Inflammatory Aspects of Type 2 Diabetes in the Andean Region

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Inflammatory Aspects of Type 2 Diabetes in the Andean Region

Inflammatoire aspecten van type 2 Diabetes in de Andes

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To my reason for living, Gabriel

CONTENTS

Chapter 1

General Introduction

GENERAL INTRODUCTION

This thesis deals with the immune inflammatory aspects of obesity, the metabolic syndrome (MetS), insulin resistance and type 2 diabetes (T2D) in the Andean region, more precisely in Quito, Ecuador. To understand the research questions a short introduction in the immune system is presented first; followed by a brief description of the pathophysiological and immune aspects of obesity, insulin resistance, the MetS and T2D.

THE IMMUNE SYSTEM

The immune system in general

The immune system is composed of cells, tissues and molecules aimed at responding to disturbances of the homeostatic condition, either from external or internal origin. Thus, the immune system has various beneficial functions such as defense against pathogens and tumors via an inflammatory reaction, followed by the resolution of inflammation and tissue repair. In contrast to these beneficial functions, abnormally directed and excessive inflammatory immune responses are the cause of allergies and auto-inflammatory and autoimmune diseases, such as atopic diseases, autoimmune thyroid disease, type 1 diabetes (T1D), Sjögren's disease, rheumatoid arthritis, Celiac Disease and Crohn's disease.

Innate immune system

The innate immune system is the first line of defense against tissue damage, and pathogens in particular and provides a fast response with a limited specificity. The key players are: 1) barriers, such us tears, saliva and skin; 2) defense cells, which comprise neutrophils, cells of the Mononuclear Phagocyte System (MPS) (monocytes and macrophages), natural killer and mast cells; and 3) proteins (complement, cytokines and chemokines). If the innate system fails to resolve the attack and subsequent infection with and/or invasion by microbes, the adaptive immune system will get triggered by the cells of the innate immune system. This process of triggering is considered as the inter phase between the innate and adaptive immune response (see Fig.1).

The interphase between the innate and adaptive immune response

The cells responsible for the activation of the adaptive immune response and thus the key players of the interphase are the various antigen presenting cells (APCs). Accessory macrophages are an example of these APCs, but the most specialized APCs are the dendritic cells (DC), which can be considered as part of the MPS and in many instances are derived from monocytes.

DC pick up antigen at distant sites where the pathogens have invaded, or otherwise damage has occurred, travel through the lymphatics to the lymph node (as so-called veiled cells) where they present the collected antigens to lymphoid cells of the adaptive immune system. With the presence of MCH class II molecules and various costimulatory molecules on their cell surface, DC are well equipped to stimulate the naïve cells of the adaptive immune system, present in the lymph nodes. These latter cells will subsequently proliferate and be activated to play their role in the combat against the invading pathogens.

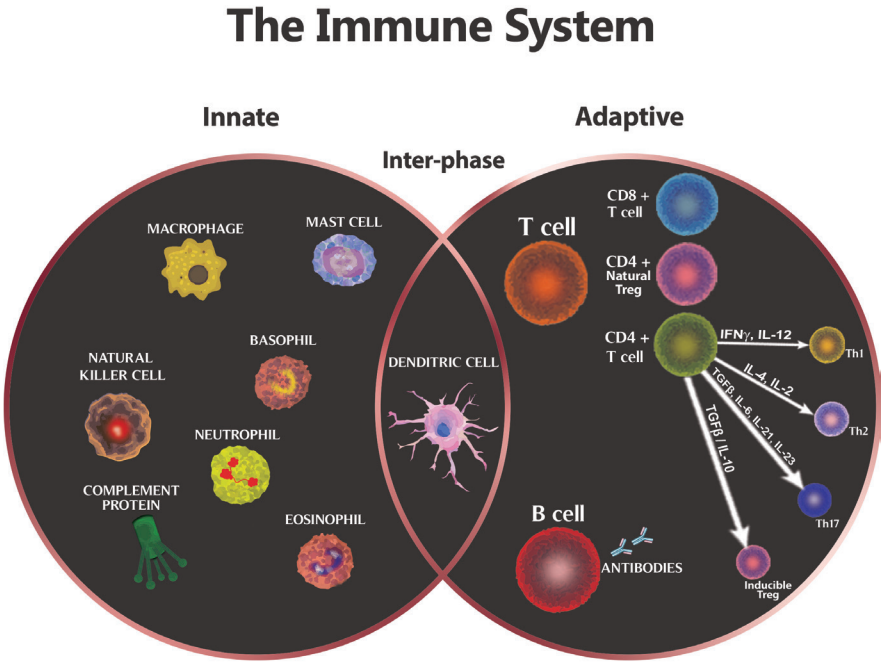

Figure 1. Key elements of the Immune system. See further text.

Adaptive immune system

The adaptive immune response is highly antigen specific, provides a memory and is typically activated a few days later than the innate system. The cellular contributors are the T cells and the B cells. B cells, which transform into plasma cells upon final maturation, are responsible for the production of antibodies. Antibodies neutralize extra cellular bacteria, viruses and toxins, activate complement and facilitate pathogen phagocytosis. T cells are responsible for so-called T cell cell-mediated immunity. T cells form a heterogeneous group of cells comprising effector cells, regulatory cells and memory cells which are

typically divided into CD4+ and CD8+ T cells. The effector CD8+ T cells are cytotoxic and important in killing other body cells infected with virus, intra-cellular bacteria or cells which are otherwise damaged or dysfunctional. The effector CD4+ T cells are important in providing help to other immune cells and are therefore called T helper cells (Th). A naïve T cell (a cell which has not encountered antigen yet presented by an APC) can differentiate into at least three Th subtypes, namely Th1, Th2 and Th17 cells, upon stimulation by an APC. Th1 cells are capable of activating macrophages via Interferon γ (INF-γ), and are in this way essential in the elimination of intracellular pathogens. Th2 cells are activated by IL-4 and IL-2 cytokines and play a central role in the transformation of B cells into plasma cells for the production of antibodies, activation of eosinophils and alternative activation of M2 macrophages (see later). The third member of Th cells, the Th17 differentiates from naïve Th cells upon activation by TGF-β, IL-6 and IL-23. Recent studies show that Th17 cells that have been activated mainly by TGFβ and IL-6 are prone to maintain barrier tissue integrity and are crucial in host defense against extra-cellular pathogens (e.g. bacteria and fungi) [1-3]; whereas IL-23 activated TH17 cells induce chronic tissue inflammation and play an important role in mediating autoimmune pathologies [4-7].

Next to these effector T helper cells, there are also CD4+ T cells with regulatory functions as the immune system needs to be tightly regulated; it is well equipped to damage the host lethally if control is lost over the immune system. The best known CD4+ T regulatory cell population is the natural T regulatory cell population, which is formed by CD4+CD25high T cells. Naturally occurring CD4+CD25high Treg cells are defined by the constitutive high expression of the receptor of IL-2 (CD25 is the IL-2 receptor α chain; IL-2 is indeed required for triggering their suppressive function) and the key transcription factor FOXP3 that is required for their development, maintenance and function [8-10]. In addition to CD25 and FOXP3 expression these Tregs are characterized by high surface expression of CTLA-4. A feature of CD4+CD25+FOXP3+ T cells is their striking lack of proliferation following TCR engagement, leading to the characterization of this population as naturally "anergic" [11-13]. Natural T reg cells are spontaneously formed in the thymus from where they are exported and recirculate through secondary lymphoid tissues as 'central' T reg cells. Activation signals involving T cell receptor (TCR) ligation, CD28 co-stimulation and/or interleukin 2 (IL-2) induce the upregulation of expression of interferon regulatory factor 4 (IRF4), which orchestrates their differentiation into 'effector' T reg cells. Further effector T reg cell differentiation involves BTB and CNC homologue 2 (BACH2) downregulation and B lymphocyte-induced maturation protein 1 (BLIMP1) upregulation [13].

Other known regulatory CD4+ T cell are the induced T regulator cells, e.g. Tr1 cells (defined by high production of the anti-inflammatory cytokine IL-10) and the Th3 cells (defined by a high production of the anti-inflammatory cytokine TGF-β) [9, 14-17] (see Fig. 1). These cells are not spontaneously formed in the thymus, but are induced from naïve

CD4+ T cells in the periphery [13]. Typically, these regulatory populations do not express FOXP3 and in contrast to natural Treg cells, induced Treg cells often have a restricted specificity for particular cell types, tumors or foreign antigens [17]. Therefore, induced T reg cells may be important to keep immune reactions to infectious agents under control [17].

Interleukin-10 (IL-10), transforming growth factor-β (TGF-β), and interleukin-35 (IL-35) have been described to play an essential role in Treg function [11, 13, 18, 19]. However, the expressions of these cytokines depend on the Treg subset and are tissue-specific. IL-10 can directly suppress T cell proliferation, decrease the expression of MHC-II, decrease costimulatory molecules on DCs, and regulate the function of mast cells and eosinophils [9, 19-21]. TGF-β is also an important factor secreted by Treg cells, which has been show to mediate the regulatory functions of auto reactive T cells. [13, 19, 22-24]. TGF-β knockout mice develop an early fatal multiorgan inflammation and T-cell hyper activation [23]. Recent studies have indicated that the cytokine IL-35, composed of the IL-12 α-chain and IL-27 β-chain, is an important factor for Treg function acting as an effector cytokine and also regulating Treg homeostasis [19, 25-27].

Not only these soluble factors have an important role in the regulatory capacity of Tregs, but also specific cell-surface molecules appear to be responsible for the suppressive effects. Thus, expression of CTLA-4 on Tregs keeps them in their hyporesponsive state and is crucial for maintaining their suppressive function [13, 19, 28, 29]. The CD4-related protein LAG-3 has been implied to contribute to Treg suppressive function via direct Treg-Teff interactions and modulating APC function [30, 31]. Finally, reports show that the Ig-like type I transmembrane protein CD83 expressed on the surface of Tregs can also directly contributes to contact dependent suppression [32, 33].

Monocytes, macrophages in detail

This thesis focusses in particular on monocytes in patients with T2D and therefore we discuss this group of cells in some more detail. Monocytes belong to the "Mononuclear Phagocyte System (MPS)". The MPS includes the promonocytes and their precursors in the bone marrow, the monocytes and dendritic cells in the peripheral blood, and the macrophages in the tissues [34, 35]. This group of hematopoietic cells has diverse characteristics and origins. Many of these cells originate in the bone marrow and migrate as monocytes through the blood to peripheral tissues. The main descendants of circulating monocytes, the macrophages and DC, occur in virtually all organs. Local myeloid precursors for macrophages and DC are also present in various organs. Each organ contains multiple different macrophage and DC subpopulations characterized by the expression of their specific cell surface and intracellular molecules. The best-known function ascribed to macrophages is clearance of pathogens and dead cells by phagocytosis and digestion. The best-known function of DC is their role in the primary activation of the adaptive immune

responses. Both DC and macrophages are capable of up and down regulating immune responses. An important characteristic of these cells is the expression of innate antigen receptors, such as toll-like receptors (TLRs) that can bind to both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)[36], which can be found on microbes and damaged body cells. In such 'danger' situations, when the cells of the MPS are triggered via such receptors, the cells of the MPS become pro-inflammatory, and then guide an effective T cell response to eliminate the danger-evoking signal. In the normal "steady state" situation without any danger the MPS cells stay in their "innate steady state", which is tolerogenic, with other words the DC are active, take up primarily auto-antigens and do interact with T cells, primarily the natural T regulatory cells, to serve tolerance induction and maintenance.

Monocytes are part of the MPS and thus part of the innate immune system and constitute 2-10% of all leukocytes in the peripheral blood [36, 37]. Morphologically, they have an amoeboid shape, clear cytoplasm, a large horseshoe-shaped nucleus, a well-developed Golgi complex and various intracytoplasmic lysosomes. Typically, human monocytes express cell surface markers such as CD14 and, dependent on the subset, CD16 [36, 38]. In response to chemotactic signals, monocytes can move relatively quickly (approx. 8–12 hours) to sites of infection in tissues and divide/differentiate into macrophages and DC to elicit an adaptive immune response [36, 37].

