atherosclerose. Monocyten zijn belangrijke spelers in de ontwikkeling en verergering van atherosclerose, onze vraag was dus of de monocytose bij kinderen met obesitas door de jaren heen zou kunnen bijdragen aan atherogenese. Obesitas bij kinderen hangt samen met een toenemend aantal circulerende klassieke CD14 + + CD16- en CD14 + CD16 + -monocyten. We analyseerden het monocyt-genexpressieprofiel van de obese kinderen met behulp van een Illumina-microarray platform op gesorteerde monocyten van 35 kinderen met obesitas en 16 slanke controles. Zwaarlijvige kinderen vertoonden een kenmerkend monocyt-genexpressieprofiel in vergelijking met slanke-controles. Na validatie met kwantitatieve PCR, bestudeerden we de associatie van de top 5 differentieel gereguleerde monocyt genen in obesitas bij kinderen met obesitas, evenals de complexiteit van coronaire atherosclerose (SYNTAX-score) in een cohort van 351 volwassenen met een verhoogd risico op ischemische cardiovasculaire aandoeningen. De downregulatie van monocyt IMPDH2 en TMEM134 bij kinderen met obesitas werd ook waargenomen bij obese volwassenen. Bovendien was downregulatie van monocyten TMEM134 geassocieerd met een hogere SYNTAX-score voor atherosclerose bij volwassenen. Samenvattend, obesitas bij kinderen houdt verband met monocyt-genexpressie-veranderingen die zijn geassocieerd met obesitas en verhoogde complexiteit van coronaire atherosclerose bij volwassenen.

Uiteindelijk, wordt in **hoofdstuk 6 (discussie)** de rol van cytokines, adipokines, EVs en immuun cellen in de context van chronische pediatrische inflammatie besproken. We hebben gezien dat cytokines belangrijke spelers zijn bij immuunmodulatie. Analyse en beïnvloeding van cytokines en cytokine-aktiviteit kan geïmplementeerd worden voor zowel ziekte gerelateerde biomarkers als een therapeutische interventies. Zoals hierboven vermeld, kunnen echter verschillende factoren de cytokine meting beïnvloeden, wat het moeilijk kan maken om gegevens naar de biologische situatie

te extrapoleren en gegevensvergelijking kan verwarren. Bovendien weerspiegelen de cytokine niveaus in de circulatie niet altijd de lokale situatie, er wordt weinig begrepen van het cytokine-netwerkdynamiek en tenslotte wordt redundantie in cytokine-functionaliteit niet altijd in overweging genomen bij het interpreteren van gegevens. Het is daardoor belangrijk dat al deze factoren meegenomen worden in het gebruiken van cytokines in het bestrijden van chronische ontstekingen. Zoals eerder vermeld, kunnen EVs zowel immuun activatie als suppressie veroorzaken, maar de vesikel isolatietechnieken zijn een van de belangrijkste zwakheden in het bepalen van de functie van deze deeltjes bij chronische ontsteking. Toekomstige technieken moeten dus ondersteuning bieden voor de analyse van afzonderlijke deeltjes met hoge resolutie, dit zal het mogelijk maken dat de vesikel inhoud en -dynamiek beter kunnen worden begrepen. Het is hoopvol dat de technologieën voor de isolatie en manipulatie van immuun cellen voortdurend wordt verbeterd. Het vermogen om afzonderlijke cellen en specifieke populaties te isoleren met behulp van FACS-sorteringstechnologie betekent dat bepaalde ziekte gerelateerde populaties kunnen worden bestudeerd en begrepen. Ons werk illustreert echter dat het ontcijferen van de rol van immuun cellen bij chronische ontsteking en atherogenese problematisch is met alleen FACS-technologie. Cellulaire markers vertegenwoordigen niet op zichzelf de immuun cel functie, aangezien factoren zoals immuun cel metabolisme, cytokineproductie en verschillende cellulaire processen ook bijdragen aan immuun cel activiteit. Uitgebreide fenotypering van de immuun cel is, in combinatie met functionele testen, daarom noodzakelijk om de rol van immuun cellen in de context van chronische ontsteking te bepalen.