Macrophages were first identified by Elie Metchnikoff in 1883. These cells are considered the key players of the innate immune system [34]. Nowadays, they are recognized as important pathophysiologic agents in wide-spread disease processes associated with aging (cancer) and chronic inflammation (e.g. the MetS, T2D, and atherosclerosis)[39, 40]. Macrophages have two main characteristics that are diversity and plasticity. Depending of the triggering factor macrophages can polarize and skew towards two polar (M1 or M2) phenotypes. The M1 and M2 phenotype are in fact caricatures, and in-between forms exist as well as polarities in other directions , which are characterized by the production of specific growth factors, and play a role in vascular repair [41-43] or neuronal growth [44-46]. Also M1 and M2 macrophages can be converted into each other in their specific microenvironments [47,48]. M1 macrophages (also called classically activated macrophages, typified by those that have been stimulated with a TLR-ligand such as LPS and IFN-γ) are pro-inflammatory and have a central role in host defense against pathogens and promoting inflammation, while M2 macrophages (also called alternatively activated macrophages) are associated with responses to anti-inflammatory reactions, tissue repair and remodeling [40, 47-50]. M1 phenotype macrophages express numerous pro-inflammatory mediators including TNF-α, IL-1, IL-6, reactive nitrogen and oxygen intermediates, which have a strong microbicidal and tumoricidal activity, while M2 macrophages express molecules

including resistin-like-α (Fizz1), Arginase1, chitinase 3-like 3 (Ym1), IL-10 and Mrc1 (CD206), which are involved in immunoregulatory functions [47, 48, 51] (see Fig.2).

IFN-γ, which is mainly produced by Th1 cells and natural killer (NK) cells, is the main cytokine associated with M1 polarization. IFN-γ induces gene expression of cytokine receptors (CSF2RB, IL15 receptor alpha, IL2RA, and IL6R), cell activation markers (CD36, CD38, CD69, and CD97), and cell adhesion molecules (ICAM1, integrin alpha L, ITGA4, ITGbeta-7, mucin 1, and SIAT1) [51-53]. IFN-γ knockout (KO) mice show impaired production of antimicrobial products, and these mice are susceptible to bacterial infections [52, 54, 55]. On the other hand, IL-4 which is mainly produced by Th2 cells, eosinophils, basophils, and macrophages is the main cytokine associated with M2 polarization [52, 56]. IL-4 activates several transcription factors such as JAK1, JAK3, c-Myc and IRF4, Krüppel-like factor 4 , and the signaling modulators CISH and SOCS1 [52, 53, 57, 58]. IL-4 KO mice have defects in the immune response against some viral infections and nematodes [52].

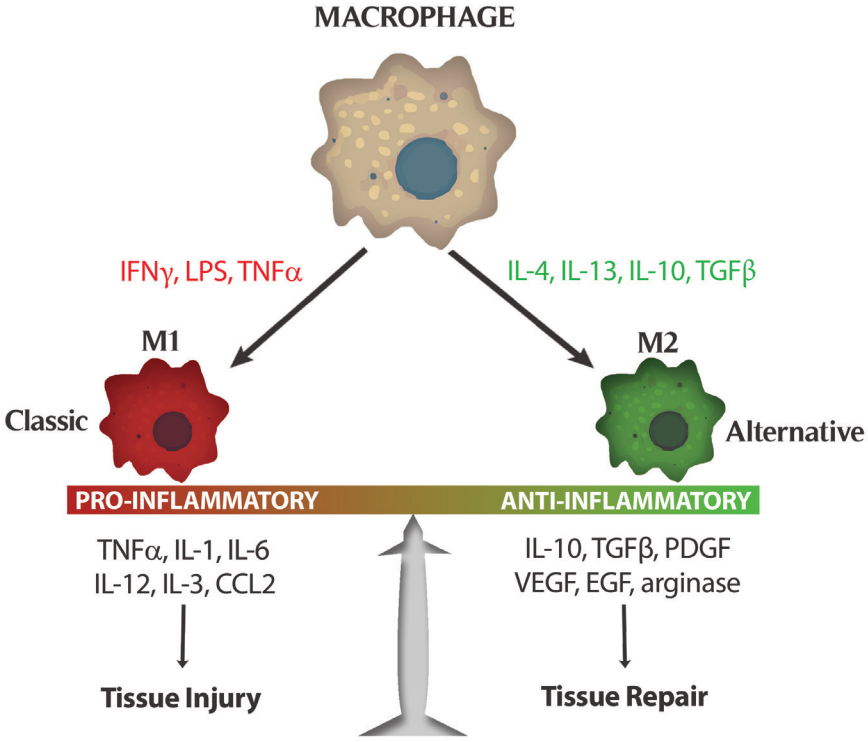

Figure 2. The prototypical M1 and M2 macrophages.

The vascular repair function of monocytes

Classically it is thought that monocytes/macrophages only play a vascular destructive role in the process of atherosclerosis (as M1-polarized cells). However specific monocyte/macrophage populations have the potential to repair the blood vessel wall upon injury [59]. In general endothelial repair is mediated by proliferation and differentiation of local endothelial cells stimulated via the production of growth factors and cytokines that aid in the repair process. In this process, also circulating pro-angiogenic cells (PACs) are critical [60-62]. PACs are recruited from the bone marrow and are a heterogeneous cell population expressing endothelial characteristics when stimulated [59]. Nowadays it is recognized that different cell populations derived from the bone marrow are involved in neovascularization and re-endothelialization [59, 63-65]. PACs are closely linked to monocytes/macrophages, and can be derived from the fraction of circulating CD14 + cells [60, 61, 63] . In situations of vascular injury, the CD14 + cells may develop an anti-inflammatory phenotype and become pro-angiogenic cells [60, 64].

ROLE OF IMMUNITY IN OBESITY AND INSULIN RESISTANCE

Fat as a chronically inflamed tissue in obesity

Adipose tissue is an active metabolic and endocrine organ. Loss of immune regulation in this tissue is the major factor contributing to the development/progression of insulin resistance and T2D [66]. Chronic mild Inflammation of adipose tissue, in particular visceral adipose tissue, causes production of proinflammatory cytokines/chemokines which are capable of inactivating the insulin receptor via induction of serine phosphorylation; this has as result the blockade of insulin receptor signaling in multiple cell types, including muscle and liver cells.

There is accumulating evidence to suggest that both innate and adaptive immune cells are skewed towards a proinflammatory set point in visceral adipose tissue of obese subjects [67-72]. The consequent adipose tissue dysfunction is manifest not only for the secretion of cytokines (e.g. of TNF, IL-6, IL-1β, PAI1) and chemokines (CCL2, CCL4, CCL20, CXCL14), but also for the secretion of proinflammatory adipokines (e.g. leptin) [67, 73-75]. This chronic proinflammatory secretion pattern in adipose tissue induces a systemically low grade of inflammation which perpetuates the positive feedback loop of inflammation [71].

It is unknown which are the trigger factors that activate the immune cells in adipose tissue. A combination of different endogenous and exogenous danger signals can be the initiators. It is well described that saturated fatty acids (e.g. palmitic acid), specific cytokines (e.g. TNF) and also pathogen-associated molecular patterns (PAMPs) (e.g. LPS)

can directly activate the NLRP3 inflammasome, inducing the activation of inflammatory cells in adipose tissue and the secretion of IL-1β and other inflammatory cytokines [76-78]. Also, some reports show that fatty acids can activate TLR4 and TLR2 in macrophages and adipocytes [79, 80].

Interestingly, there is evidence that suggest that a high-fat and high-carbohydrate diet by itself could result in chronic innate immune-driven inflammation. Wang et al, showed that the intestinal epithelial cell barrier of animals on a high fat diet (HFD) could be disrupted, consequently gut antigens and gut microbial PAMPs, like LPS, can circulate and stimulate inflammatory activation of innate and adaptive immune cells in visceral adipose tissue (VAT) [81].

Role of the innate immune system in obesity and insulin resistance

In lean individuals in and surrounding the adipose tissue there is a predominance of alternatively activated (M2-like) macrophages which have anti-inflammatory (IL-10 secretion) and repair functions. In the initiation of obesity there is a progressive accumulation of classically activated (M1) macrophages which have pro-inflammatory functions [52, 53]. In chronic obesity, the infiltration of M1 macrophages into obese adipose tissue is important, forming "crown-like structures" [82, 83]. These M1 macrophages release inflammatory cytokines and chemokines such as IL-1β, IL-6, TNFα and CCL2 [52, 72, 84, 85]. Notably, under these conditions adipocytes increase not only in number but also in size, this stimulates them to secrete inflammatory products inducing a further positive feedback loop of inflammation. Importantly, Patsouris et al show that the ablation of CD11c-positive cells, which is expressed by classically activated macrophages, normalizes insulin sensitivity in obese insulin resistant animals [86]. It is known that the chemokine CCL2 plays a prominent role in macrophage infiltration. In accordance, systemic deletion of CCL2 prevents inflammatory macrophage accumulation in adipose tissue and diminishes insulin resistance [85, 87, 88].

As previously mentioned in normal conditions adipose tissue is infiltrated and surrounded by M2 macrophages. The cytokines that stimulate the differentiation of these macrophages are IL-4 and IL-13 [56, 57] . Notably, 90% of IL-4–expressing cells in adipose tissue are eosinophils suggesting that these cells have an important role in fat immune regulation under normal, lean conditions [89, 90].

Additionally, other cells of the innate system such as mast cells are importantly increased in visceral adipose tissue, these cells can also promote and regulate inflammation [91, 92]. On the other hand, the invariant NKT (iNKT) cells are importantly reduced [90, 93, 94]. Lynch et al reported that HFD mice lacking iNKT cells show increased insulin resistance and weight gain [93]. Furthermore, evidence shows that neutrophils infiltrate adipose tissue as early as 3 days after the initiation of a HFD in animal models and neutrophil elastase

production can contribute to inflammation [90, 95, 96]. Importantly, mice deficient in neutrophil elastase are protected against HFD-induced obesity and insulin resistance. Increased activity of neutrophil elastase has also been described in the serum of obese human subjects [97] (see Fig. 3).

In sum, a plethora of innate immune cells and innate immune cell products regulate the inflammatory state of adipose tissue and consequently the insulin resistance state.

Role of the adaptive immune system in obesity and insulin resistance

Recent evidence shows that the adaptive immune system also plays an important role in obesity, insulin resistance and T2D [72, 90, 98] . It is thought that T cells infiltrate adipose tissue before macrophage accumulation [74, 99]. Infiltrating cytotoxic T-cells are capable of secreting IFN-γ promoting an overactive Th1 cell response and the recruitment and activation of M1 macrophages increasing adipose tissue inflammation [74, 98, 100]. Additionally, infiltrating CD8+ T cells can also produce elevated levels of chemokines such as CCL5 and CXCL1 which are contributing factors to the recruitment of macrophages into adipose tissue [90, 101, 102] .

Bertola et al, showed $CD11c^{high}$ $F4/80^{low}$ dendritic cell infiltration of adipose tissue in obese mice and these cells were capable of inducing the differentiation of proinflammatory Th17 cells from naïve T cells [103].

Not only T cells, but also B cells contribute to obesity-associated inflammation in adipose tissue [90, 98-100]. This has been shown in mice rendered B cell-deficient via genetic means or Ab treatment. Also the transfer of IgG2c from HFD mice can induce insulin resistance in recipient mice [100].

Importantly, the suppressive function of immune cells is affected in obesity as well [90, 104]. The function and number of T regulatory cells are altered in adipose tissue of obese mice [93, 105-107].

Depletion of Tregs in animal models induced insulin resistance [90, 106]. Tregs from obese HFD mice have reduced expression of IL-10 [90, 108] . Also, follicular B cells have reduced IL-10 production [99] (see Fig.3).

However it must be noted that in a recent study of our group morbidly obese subjects had a selective increase in peripheral circulating blood CD4+ naive, memory, natural CD4+CD25+FoxP3+ Treg and Th2 T cells, whereas CD8+ T cells were normal [109]. CD4+ and CD8+ T-cell proliferation was increased, whereas the TCRB repertoire was not significantly altered. Plasma levels of cytokines CCL5 and IL-7 were elevated. Total CD4+ T-cell numbers correlated positively with fasting insulin levels. We thus found the peripheral blood T-cell compartment of morbidly obese subjects characterized by increased homeostatic T-cell proliferation to which the cytokines IL-7 and CCL5, among others, probably contributed. This homeostatic T-cell proliferation was associated with increased CD4+ T cells numbers,

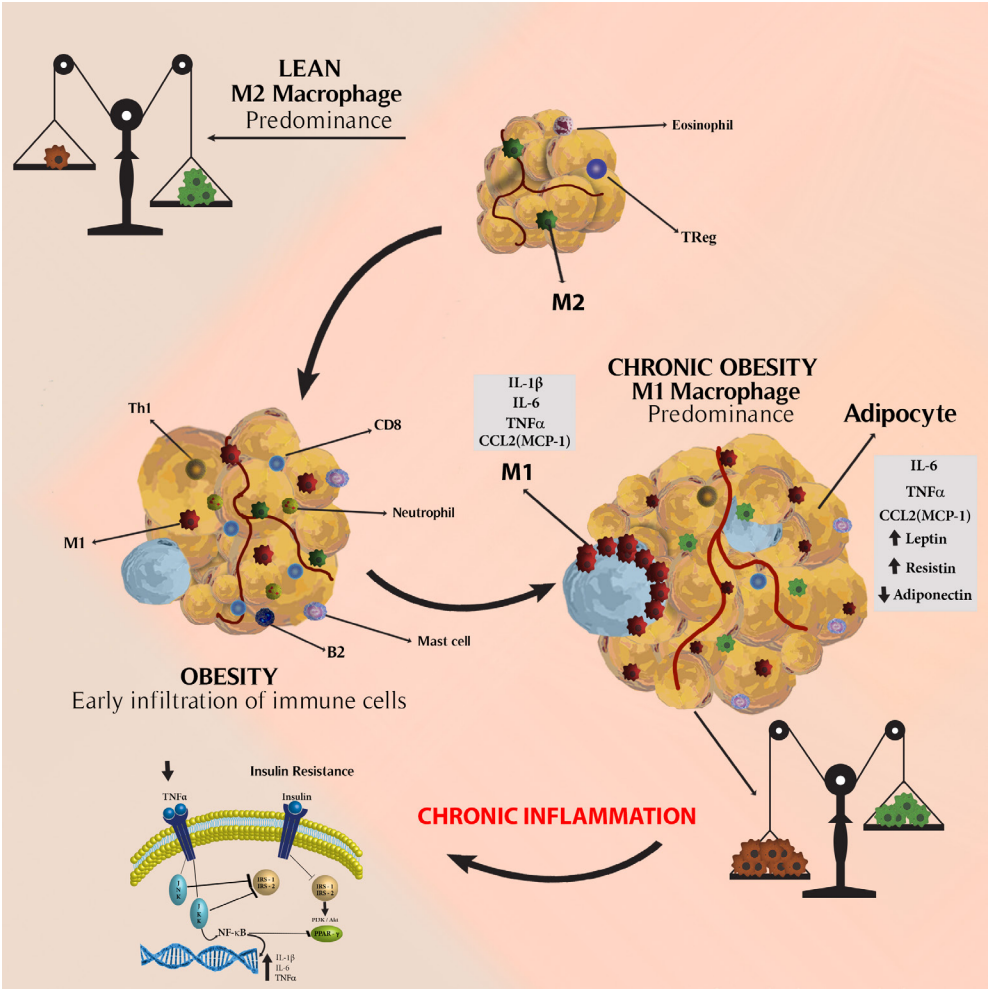

Figure 3. Immunity in Obesity and Insulin Resistance.

with a skewing toward a T regulatory and Th2-dominated phenotype, suggesting a more anti-inflammatory T cell set point of morbidly obese subjects in the peripheral blood [109].

Without a doubt, obesity induces several alterations in the innate and adaptive immune system characterized by a combination of local hyper activation of immune cells, as well as a gain or loss of immune regulation (M2 macrophages, Tregs, and B cells) in different tissue compartments.

TYPE 2 DIABETES

Disease in general

T2D comprises a group of metabolic disorders characterized by a disturbance in glucose homeostasis, resulting in increased blood glucose levels, due to a low responsiveness of target tissues (skeletal muscle, adipose tissues, liver etc.) to insulin and insufficient insulin production/secretion by pancreatic β cells. T2D is therefore characterized by three pathophysiological abnormalities: 1) peripheral insulin resistance, that is the inability of the target tissues (mainly skeletal muscle, liver and adipose tissues) to respond to raised blood concentrations of insulin; 2) impaired insulin secretion, that is the incapacity of the β cells to compensate this resistance (which is effective in the phases before the overt hyperglycemia) and, finally, 3) excessive hepatic glucose production [110]. No single etiologic factor has been defined as the cause of T2DM, although important risk factors include age, ethnicity, and family history together with obesity.

The prevalence of the Metabolic Syndrome (MetS) and T2D is increasing exponentially worldwide reaching almost epidemic proportions. The MetS is defined as having central obesity (defined as waist circumference with ethnicity-specific values) plus any two of the following four factors: raised triglycerides ≥ 150 mg/dL (1.7 mmol/L); reduced HDL cholesterol < 40 mg/dL (1.03 mmol/L) in males and < 50 mg/dL (1.29 mmol/L) in females; raised blood pressure systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg; raised fasting plasma glucose (FPG) ≥ 100 mg/dL (5.6 mmol/L) [111]. The International Diabetes Federation reported that worldwide currently 371 million people have diabetes, with a prevalence of 8.3%. In 2014, 4.8 million people died from this cause. Despite all efforts about 187 million people are estimated to be unaware of their diabetic condition. With this trend, it is estimated that in 2030, 552 million people will be affected [112, 113]. The developing countries are most vulnerable; four out of five people with diabetes live in low-income countries. Currently, in South and Central America 26 million people suffer from this disease, however 45.5% of cases have not yet been diagnosed. Projections indicate that by 2025 the disease will rise to 40 million individuals [112, 114, 115].

Also dyslipidemia plays an important role. This dyslipidemia in diabetes is characterized by high levels of (oxidized) low-density lipoprotein (Ox–LDL) and low high-density lipoprotein (HDL) levels; while increased circulating fatty acids may interfere with the signaling cascade of insulin through a mechanism called lipotoxicity [116-118]. Macro vascular problems (vascular coronary disease, peripheral vascular disease and brain vascular disease) as well as microvascular damage (retinopathy, neuropathy and nephropathy) often affect these dyslipidemia patients. In particular the ability of monocytes/macrophages to differentiate into pro-angiogenic cells (PACs), which are crucial support cells in repair of vascular destructive alterations, is importantly impaired in T2D. Ultimately, this leads to

increased vascular damage, a characteristic problem in long-standing and poorly regulated diabetes [60, 64, 119, 120].

T2D in Ecuador

Similar to what is happening worldwide, diabetes has become a public health problem in Ecuador. Already in 2007 the Ecuadorian Institute of Statistics and Census (INEC) reported that diabetes mellitus was the first cause of death [121]. In 2013, 563,840 cases of diabetes were reported causing 5,492 deaths in that year [112].

Several environmental and genetic factors contribute to the development of diabetes. It is recognized that T2D has a strong genetic component. In fact there is a concordance of 95% in homozygous twins [122]. A number of genetic variants associated with T2D have been described across different populations although a diverse picture emerges from different studies. For instance, one of the most robust associations between common genetic variation and type 2 diabetes risk, reported in European and Asian populations, involves intronic single nucleotide polymorphisms (SNPs) in the CDKAL1 gene, encoding the CDK5 regulatory subunit associated protein 1-like 1, a methylthiotransferase with so far unknown role in T2D [123]. Contrary, Locke et al did not find this association in a white UK cohort [124]. Interestingly, another genome-wide association study for T2D has highlighted multiple genes implicated in adipo-cytokine signaling pathways (mostly adiponectin, leptin and TNF-α signaling) and cell cycle regulation [125].

Next to genetic determinants, social factors seem to be inducing factors for the development of this disease in developing countries. One of the factors contributing to the development of diabetes is the rapid urbanization. In recent years an increased number of people have migrated from the countryside to the cities. Presently about 66% of Ecuador's total population is urban. A recent study in a national sample of Ecuador, reported an overall prevalence of DM2 of 2.7% in the population of 10-50 years. However, the prevalence in Quito is one of the highest (4.8%), followed by the urbanized areas of the coast (3.8%). The lowest prevalence was found in the rural Sierra and Amazonian areas (about 1%). The prevalence increased with age and is particularly high in the older population, averaging 10% in groups of 50-59 years [91]. Overall, the prevalence of diabetes in urban areas ranges between 7 and 8%, while in rural areas it is only 1 to 2% [112, 113].

The recent longevity in Ecuador also contributes to increased numbers of diabetic cases. In most Latin American countries the annual growth rate of the population over 60 years is approximately 3 to 4% compared with 1 to 2% in previous years [112]. Also certain ethnic groups such as the American Hispano have a high propensity to develop insulin resistance and diabetes. [113, 126, 127]. Mao et al, showed significant differences in gene expression and signaling pathways of blood leukocytes of diabetic patients of African American origin

as compared to Caucasian diabetic patients [128]. Ecuador is in this respect particularly vulnerable being a multi-ethnic country.

The shift from traditional eating habits of the indigenous population of low-calorie diets, especially derived from plants, to diets high in carbohydrates and animal fat (Western diet) seems to be a crucial factor in the development of metabolic disorders in the population, mainly characterized by altered lipid profiles and increased body weight [129, 130]. This hyper-caloric excess has caused an increase in the prevalence of overweight and obesity in the Ecuadorian population. In 2005, the World Health Organization (WHO) reported that 40% of men and 50% of women in Ecuador were overweight (body mass index greater than 25) and 6% of men and 16% of women were obese (BMI greater than 30)[131]. Obesity is the dominant factor for the development of diabetes. Specifically abdominal obesity (> 80 cm in women and> 90 cm in men) is considered as a reliable predictor of MetS and T2D [113, 132-135].

MONOCYTE DYSFUNCTION IN METS AND T2D

There is evidence that monocytes are affected at the molecular level in MetS and T2D. Cellular changes due to different environmental conditions are tightly regulated by cellular sensors and signaling processes that modulate monocyte function [36, 66]. Several reports show that TLR-2 and TLR-4 expression is increased in monocytes of patients with the MetS and T2D [136-138]. Interestingly, Miller et al. described that Ox-LDL can trigger TLR-4 signaling [139]. The increased activity of TLR4 induces the activation of downstream transcription factor nuclear factor-κB (NF-kB) and the secretion of inflammatory molecules (e.g. IL-1β, IL-6, IL-8)[138, 140, 141]. In fact the importance of TLR4 in the MetS and T2D has been demonstrated in animal models [142]. Jialal et al, showed that genetic deletion of TLR2 and TLR4 in mice remarkably diminishes inflammation, and insulin resistance [143].

Other cellular pathways in monocytes importantly interact with inflammatory signaling cascades, thus contributing to T2D pathogenesis. For instance, the interleukin-1 receptor-associated kinase-3 (IRAK3) is a negative regulator of TLR2/NF-κB-mediated inflammation. Interestingly, adiponectin level seems to directly regulate IRAK3 expression directly. In the MetS and T2D the secretion of adiponectin is importantly reduced, as well as the expression of IRAK 3, leading to a stimulation of which favor the inflammation process[144]. In addition, abnormalities in CD40-CD40L interactions in monocytes of MetS patients have been described [145-147]. As a result, the monocytes become excessively activated secreting proinflammatory cytokines (IL-1, IL-6), matrix metalloproteinases (MMPs), cyclooxygenase-2 (COX-2) and tissue factor [148, 149].