Samengevat, het combineren van oude kennis, het optimaliseren van technologieën, het voortzetten van het huidige werk en het implementeren van de toekomstige stappen die in dit proefschrift worden gepostuleerd, zou nieuwe manieren kunnen tonen om de chronische ontstekingsfase te begrijpen en te manipuleren. Deze geïnformeerde manier om ontstekingen te behandelen is met name relevant in de pediatrische populatie, aangezien de behandelingen die worden toegepast bij kinderen hun kwaliteit van leven waarborgen en, in het bijzonder, zou moeten bijdragen aan een ziektevrije levensduur bij deze populatie.

Curriculum Vitae

Genoveva was born on August the 4th 1989 in Blantyre, Malawi where she attended Primary (Phoenix int.) and High School (Saint Andrews Int.) till she moved to the Netherlands in 2007. Between 2007 and 2010 she completed her Bachelor's degree (Hons.) at University College Utrecht with a Pre-med focus. Additional courses she took during this time also included psychology, philosophy and Dutch. During these years Genoveva came into contact with the field of Infection and Immunity, a multidisciplinary field in which physiology, biology, medicine and out of the box thinking came together. As a result of this exciting and evolving arena, Genoveva enrolled in the Infection and Immunity Master program of Utrecht University (2010-2012). For her Major research project (internship) Genoveva looked to work with the pediatric population, a demographic she has always been passionate about. Through this search, she came into contact with the group of Berent Prakken (where she would eventually perform her PhD). This internship bolstered her passion for understanding and altering immune system functionality with the hopes of curing and curbing disease pathology in the pediatric population. In 2012, Genoveva began her PhD under the supervision of a super team: Prof. Berent Prakken, Prof. Gerard Pasterkamp, Wilco de Jager (PhD) and Dr. Henk Schipper (PhD), looking at inflammatory mediators in childhood chronic diseases. During her PhD Genoveva additionally participated in extracurricular activities by joining (2013) the Graduate School of Life Science PhD Council and the Infection and Immunity Education Committee. In June 2014 she became Chair of the PhD council and filled this role with great pleasure till May 2016. In August 2016 Genoveva completed her laboratory work and in September 2016 she began working part time as Research Project Coordinator of the Utrecht University Graduate School of Life Sciences. In March 2017 she switched departments within the Education Center and began working as an Advisor for Doctoral Education for the Utrecht University Graduate School of Life Sciences. During her free time Genoveva also works as a CrossFit Trainer as well as a Functional Coach. In February 2018 Genoveva decided to combine her passion for helping people, her analytical mind, her joy in presenting and connecting and her skills in training and coaching to start her own company: KEUS.

List of Publications

Keustermans G, van der Heijden L.B, Boer B, et al. Differential adipokine receptor expression on circulating leukocyte subsets in lean and obese children. Appel S, ed. *PLoS ONE*. 2017;12(10):e0187068. doi:10.1371/journal.pone.0187068.

Keustermans G.C, Kofink D, Eikendal A, et al. Monocyte gene expression in childhood obesity is associated with obesity and complexity of atherosclerosis in adults. *Scientific Reports*. 2017;7:16826. doi:10.1038/s41598-017-17195-3.

Keustermans G.C, Hoeks S.B, Meerding J.M, Prakken B.J, de Jager W. Cytokine assays: An assessment of the preparation and treatment of blood and tissue samples. *Methods.* 2013; 15;61 (1):10 http://www.ncbi.nlm.nih.gov/pubmed/23603216

Isolating extracellular vesicles from juvenile idiopathic arthritis synovial fluid; a feasibility study for functional assays. Genoveva Keustermans, Nienke Kettelarij, Rianne Scholman, Belinda van't land, Henk S. Schipper, Berent Prakken, Wilco de Jager. *(In preparation)*

Dankwoord

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