Furthermore, in MetS and T2D the expression of Fc gamma receptors (CD32 and CD64) and chemokine receptor CCR5 are elevated [36, 150-152]. In parallel to CCR5 expression, its ligands CCL11/eotaxin-1 and CCL4/MIP-1β are also overexpressed [75]. Contrary, significant down-regulation of Peroxisome Proliferator Activator Receptor (PPAR) α and PPARδ have been shown [75]. This probably contributes to a pro-inflammatory profile of monocytes in MetS and T2D as signaling via these nuclear receptors is important for the induction and maintenance of an anti-inflammatory M2 phenotype of monocytes/macrophages [153, 154]

Finally, in monocytes of MetS and T2D patients endoplasmic reticulum (ER) stress is well described, and may be induced by elevated glucose levels and dyslipidemic conditions [155-157] . ER stress is characterized by the increased secretion of various oxidative biomarkers such as ox-LDL, nitrotyrosine and superoxide anion [70, 158-162].

Important for the studies in this thesis is that we previously reported a higher inflammatory gene expression profile in monocytes of diabetic patients, also in patients with T2D [163]. We studied patterns of inflammatory gene expression in CD14+ monocytes of patients with type 1 diabetes (juvenile onset and adult onset), latent autoimmune diabetes of the adult (LADA), type 2 diabetic patients and non-diabetic control subjects using quantitative PCR. We tested 25 selected genes: 12 genes detected in a pre-study via whole-genome expression analyses plus an additional 13 genes identified as part of a monocyte inflammatory signature previously reported in auto-inflammatory conditions. We identified two distinct monocyte gene expression clusters in diabetes. The first cluster comprised 12 pro-inflammatory cytokine/compound genes (IL-1B, IL-6, TNF, TNFAIP3, PGS2, CCL20, PTX3, PDE4B, DUSP2, ATF3, CXCL2 and BCL2A1); the second cluster comprised 12 chemotaxis, adhesion, motility, and metabolism genes (CCL2, CCL7, MAPK6, NAB2, CD9, STX1A, EMP-1, CDC42, PTPN7, DHRS3, FABP5, HSPA1A). Different gene expression profiles of these two gene clusters typified the circulating monocytes of LADA patients, T1D and T2D patients. [163]. We use the gene profiles as described by Padmos et al in our studies described in Chapter 3 and 5.

MICRORNAS IN GENERAL

MicroRNAs represent a class of small non-coding RNAs and have been identified as important regulators of translation and stability of messenger RNA (mRNA) [164, 165]. MicroRNAs negatively regulate gene expression at post-transcriptional level by mediating translational repression (through an imperfect pairing to the target mRNA, directs RISC to bind the 3‟ untranslated regions (3‟ UTRs) of the targets) or degradation of the mRNA

targets [166]. The microRNAs have been highly conserved during evolution, strengthening the notion that they playing a key role in gene regulation. So far, around almost 2000 mature microRNAs have been identified in human (1881 miRNAs at the time of writing reported in miRBase version 21); they can modulate the expression of at least one third of all encoded mRNAs [167, 168]. The micro-RNA's mature form is a single stranded RNA, 19-22 nucleotides long, derived from a primary transcript, whose which maturation steps take place in part in the nucleus and in part in the cytoplasm (see Fig 4) [166, 168].

Transcription of a microRNA gene is mediated by RNA polymerase II or III, which produce a primary transcript, called primary microRNA [165]. PrimiRNAs are long primary transcripts that contain a local stem-loop structure. This stem-loop structure is cleaved in the nucleus by the RNase III Drosha to release the precursor of microRNA (pre-miRNA). The Drosha product pre-miRNA needs to be exported to the cytoplasm. Export of pre -miRNA is mediated by exportin-5 (Exp5) [169, 170]. Once in the cytoplasm, pre-miRNAs are processed into 22-nt microRNA duplexes by the cytoplasmic RNase III Dicer. The cleavage products [22-nt 5p- and 3p-microRNA/miRNA star (miRNA/miRNA*) duplexes] are thought to be quickly unwound by heli-case (Argonaute protein), and a single mature strand, preferentially the most thermodynamically stable, can be asymmetrically incorporated into the RNA-induced silencing complex (RISC) where it can then act by translational

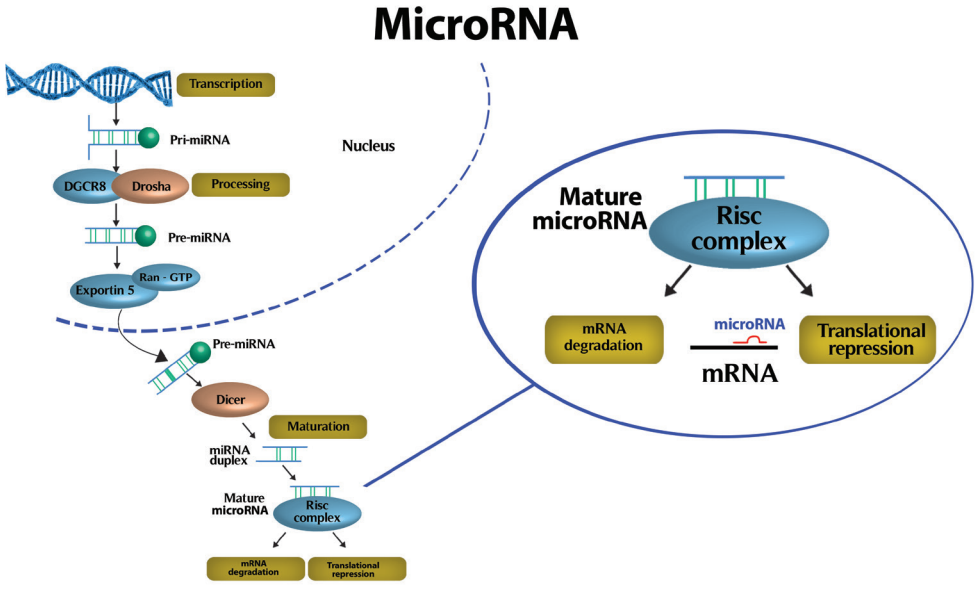

Figure 4. MicroRNA biogenesis and function.
Adapted from Urbich, et al, Cardiovascular Research (2008)

repression (by a cleavage-incompetent RISC) or mRNA degradation (by a cleavage-competent, Slicer-containing RISC). The counterpart of the mature miRNA from the duplex that is generally regarded as a passenger strand, previously indicated as 'star' strand, called miR-NA* (miRNA "star"), whose regulatory capacity has not been systematically examined, is usually degraded (see Fig. 4) [164-166, 168, 171].

MicroRNA alteration of peripheral blood mononuclear cells (PBMC) and monocytes in T2D

MicroRNAs have been associated with the development of various diseases, including diabetes [172-176] playing for instance important roles in the normal differentiation and maturation of hematopoietic cells [177]. Various inflammatory triggers appear to induce the selective expression of microRNAs in monocytes/macrophages, which in turn functionally affect the expression of proteins involved in the inflammatory cascade [178-181]. The dysregulation of microRNAs has been implicated in MetS and diabetes, although there is a relative paucity of literature on PBMC/ monocyte miRNA alterations in type 2 diabetes. Here we present the available literature (summarized in Table 1).

MiR-146a and miR-155 expression levels have been found to be significantly decreased in the PBMC of patients with T2D as compared to control subjects [182, 183]. Expression values correlated negatively to parameters of metabolic control (Hb1Ac, glucose) and signs of inflammation (NFκB mRNA levels in PBMC and circulatory levels of pro-inflammatory cytokines) [182, 183]. Tome-Carneiro et al, carried out a study of anti-inflammatory supplementation in a group of male T2D patients. They found downregulation of miR-21, miR-181b, miR-663 and miR-30c2 in PBMCs. MiR-155 and miR-34a were slightly upregulated after treatment, but did not reach statistical significance. Mocharla et al, described that miR-126 was the most differentially expressed miRNA in CD34+ hematopoietic progenitor cells as compared to CD34- PBMC subsets [184]. Finally, Collares et al studied the microRNA expression profile of PBMC from type 1 (T1D), type 2 (T2D), and gestational diabetes (GDM) patients using a microarray platform. They reported that nine miRNAs (miR-126, miR-1307, miR-142-3p, miR-142-5p, miR-144, miR-199a-5p, miR-27a, miR-29b, and miR-342-3p) were shared among T1D, T2D and GDM, and additional specific microRNAs were identified in T2D patients (miR-140-3p, miR-199a-3p, miR-222, miR-30e and miR-451) [185].

Table 1. MicroRNA dysregulation of peripheral blood mononuclear cells (PBMC) in T2D.

microRNAs		Cell type	Reference
Up-regulated	**Down-regulated**		
	miR-146a	PBMCs	Balasubramanyam M, Aravind S et al: Impaired miR-146a expression links subclinical inflammation and insulin resistance in Type 2 diabetes. Molecular and cellular biochemistry 2011, 351(1-2):197-205.
	miR-155, miR-146a	PBMCs	Corral-Fernandez NE, Salgado-Bustamante M,et al: Dysregulated miR-155 expression in peripheral blood mononuclear cells from patients with type 2 diabetes. Experimental and clinical endocrinology & diabetes: official journal, German Society of Endocrinology [and] German Diabetes Association 2013, 121(6):347-353.
miR-34a, miR-155 (NS)	miR-21, miR-181b, miR-663 and miR-30c2	PBMCs	Tome-Carneiro J, Larrosa M, et al: One-year supplementation with a grape extract containing resveratrol modulates inflammatory-related microRNAs and cytokines expression in peripheral blood mononuclear cells of type 2 diabetes and hypertensive patients with coronary artery disease. Pharmacological research: the official journal of the Italian Pharmacological Society 2013, 72:69-82.
miR-126		CD34+ cells	Mocharla P, Briand S, et al: AngiomiR-126 expression and secretion from circulating CD34 (+) and CD14 (+) PBMCs: role for proangiogenic effects and alterations in type 2 diabetics. Blood 2013, 121(1):226-236.
miR-126, miR-1307, miR-142-3p, miR-142-5p, miR-144, miR-199a-5p, miR-27a, miR-29b, and miR-342-3p, miR-140-3p, miR-199a-3p,miR-222,miR-30e and miR-451		PBMC	Collares CV, Evangelista AF, et al: Identifying common and specific microRNAs expressed in peripheral blood mononuclear cell of type 1, type 2, and gestational diabetes mellitus patients. BMC research notes 2013, 6:491.

MicroRNA alterations in the circulation (serum/plasma) of patients with T2D

Serum/plasma circulating microRNA expression patterns have been suggested to have a predictive value as potential biomarkers in a variety of diseases including diabetes [186, 187]. It has been demonstrated that some microRNAs can exist in serum stably, suggesting a potential utility of microRNAs as clinical biomarker that are minimally invasive, life-style independent and informative [188].

The literature about dysregulation of microRNAs in serum of T2D is increasing daily (summarized in Table 2). For instance, Wang et al, determined 14 circulating microRNAs in Swedes and Iraqis with and without T2D. The levels of miR-24 and miR-29b were significantly different between T2D patients and controls [189]. Another report has shown that miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, and miR-486 are downregulated in plasma of T2D, whereas miR-28-3p was upregulated. Importantly, reduced miR-15a, miR-29b, miR-126, miR-223, and elevated miR-28-3p levels preceded the manifestation of disease [190]. Prabu et al, performed global serum miRNA profiling of 'Asian Indian' people. They showed that 4 microRNAs (miR-128, miR-130b-3p, miR-374a-5p and miR-423-5p) were differentially expressed in T2DM patients compared to controls. Interestingly, miR-128 had never been described in previous studies/populations and appeared to be a 'new lead' in the Indian population [191]. Using qPCR Kong et al, report elevated levels of serum miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a and miR-375 in the serum of newly diagnosed T2D patients [192]. Higuchi et al. performed Illumina sequencing of microRNAs in T2D serum. MiR-101, miR-375, and miR-802, were significantly increased in T2D patients. Interestingly, levels of HbA1c and HDL-C were identified as significant determinants [193]. Recently Wu et al, using q-PCR profiled miRNAs from serum of obese, T2D and healthy controls. MiR-593 was significantly lower in T2D patients when compared to controls. MiR-17 and miR-152 were also significantly lower but when compared to obese subjects. Contrary, miR-138 was significantly upregulated [194].

In sum, there is a large list of circulating candidate microRNAs that might be useful as clinical biomarkers of T2D. However, a clear picture or a lead microRNA has not been discovered. Moreover most of the microRNAs have not yet been validated in sufficiently powered and longitudinal studies for specificity for T2D [186]. In addition, ethnicity and other confounding factors, such as age, gender, obesity, dyslipidemia, smoking, use of contraceptives and other drugs capable of influencing the inflammatory state and vascular disease probably influence the profile of circulating microRNAs and need to be taken into consideration. Needless to say that lot of work still needs to be done. Studies reported in this thesis belong to the first steps in addressing these issues.

Table 2. MicroRNA dysregulation of serum/plasma of T2D patients.

microRNAs		Compartment	Reference
Up-regulated	**Down-regulated**		
miR-28-3p	miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, miR-486	Plasma	Zampetaki A, Kiechl S, et al: Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. Circulation research 2010, 107(6):810-817.
miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a, miR-375		Serum	Kong L, Zhu J, et al: Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study. Acta diabetologica 2011, 48(1):61-69.
miR-24, miR-29b		Plasma	Wang X, Sundquist J, et al : Determination of 14 circulating microRNAs in Swedes and Iraqis with and without diabetes mellitus type 2. PloS one 2014, 9(1):e86792.
miR-128, miR-130b-3p, miR-374a-5p miR-423-5p		Serum	Prabu P, Rome S, et al: Circulating MiRNAs of 'Asian Indian Phenotype' Identified in Subjects with Impaired Glucose Tolerance and Patients with Type 2 Diabetes. PloS one 2015, 10(5):e0128372.
miR-101, miR-375, miR-802		Serum	Higuchi C, Nakatsuka A, et al: Identification of circulating miR-101, miR-375 and miR-802 as biomarkers for type 2 diabetes. Metabolism: clinical and experimental 2015, 64(4):489-497.
miR-138	miR-593 miR-17, miR-152	Serum	Wu L, Dai X, Zhan J, et al: Profiling peripheral microRNAs in obesity and type 2 diabetes mellitus. APMIS : acta pathologica, microbiologica, et immunologica Scandinavica 2015.

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Chapter 2

Experimental aims of this thesis

AIMS

The **overall aim of this thesis is** to establish a putative dysregulation of microRNAs in monocytes and serum, and to correlate the dysregulated microRNA expression to inflammatory markers in monocytes and serum. The unravelling of a dysregulation of microRNAs and inflammatory markers in monocytes and serum is the basis to understand better the molecular pathogenic processes playing a role in T2D. Moreover, the dysregulated microRNAs and inflammatory compounds could serve as biomarkers of T2D.

We firstly identified a microRNA signature capable of distinguishing patients with T2D from individuals not suffering from T2D. In **the first part of Chapter 3** we describe that, in a study on the monocytes of 34 European and Ecuadorian patients and of 25 non-diabetic controls, we were able to identify 142 significantly differentially expressed microRNAs, 15 having the strongest power to discriminate T2D patients from controls. Indeed, this approach showed that subject clusters could be identified with a first cluster containing 24 T2D cases and only 2 non-diabetic controls, and a second mixed cluster comprising 12 cases and 23 non-diabetic controls (sensitivity 66%, specificity 90%). Thus, using microRNA expression in monocytes, we found that a partial separation could be made between T2D cases and non-diabetic controls. These monocyte prediction signature microRNAs, however, appeared less useful to validate as microRNAs that can be clinically used as discriminating parameters between T2D patients and controls using qPCR as an independent technique, since the expression fold changes observed for these microRNAs were generally too low to allow reliable confirmation within the technical limitations of qPCR.

We thereafter decided to use the microRNAs as biomarkers to test the biological function and inflammatory state of the circulating monocytes in patients with T2D, since there is a paucity of studies focusing on the inflammatory state of these circulating cells in T2D. Therefore, we chose to select from the differentially expressed microRNAs those with the highest fold changes (FC) between cases and controls with FC of >1.4 or <0.6. Another criterion for selection was that TaqMan probes and primers needed to be available. From the 142 differentially expressed microRNAs found in Exiqon, 5 microRNAs fulfilled the selection criteria: miR-138; miR-34c-5p; miR-410; miR-574-3p and miR-576-3p. Additionally, we tested microRNAs-146a and -155 in TaqMan analyses, since these microRNAs are well-known regulators of inflammation, and have been identified in T2D PBMC by others .

In **the second part of Chapter 3** we describe the outcomes of these TaqMan studies and the correlation in expression between the tested microRNAs and genes previously found abnormally expressed in monocytes of T2D patients. We tested for 24 selected genes forming two mutually correlating gene clusters. The first cluster comprised 12 pro-inflammatory cytokine/compound genes (IL-1B, IL-6, TNF, TNFAIP3, PGS2, CCL20, PTX3, PDE4B, DUSP2, ATF3, CXCL2 and BCL2A1); the second cluster comprised 12 chemotaxis, adhesion,

motility, and metabolism genes (CCL2, CCL7, MAPK6, NAB2, CD9, STX1A, EMP-1, CDC42, PTPN7, DHRS3, FABP5, HSPA1A). Different gene expression profiles of these two gene clusters typified the circulating monocytes of LADA patients, T1D and T2D patients. We used the monocytes of a series of 64 Ecuadorian patients and 44 non-diabetic Ecuadorian controls. We found the microRNA and gene expression profile of the monocytes to indicate an anti-inflammatory and motile/adhesive potential of the cells and we hypothesized that the monocytes in the T2D patients might be pro-angiogenic cells.

Since HGF is a well-known T2D related molecule and since HGF is used as a marker for pro-angiogenic cells we also tested HGF in the monocytes of the series of 64 Ecuadorian patients and 44 non-diabetic Ecuadorian controls and related the expression of HGF to the other genes and the microRNAs in the monocytes. These experiments are described in **the first part of Chapter 3**. HGF was indeed over expressed in the monocytes and primarily related to the cluster 2 genes

We also tested microRNAs-146a and -155 and the above described "monocyte microRNAs", i.e. miR-138; miR-34c-5p; miR-410; miR-574-3p and miR-576-3p, in the serum of the Ecuadorian patients and the non-diabetic Ecuadorian controls. We compared data to the serum level of a commercially available series of 12 pro-inflammatory and growth factors (TNFα, IL-1β, IL-6, NGF, HGF, PAI, Resistin, CCL2, Adiponectin, Leptin, IL-8, and CCL4), to see if the microRNAs had an expression pattern similar to these inflammatory/immune activation compounds. We also compared the serum level of the microRNAs and the inflammatory/immune activation compounds to the expression levels found in the circulating monocytes of T2D patients, to see if there are indications for the idea that the circulating monocytes are the source of the compounds in serum.

Outcomes of the experiments are described in **the second part of Chapter 4 and in Chapter 5**.

Chapter 3

Type 2 diabetes monocyte microRNA and mRNA expression: Dyslipidemia associates with increased differentiation-related genes but not inflammatory activation

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ABSTRACT

There is increasing evidence that inflammatory macrophages in adipose tissue are involved in insulin resistance of type 2 diabetes (T2D). Due to a relative paucity of data on circulating monocytes in T2D, it is unclear whether the inflammatory changes of adipose tissue macrophages are reflected in these easily accessible cells.

Objective. To study the expression pattern of microRNAs and mRNAs related to inflammation in T2D monocytes.

Design. A microRNA finding study on monocytes of T2D patients and controls using array profiling was followed by a quantitative Real Time PCR (qPCR) study on monocytes of an Ecuadorian validation cohort testing the top over/under-expressed microRNAs. In addition, monocytes of the validation cohort were tested for 24 inflammation-related mRNAs and 2 microRNAs previously found deregulated in (auto)-inflammatory monocytes.

Results. In the finding study, 142 significantly differentially expressed microRNAs were identified, 15 having the strongest power to discriminate T2D patients from controls (sensitivity 66%, specificity 90%). However, differences in expression of these microRNAs between patients and controls were small. On the basis of >1.4 or<0.6-fold change expression 5 microRNAs were selected for further validation. One microRNA (miR-34c-5p) was validated as significantly over-expressed in T2D monocytes. In addition, we found over expression of 3 mRNAs (CD9, DHRS3 and PTPN7) in the validation cohort. These mRNAs are important for cell morphology, adhesion, shape change, and cell differentiation. Classical inflammatory genes (e.g. TNFAIP3) were only over-expressed in monocytes of patients with normal serum lipids. Remarkably, in dyslipidemia, there was a reduction in the expression of inflammatory genes (e.g. ATF3, DUSP2 and PTGS2).

Conclusions. The expression profile of microRNAs/mRNAs in monocytes of T2D patients indicates an altered adhesion, differentiation, and shape change potential. Monocyte inflammatory activation was only found in patients with normal serum lipids. Abnormal lipid values coincided with a reduced monocyte inflammatory state.

Key words. Type 2 diabetes, monocytes, miR-34c-5p, inflammation, dyslipidemia

INTRODUCTION

There is increasing evidence that monocytes, macrophages and related cells are closely involved in the pathogenesis of the metabolic syndrome (MetS) and type 2 diabetes (T2D). Importantly, in obesity the number of macrophages increases from 10-15% to 50-60% of total cells in adipose tissue [1,2] The increased secretion of leptin and decreased secretion of adiponectin, by metabolically stressed adipocytes in obesity, results amongst others in the accumulation of macrophages in adipose tissue [3,4] The increase in macrophage number is accompanied by a hyper activation of the cells and leads to a pro-inflammatory state of the macrophages (so-called M1 type or classically activated macrophages). M1 type macrophages in adipose tissue secrete pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, CCL-4) and chemokines (CCL2), which spill over in the circulation causing a chronic low-grade inflammation [1,3,5-8] The pro-inflammatory cytokines and chemokines play an important causative role in the insulin resistance of T2D [1,9]

Monocytes are bone marrow-derived and considered to be important circulating precursors for the macrophages in adipose tissue [10-12]. There is however a relative paucity in reports on the state of activation of circulating monocytes in patients with the MetS [13,14] and T2D [15] . In general this state of activation is thought to be pro-inflammatory and increases in pattern recognition receptors (TLRs, NOD-like receptors), oxidative stress and the machinery for the production of pro-inflammatory cytokines have been described [16,17]. The combination of dyslipidemia and chronic hyperglycemia is thought to play a role in this inflammatory activation of the circulating monocytes in MetS and T2D [18,19] . The view has been expressed that further studies on monocyte biology are needed to define the pathogenic role of monocytes/macrophages in MetS and T2D, given that this circulating population is easily accessible and that further clarification of the inflammatory pathophysiology of T2D is needed [20].

Previously, our group carried out such studies on monocyte biology in Latent Onset Diabetes of the Adult (LADA), T2D and Type 1 diabetes (T1D) and reported that diabetic patients exhibit abnormal monocyte gene expression profiles when monocytes were tested for 24 inflammation-related genes (these genes had been detected in earlier carried out gene profiling studies [21]). Two mutually correlating sets of genes (clusters) were found abnormally expressed in the monocytes. A first set (cluster 1) consisted of 12 inflammatory cytokine/compound genes (IL-1B, IL-6, TNF, TNFAIP3, PGS2, CCL20, PTX3, PDE4B, DUSP2, ATF3, CXCL2 and BCL2A1), while a second set (cluster 2) consisted of 12 genes mainly involved in cell motility, chemotaxis, adhesion, differentiation and metabolism (CCL2, CCL7, MAPK6, NAB2, CD9, STX1A, EMP-1, CDC42, PTPN7, DHRS3, FABP5, HSPA1A). Both gene clusters were up-regulated in monocytes of LADA and T2D patients; in juvenile T1D

patients only cluster 2 genes were up regulated [21]. Up-regulations of cluster 1 and 2 genes have also been found in the monocytes of other (auto)-inflammatory conditions, such as autoimmune thyroiditis [22] and major mood disorders [23].

Gene expression is partly regulated by a newly discovered level of control, the microRNA system. There is extensive literature indicating that two microRNAs, i.e. miR-146a and miR-155, are key regulators of inflammatory processes [24-32]. An altered expression of these microRNAs has been described in monocytes/macrophages during inflammatory and autoimmune conditions [33-37]. Dysregulation of these microRNAs in peripheral blood mononuclear cells (PBMC) has also been implicated in diabetes [38-40], i.e. miR-146a and miR-155 expression levels were found to be significantly decreased in the PBMCs of patients with T2D compared to control subjects. Moreover, expression values correlated negatively with parameters of metabolic control (Hb1Ac, glucose) and signs of inflammation (NFκB mRNA levels in PBMC, circulatory levels of pro-inflammatory cytokines) [39].

Here we firstly report on a search for abnormally expressed microRNAs in purified monocytes of a cohort of 34 German/Ecuadorian T2D patients using Exiqon array profiling. We tested the found 142 significantly differently expressed microRNAs for discriminating power between patients and non-diabetic controls. Thereafter we selected from the 142 microRNAs 5 microRNAs which were the highest over- or under-expressed with fold changes >1.4x or <0.6x as compared to the non-diabetic controls. We took these 5 microRNAs (miR-138; miR-34c-5p; miR-410; miR-574-3p and miR-576-3p) plus miR-146a and miR-155 for further TaqMan qPCR monocyte studies in a validation cohort of 64 Ecuadorian T2D patients and 44 non-diabetic Ecuadorian controls. In the monocytes of this validation cohort we also tested via qPCR the expression level of the 12 cluster 1 and 12 cluster 2 genes of the earlier reported monocyte inflammatory signature. We finally correlated the expression levels of the tested microRNAs and genes in the validation study to each other (cluster analysis) and to clinical variables, such as age, gender, Hb1Ac and dyslipidemia and studied their discriminative power in distinguishing between patients and controls.

MATERIALS AND METHODS

Subjects for microRNA expression profiling (finding) cohort

Thirty-four subjects diagnosed with type 2 diabetes (T2D), according to the criteria of The Expert Committee on the diagnosis and classification of Diabetes Mellitus [41], were recruited in the German Diabetes Center, Düsseldorf, Germany (Dr Nanette Schloot, n =

10) and from three medical centers in Quito, Ecuador (Eugenio Espejo Hospital, Club de Leones Sur, and Fundación de la Psoriasis; n = 24 subjects) in 2009. A total of 25 healthy controls (n =9 from Germany and n =16 from Ecuador) with similar ethnic and social background, neither suffering from T2D, nor from other medical disorders (including acute infection), were recruited as well. Studies were performed with approval of the local ethical committees and after informed consent.

Subjects for the qPCR validation studies

The validation was performed using a new cohort of 64 subjects diagnosed with type 2 diabetes (T2D), according to the criteria of The Expert Committee on the diagnosis and classification of Diabetes Mellitus [41]. Patients were recruited in 4 medical centers of Quito, Ecuador (Eugenio Espejo Hospital, Club de Leones Sur, Fundación Oftalmológica del Valle and Fundación de la Psoriasis) from 2009 until 2012. For demographic and clinical details see Table 1. At the same time, 44 healthy controls with similar ethnical and social background, neither suffering from T2D nor other important medical disorders (including acute infection) served as controls. Controls had to be over 30 years of age (considering the age dependency of T2D). For practical reasons, not in all instances all cases and controls could be tested in the qPCR studies. Exact numbers of tested individuals are indicated.

In all cohorts, patients and healthy controls with other immune disorders, other serious medical illnesses, recent infections (last 2 weeks), obvious vascular complications such as diabetic foot and ulcers, fever, pregnancy/postpartum and LADA patients (patients positive for GAD-65 Abs) were excluded. None of the patients used statins. The Medical Ethical Review Committee of the Ecuadorian Corporation of Biotechnology Quito, Ecuador and the Ethic Committee of the Central University of Quito approved the study. Written informed consent was obtained of all subjects participating in the study. The Ecuadorian Ministry of Health (MSP) gave the permit to export and process the samples in Erasmus MC, Rotterdam, The Netherlands.

Blood collection and preparation

Blood (drawn in the morning) was collected in tubes containing sodium-heparin for immune cell preparation. From the heparinized blood, peripheral blood mononuclear cell (PBMC) suspensions were prepared in the afternoon by low-density gradient centrifugation, as previously described in detail [42] within 8 h to avoid activation of the monocytes. PBMCs were frozen in 10%-dimethylsulfoxide and stored in liquid nitrogen. This enabled us to test patient and control immune cells in the same series of experiments later.

Table 1. Demographic details and clinical characteristics of the validation cohort of Ecuadorian T2D patients and controls.

	T2D			NDC			T2D vs. NDC
Group size n	64			44			
Age mean (range)	61 (37-85)			53 (32-87)			**0.00****
BMI mean (range) %	29.5 (22-49)	Normal	16.1%	28.7 (23-42)	Normal	18.2%	0.405
		Overweight	40.3%		Overweight	45.5%	
		Obese	43.5%		Obese	36.4%	
Gender							
Female n (%)	40 (62.5%)			31 (70.5%)			NA
Male n (%)	24 (37.5%)			13 (29.5%)			NA
Glucose state							
Fasting Glucose mg/dL	146 (69 - 397)	Normal	45.3%	88 (60.9- 180.5)	Normal	88.6%	**0.00****
mean (range) %		High	54.7%		High	11.4%	
HbA1C	7.0 (3.2 - 12.5)	Normal	35.7%	5.6 (3.9 - 6.9)	Normal	81.8%	**0.00****
mean (range) %		High	62.5%		High	18.25%	
Lipid Profile							
Cholesterol mg/dL	237 (143- 465)	Normal	37.5%	237 (131-328)	Normal	31.8%	0.99
mean (range) %		High	62.5%		High	68.2%	
TG mean mg/dL	205 (76 - 628)	Normal	60.9%	194 (85 -547)	Normal	63.6%	0.56
mean (range) %		High	39.1%		High	36.4%	
HDL mean mg/dL	43 (17 -85)	Normal	57.8%	43 (27- 87)	Normal	54.5%	0.81
mean (range) %		Low	42.2%		Low	45.5%	
LDL mg/dL	158 (77- 395)	Normal	56.3%	158 (78 - 266)	Normal	50%	0.95
mean (range) %		High	43.8%		High	50%	
Hepatic Profile							
ASAT mean mg/dL	33.3 (6.0 - 78)	Normal	70.8%	41.3 (19 -95)	Normal	48.7%	**0.01***
mean (range) %		High	29.2%		High	51.3%	
ALAT mean mg/dL	38.8 (7.0 -131)	Normal	64.6%	44.7 (10- 135)	Normal	47.4%	0.252
mean (range) %		High	35.4%		High	52.6%	
Medication							
Oral Anti-diabetics	70%	0%					
Insulin treatment	30%	0%					
Statins (%)	0%	0%					

Values in bold denote a significant difference between two groups.
*p0.01, **p0.001

Table 1 shows sample sizes, distributions of age, gender, comorbidities, HbA1c/hyperglycemia, BMI, hepatic profile, lipid profile, and medication use of the patient and control groups.

Isolation of monocytes

CD14-positive (CD14+) monocytes were isolated from thawed PBMCs by a magnetic cell sorting system (MACS; Miltenyi Biotec, Auburn, California). The purity of monocytes was >95% (determined by morphological screening after Trypan Blue staining and flow cytometric analysis). As previously reported; the positive vs. negative selection of immune cells did not influence gene expression profiles [43].

MicroRNA microarray hybridization

Total RNA was extracted from purified monocytes using a mirVana miRNA isolation kit (Ambion) according to the manufacturer's protocols. RNA was labeled using a ULS RNA labeling kit (KreatechDiagnostics, Amsterdam). To that end, 1.5 µg of total RNA was incubated with Cy3-ULS for 30 min at 85°C and purified to remove unbound Cy3-ULS. Labeled RNA was hybridized on miRCURY LNA microRNA arrays (probe set 10.0; Exiqon, Vedbaek, Denmark) at 60°C for 16h using a Tecan 4800 hybridization station. Slides were washed and immediately scanned using a Tecan LSRe loaded microarray laser scanner.

microRNA RT qPCR assays

Total RNA was isolated from purified monocytes using the mirVana miRNA Isolation Kit (Ambion) as described by the manufacturer's manual. Purity and integrity of the RNA were assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip reagent set (Agilent Technologies, Santa Clara, CA, USA) and the RNA was then stored at −80 °C. Subsequently, specific stem-looped reverse transcription primers were used to obtain cDNA for mature microRNAs. The RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit from Applied Biosystems, The Netherlands (ABI). PCR was performed using pre-designed TaqMan microRNA assays and TaqMan Universal Master Mix, NoAmpEraseUNG, with an ABI 7900 HT real-time PCR machine. The PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15s at 95°C, and 1 min at 60°C.

mRNA expression analysis in monocytes via TaqMan Array Cards

One µg of RNA was reverse-transcribed using the High Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed using custom TaqMan Arrays, format 48 (Applied Biosystems), according to the manufacturer's protocol and validated against the single RT-qPCR method. Per fill port, 400 ng of cDNA (converted from total RNA) was loaded. PCR amplification was performed using an Applied Biosystems Prism 7900HT sequence detection system with TaqMan Array block. Thermal cycler conditions were 2 min at 50°C, 10 min at 94.5°C, and then 30s at 97°C, and 1 min at 59.7°C for 40 cycles. Relative to the housekeeping gene ABL1, the expressions of ATF3, BCL2A1, CCL20, CCL2, CCL4, CD9, CDC42, CXCL2, DHRS3, DUSP2, EMP1, FABP5, HSPA1A/HSPA1B, IL-1B, IL-6,

MAPK6, NAB2, PDE4B, PTGS2, PTPN7, PTX3, STX1A, TNF, and TNFAIP3 were determined and values were calculated using the comparative threshold cycle (Ct) method. ABL was chosen as a reference gene because it was previously shown that ABL was the most consistently expressed reference gene in hematopoietic cells [44] . The quantitative value obtained from qPCR is a cycle threshold (Ct). The fold change values between different groups were determined from normalized Ct values (Ct gene – Ct housekeeping gene), by the ΔΔCt method.

Data analysis microRNA microarray

Microarray data extraction and normalization was carried out as described previously [45]. We analyzed 711 microRNAs using Empirical Bayesian method for assessing differential expression (R package limma) to detect microRNAs differentially expressed between cases and controls. For outlier detection, we used Grubb's test for individual microRNA (threshold for significance 0.05). Outliers were replaced by a median expression value. The Benjamin-Hochberg method (5% false discovery rate) was applied to correct for multiple testing. Target genes of the identified microRNAs were predicted using miRecords (http://www.mirecords.bioled.org). Functional annotation of the predicted genes was performed using Ingenuity Pathway Analysis (Ingenuity Systems).

Data analysis RT qPCR

The SDS software (ABI) was used to collect the data and the RQ Manager Program (ABI) was used to assign, check, and standardize Ct values. Data Assist software (ABI) was used to normalize the data to ABL for mRNA expression and RNU44 for microRNA expression. For threshold cycles below 40, the corresponding microRNA was considered detected, higher cycle numbers were not included in calculations. The results were quantified using the ΔΔCt method ($2^{-\Delta\Delta Ct}$, User Bulletin 2, ABI). Statistical analysis was performed using the SPSS (IBM, Inc.) package for Windows. Data were tested for normal distribution using the Kolmogorov-Smirnov test. The Grubbs' test for outlier detection was applied (http://graphpad.com/support/faqid/1598/). Depending on the distribution pattern and the total number of subjects, parametric (normal distribution, independent t test) or nonparametric group comparison (Mann-Whitney U test) were applied. Correlations were determined by Spearman's correlation coefficient. Levels of significance were set at $p \leq 0.05$ (two tailed). A dendrogram visualizing associations was constructed in SPSS using hierarchical cluster analysis of the genes and microRNA expression using the between-groups linkage method. Graphs were designed with Illustrator CS6 for Windows.

RESULTS

Exploratory search for T2D-related monocyte micro-RNAs using Exiqon arrays.

To investigate T2D-related monocyte microRNA profiles, we profiled the monocytes of 34 T2D patients (age: 22-77 years, mean 55 years) and of 25 non-diabetic controls (age: 31-71 years, mean 49 years) of the finding cohort. After correction for multiple testing (Benjamin-Hochberg method), we detected 142 microRNA differentially expressed in T2D patients compared to controls. From the 142 microRNAs, 49 microRNAs (35%) were down-regulated and 93 microRNAs (65%) were up-regulated. The list is available in the supporting information files of this article (S1 Table). Using Ingenuity pathway analysis with inclusion of only literature-confirmed targets of the identified microRNAs, we found that SOCS4 and SOCS6 genes ranked highest as potential targets of these differentially expressed microRNAs in monocytes, suggesting that especially inflammatory networks were regulated by these microRNAs.

Additionally, computational class prediction analysis was performed with the 142 significantly different expressed microRNAs using the LASSO model of penalized prediction. This showed that 15 microRNAs indicated an optimal prediction signature (underlined in S1 Table). Using the data on expression of these microRNAs as determined in array, we clustered patients and controls of the finding cohort by unsupervised hierarchical clustering (S1 Fig). Indeed, this approach showed that subject clusters can be identified with a first cluster containing 24 T2D cases and only 2 healthy controls, and a second mixed cluster comprising 12 cases and 23 healthy controls (sensitivity 66%, specificity 90%). Thus, using microRNAs, we found that a partial separation can be made between T2D cases and controls. These prediction signature microRNAs, however, appeared less useful to validate as microRNAs that can be used as discriminating parameters between T2D patients and controls in a separate cohort using qPCR as an independent technique, since the expression fold changes observed for these microRNAs were generally too low to allow reliable confirmation within the technical limitations of qPCR. Therefore, we chose to select from the differentially expressed microRNAs those with the highest fold changes (FC) between cases and controls with FC of >1.4 or <0.6. Another criterion for selection was that TaqMan probes and primers needed to be available.

Validation studies of 5 selected T2D-related monocyte microRNAs using qPCR

From the 142 differentially expressed microRNAs found in Exiqon, 5 microRNAs fulfilled the selection criteria: miR-138; miR-34c-5p; miR-410; miR-574-3p and miR-576-3p. Additionally, we tested microRNAs-146a and -155 in TaqMan analyses, since these microRNAs are well-known regulators of inflammation, and have been identified in T2D PBMC by others [38,39].

The table of Fig. 1 shows (apart from other data) the expression level of the 5 microRNAs in the monocytes of the Ecuadorian validation cohort of type 2 diabetic patients and non-diabetic controls (in total 48 patients and 34 controls could be used). Of the microRNAs, miR-155 was not or hardly detected in the monocytes using TaqMan qPCR array techniques, and therefore data are not given. The table of Fig. 1 shows that of the tested microRNAs, MiR-34c-5p and miR-576-3p were significantly higher expressed in the monocytes of the type 2 diabetic patients as compared to the monocytes of the non-diabetic controls.

		T2D		NDC vs. T2D
	Genes / miRNA	Mean (FC)	SEM	p Value
CLUSTER A	CCL4	1.12	0.236	0.78
	IL6	1.30	0.286	0.39
	TNF	1.08	0.140	0.70
	IL1B	0.70	0.089	0.18
	CCL2	0.96	0.189	0.89
	CCL20	1.07	0.180	0.85
	TNFAIP3	1.11	0.127	0.54
	PDE4B	0.96	0.100	0.83
	miR410	1.22	0.257	0.50
	DUSP2	0.75	0.071	0.15
	PTGS2	0.60	0.061	0.05
	ATF3	0.76	0.073	0.14
	miR576-3p	1.35	0.145	**0.04***
CLUSTER B	CDC42	1.11	0.093	0.42
	PTX3	0.92	0.068	0.56
	CXCL2	1.19	0.222	0.53
	STX1A	1.49	0.258	0.12
	NAB2	1.04	0.113	0.85
	EMP1	1.21	0.109	0.18
	BCL2A1	0.84	0.082	0.49
	PTPN7	1.42	0.153	**0.02***
CLUSTER C	miR138	0.79	0.063	0.30
	miR574-3p	0.85	0.093	0.24
	miR146a	1.07	0.095	0.54
	miR34c5p	1.32	0.124	**0.03***
	FABP5	0.86	0.079	0.48
	MAPK6	0.87	0.073	0.35
	HSPA1aHSPA1b	0.91	0.074	0.46
	DHRS3	1.39	0.136	**0.02***
	CD9	1.72	0.298	**0.04***

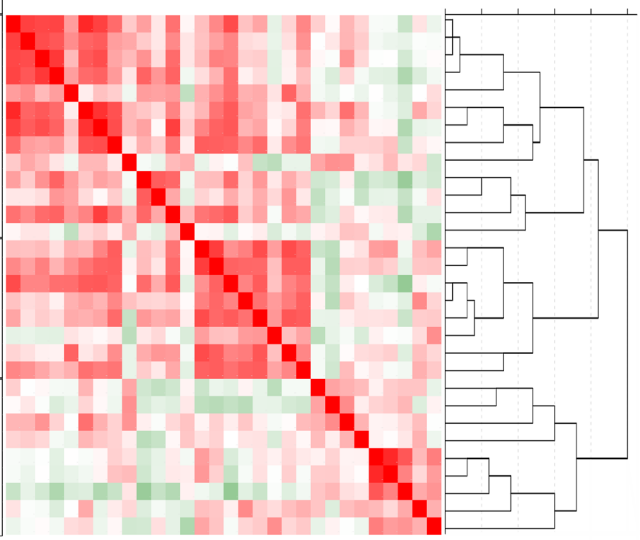

Figure 1. Hierarchical cluster analysis of the tested genes and microRNAs of the monocytes of Ecuadorian type 2 diabetic patients and controls in the validation cohort. On the left, the fold change values between the T2D group and the non-diabetic controls were determined from normalized Ct values (Ct gene/Ct reference gene ABL) by the ΔΔCt method (2−ΔΔCt, User Bulletin 2; Applied Biosystems, Foster City, CA). Data were standardized to the non-diabetic control subjects. The fold change of each gene in the non-diabetic control subjects is therefore 1. Differences between groups were tested using t tests for independent samples. This table shows that 2 microRNAs (MiR-34c-5p and miR-576-3p) were significantly higher expressed in the monocytes of the T2D patients compared to non-diabetic controls. Also, 4 genes (of the 24 tested) were significantly different expressed (PTGS2 lower, and CD9, DHRS3 and PTPN7 significantly higher).
The heatmap and dendrogram present the result of the hierarchical clustering of the genes. Three major clusters were found: Cluster A contains inflammatory compounds and includes miR-410 and miR-576-3p. Cluster B contains inflammatory compounds and factors involved with migration/differentiation/metabolism; Cluster C only consists of migration/metabolic factors. MiR-138, miR-574-3p, miR-146a and miR-34c-5p formed a sub-cluster within cluster C and strongly clustered together.

Since our patients and controls of the validation cohort differed on average 8 years in age, we took special notice of correlations of molecular parameters with age. MiR-576-3p correlated significantly and positively with age (r=.257; p=.02), whereas miR-34c-5p did not (r=.041; p=.713). It is important to note that correction for age resulted in loss of significance in the association of T2D with the expression of miR-576-3p. We therefore consider higher expression of this microRNA as related to age rather than to disease. We thereafter performed a correlation analysis between the level of miR-34c-5p and dyslipidemia, hyperglycemia and liver function. This analysis showed that the expression level of miR-34c-5p was not determined by these factors but was only associated with T2D.

Target prediction of miR-34c-5p

Since the expression of miR-34c-5p was significantly up-regulated in T2D monocytes, we asked if there were in silico indications linking miR-34c-5p expression to the regulation of inflammation. We used miRecords as a resource for microRNA-target interactions as this web-based tool integrates predicted microRNA targets produced by 11 established microRNA target prediction programs (DIANA-microT, MicroInspector, miRanda, MirTarget2, miTarget, NBmiRTar, PicTar, PITA, RNA22, RNAhybrid and TargetScan/TargetScanS, available at http://www.mirecords.bioled.org).

Minimum target gene prediction coverage of three algorithms was used to perform prediction analysis for miR-34c-5p, which resulted in 4291 hits.

Ingenuity pathway analysis (Ingenuity Systems) was used for mapping of the predicted target genes to biological functions. Interestingly, the top molecular and cellular function of the miR-34c-5p predicted target genes was "cell morphology" (S1 Text); while the second top-associated network was "cell morphology/cellular assembly and organization/cellular development". In the top canonical pathways, the STAT3 pathway was third in line .

Among the potential targets of miR-34c-5p, some of the diabetes-related signature genes identified earlier [21] were found, i.e. PTGS2, PDE4B and EMP1 were predicted as targets of miR-34c-5p in three to five algorithms. Interestingly, all our predicted targets derived from the same 6 (miRanda, Mir Target2, PicTar, PITA, RNAhybrid and Target Scan) of the 11 algorithms integrated by miRecords.

TaqMan qPCR analysis for the expression of the 24 signature mRNAs in the monocytes of the Ecuadorian validation cohort

We carried out a qPCR analysis for the expression of the 24 cluster 1 and 2 mRNAs using the monocytes of the validation cohort of the Ecuadorian T2D patients and controls. The levels of the gene data are expressed in the table of Fig 1 (which also shows the cluster analysis of the genes and the tested microRNAs, see for explanation underneath). The table shows that of the 24 genes tested, 4 genes were significantly differentially expressed in the

monocytes of the T2D patients as compared to the monocytes of the non-diabetic controls (in total material of 43 patients and 33 controls was available).

PTGS2 was significantly lower expressed in the T2D monocytes, while CD9, DHRS3 and PTPN7 were significantly higher expressed in the monocytes of the T2D patients as compared to the non-diabetic controls.

Since our patients and controls of the validation cohort differed on average 8 years in age, we again took special notice of correlations of the expression of these genes with age. There were no age- or gender-dependencies of these 4 abnormally expressed genes. We also performed correlation analyses with hyperglycemia, dyslipidemia, and liver function, but did not find statistically significant correlations.

Interdependence of microRNA and mRNA expression in T2D monocytes

To study the mutually inter-dependent state of mRNAs and microRNAs in expression, we also performed a cluster analysis on the qPCR data of both the mRNAs and microRNAs. The dendrogram of this analysis is also given in Fig 1. Three main clusters of mutually correlating genes and microRNAs could be identified; we arbitrarily called these clusters A, B and C.

Cluster A consisted predominantly of genes originally found in Padmos et al. [21] on diabetic monocytes to belong to the cluster of inflammatory genes (previously called "cluster 1" in Padmos et al, 23). These genes are well-known inflammatory compound genes, such as genes for the pro-inflammatory cytokines IL1B, IL6 and TNF, the inflammatory compound PTGS2/COX2, inflammatory chemokines (CCL20, CCL2, CCL4) and transcription factors and regulators of inflammatory pathways such as PDE4B, DUSP2 and ATF3 (Fig. 1). Also miR-410 and the age-related miR-576-3p appeared to be part of this inflammatory cluster.

Cluster B also consisted of genes earlier found in the cluster of inflammatory genes ("cluster 1"), such as CXCL2, PTX3 and BCL2A1. However, this cluster also contained genes originally found by Padmos et al. to belong to the cluster of adhesion/motility/differentiation/metabolic factors ("cluster 2") [21], such as CDC42, STX1A, NAB2, EMP1 and PTPN7.

A third cluster C consisted exclusively of genes found earlier in the cluster of adhesion/motility/differentiation/metabolic factors of Padmos et al ("cluster 2"), such as CD9, DHRS3, FABP5, MAPK6 and HSPA1. Interestingly, miR-138, miR-34c-5p, miR-146a and miR-574-3p formed a sub-cluster within cluster C and strongly clustered together.

Clustering of the diabetic patients and non-diabetic controls of the validation cohort using microRNA and mRNA expression

Using the data on expression of the mRNAs and microRNAs of the validation study, we also clustered diabetics and non-diabetics by unsupervised hierarchical clustering (Fig 2) (in 22 patients and 19 controls both mRNA and microRNA studies had been performed). This approach showed that two main subject clusters were identified with a first cluster (cluster X) containing 5 diabetics and 7 non-diabetic subjects, and a second cluster (cluster Y) comprising 17 diabetics and 12 non-diabetics, showing that a clinically useful distinction between diabetes and non-diabetes could not be made using the selected mRNAs and microRNAs.

However, it also emerged from the data that the subject clustering made a distinction on the basis of dyslipidemia, particularly in the diabetic group (see Table 2): The diabetics in the cluster X had virtually normal levels of cholesterol, LDL and triglycerides, while the diabetics of cluster Y had significantly higher levels of cholesterol, LDL and triglycerides. With regard to monocyte gene expression cluster X of diabetics with normal lipid values had pro-inflammatory monocytes, with up-regulation of many cluster A genes, which reached significance for TNFAIP3 (HSPA1 of gene cluster C was down-regulated). Remarkably, the second cluster of diabetics (Y) with high lipid values had reduced expression of most of these pro-inflammatory genes, reaching significance for DUSP2, ATF3 and PTGS2. MicroRNAs were not significantly differentially expressed in the groups (except for perhaps miR-138, which tended to be lower in the diabetics with normal lipids).

In the non-diabetic subjects, this type of clustering also made a distinction in cluster X subjects with a high expression of gene cluster A inflammatory genes and cluster Y subjects with a reduced expression of these genes. Although the latter non-diabetic subject group also had higher cholesterol and LDL levels these did not reach statistical significance.

DISCUSSION

In this study, we found 15 discriminating microRNAs in a screening study on the purified monocytes of T2D patients, a distinction could be made between T2D patients and non-diabetic controls with a sensitivity of 66% and specificity 90%. Although the specificity is acceptable, sensitivity is relatively low. Moreover, the 15 discriminating microRNAs were only marginally lower and higher expressed. We therefore chose to continue our search and to validate only discriminating microRNAs with a significant fold change of 1,4 or 0,6 versus non-diabetics in a qPCR study using the monocytes of a new series of Ecuadorian patients, of whom we had detailed clinical information.

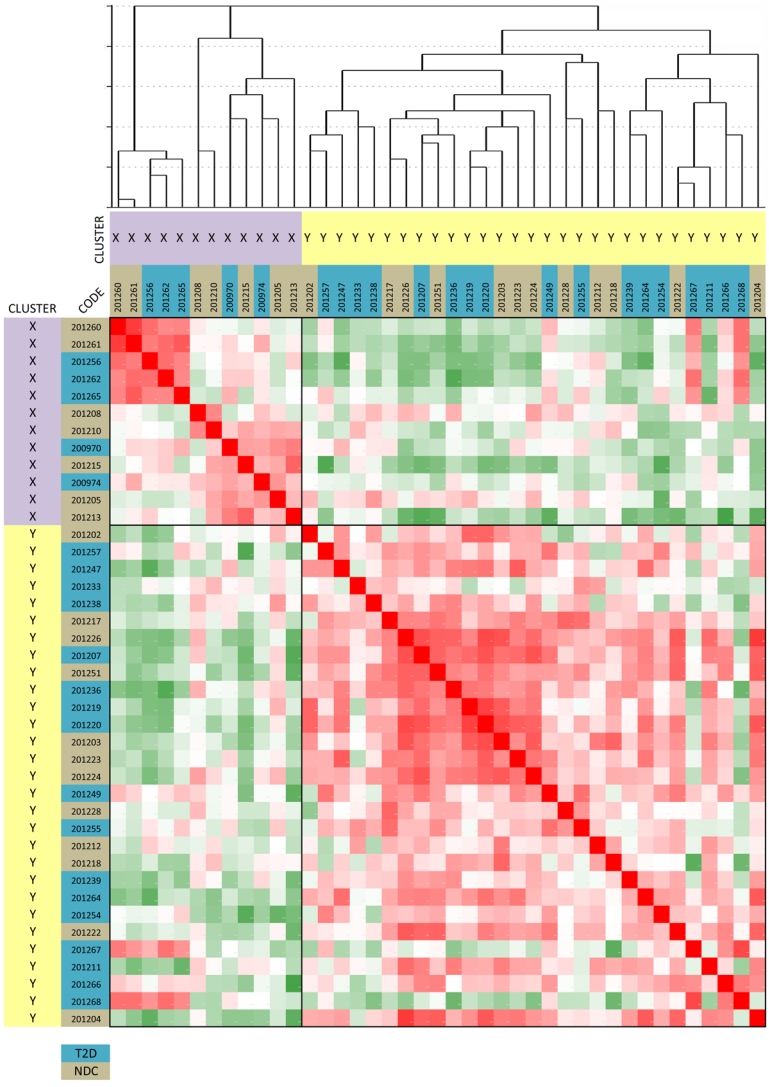

Figure 2. Dendrogram and heatmap of hierarchical clustering of T2D patients and non-diabetic controls of the validation cohort using microRNA and mRNA expression as determined by qPCR. Figure 2. Dendrogram and heatmap of hierarchical clustering of T2D patients and non-diabetic controls of the validation cohort using microRNA and mRNA expression as determined by qPCR. This figure shows that two main subject clusters (X and Y) could be identified. Cluster X contained 5 diabetics and 7 non-diabetic subjects, and cluster Y comprised 17 diabetics and 12 non-diabetics. This approach did not distinguish between T2D patients and non-diabetic controls.

Of the selected 5 microRNAs only miR-34c-5p was validated as significantly higher expressed in the circulating monocytes of the validation cohort of T2D patients, also taking confounding factors as age, gender, BMI, liver function and dyslipidemia into consideration.

With regard to previous literature on the validated expression of microRNAs in peripheral blood leukocytes in T2D, there are 5 relevant reports [38,39,46-48] . In none of these reports purified monocytes have been tested. In total 9 microRNAs were found abnormally expressed in these reports and interestingly miR-34a, which has virtually the same sequence and targets as miR-34c-5p, was found to be down-regulated in T2D patients upon resveratrol treatment [47].

In Ingenuity analysis the top molecular and cellular function of miR-34c-5p predicted target genes was "cell morphology". With regard to the literature on the role of miR-34c-5p in cell biology there are only few reports. The scarce literature shows a role of this microRNA in diverse cellular processes, such as inflammatory responses [49]; growth, apoptosis [50] [51], invasiveness of tumor cells [40], and cell morphological processes involved in the differentiation and the morphogenesis of neuronal cell projections [52].

Interestingly, a recent report shows that miR-34c-5p has a function in altering the expression of c-Met, the receptor for HGF, also known as scatter factor [53]. HGF is amongst others an angiogenic factor and plays a role in endothelial migration, proliferation and neovascularization. Also, we recently carried out a preliminary study in which we profiled T2D monocytes for microRNA expression in relation to their capacity to form pro-angiogenic cells. Pro-angiogenic cells are also known as myeloid endothelial progenitor cells and play a role in vascular repair [54]. When we compared the group of high pro-angiogenic cell formers with those with a low potential for pro-angiogenic cell formation, miR-34c appeared to be the most discriminating microRNA, being raised 10 times in the high pro-angiogenic cell formers (data to be published). With regard to the literature on pro-angiogenic cells others have found microRNAs miR-126, miR-130, miR-21, miR-27) to be lower expressed in pro-angiogenic cells of T2D patients [55]. Collectively our in silico targeting data, the literature data and our preliminary data on pro-angiogenic cells supports a view that the raised expression of miR-34c-5p in T2D monocytes might represent a molecular sign for a raised potential of the T2D monocytes for cell morphological changes and differentiation to vascular support cells to compensate increased endothelial damage in T2D.

This concept is also supported by the mRNA expression in the monocytes of the Ecuadorian validation cohort: We found a significant up-regulation of CD9, DHRS3 and PTPN7. These genes were earlier described as up-regulated in monocytes of T2D, LADA and T1D patients by Padmos et al and Beyan et al.[21,56]. CD9 is a tetraspanin and an important regulator of integrin activity and plays a role in the immunological synapse as well as in endothelial adhesion and transmigration [57,58]. DHRS3 is an enzyme involved in vitamin

Table 2. Distinction of non-diabetic controls and T2D patients on the basis of dyslipidemia.

Genes/ miRNA		T2D CI-X		T2D CI-Y			NDC CI-X	NDC CI-Y	
		Mean (FC)	p value vs N-T2D	Mean (FC)	p value vs N-T2D	p value CLX vs CLY	Mean (FC)	Mean (FC)	p value CLX vs CLY
CLUSTER A	CCL4	3.76	0.19	0.85	0.44	0.12	2.96	0.36	0.11
	IL6	4.49	0.11	0.87	0.37	0.09	2.13	0.63	**0.04**
	TNF	2.47	0.21	0.93	0.45	0.15	1.75	0.81	0.21
	IL1B	1.74	0.28	0.60	0.07	**0.02**	2.58	0.42	**0.01**
	CCL2	3.08	0.17	0.64	0.13	0.10	1.64	0.92	0.43
	CCL20	1.69	0.19	0.80	0.62	**0.04**	2.42	0.24	**0.05**
	TNFAIP3	**2.06**	0.05	1.05	0.88	**0.05**	1.71	0.73	0.10
	PDE4B	1.33	0.28	0.90	0.72	0.21	1.52	0.67	**0.02**
	miR410	1.59	0.66	1.79	0.30	0.88	0.99	1.03	0.96
	DUSP2	1.21	0.79	**0.56**	**0.03**	0.06	2.07	0.56	**0.00**
	PTGS2	0.82	0.41	**0.50**	**0.05**	0.28	2.09	0.61	0.08
	ATF3	1.50	0.33	**0.66**	**0.04**	**0.03**	1.68	0.85	0.14
	miR576-3p	1.29	0.42	1.03	0.81	0.46	0.75	1.14	**0.04**
CLUSTER B	CDC42	1.44	0.13	0.96	0.80	0.10	1.14	0.92	0.53
	PTX3	1.84	0.15	1.08	0.13	0.03	1.62	0.82	0.12
	CXCL2	3.55	0.19	0.71	0.17	0.13	2.38	0.55	0.06
	STX1A	2.98	0.34	0.89	0.62	0.31	1.66	0.81	0.12
	NAB2	1.32	0.70	0.74	0.25	0.26	1.83	0.68	0.19
	EMP1	1.64	0.52	1.12	0.92	0.53	1.23	0.99	0.63
	BCL2A1	1.07	0.87	0.72	0.28	0.13	1.64	0.83	0.42
	PTPN7	1.84	0.08	1.08	0.77	0.12	1.44	0.74	0.07
CLUSTER C	miR138	0.58	0.06	1.04	0.87	0.07	0.97	1.10	0.62
	miR574-3p	0.72	0.16	0.87	0.19	0.40	0.77	1.14	0.05
	miR146a	1.10	0.56	1.00	0.89	0.64	0.79	1.10	0.09
	miR34c5p	1.28	0.08	1.16	0.19	0.39	0.91	1.07	0.17
	MAPK6	0.58	0.07	1.03	0.93	0.07	1.08	0.96	0.70
	HSPA1aHSPA1b	**0.49**	**0.04**	0.99	0.99	**0.04**	0.91	1.04	0.42
	DHRS3	1.11	0.23	1.31	0.11	0.55	0.72	0.88	0.37
	CD9	1.32	0.54	1.93	0.13	0.37	0.67	1.22	0.24
Cholesterol		**202**		235		**0.04**	231	258	0.23
LDL		**119**		177		**0.03**	151	179	0.22
Triglycerides		**151**		238		**0.03**	220	182	0.55

Table 2 shows that the T2D patients in the cluster X had virtually normal levels of cholesterol, LDL and triglycerides. The T2D patients of cluster Y had significantly higher levels of cholesterol, LDL and triglycerides. The monocyte gene expression of cluster X (T2D with normal lipid values) had up-regulation of many pro-inflammatory genes (cluster A), which reached significance for TNFAIP3. The monocyte gene expression of cluster Y (T2D with high lipid values) had reduced expression of most of the pro-inflammatory genes (cluster A), reaching significance for DUSP2, ATF3 and PTGS2. MicroRNAs were not significantly differentially expressed in the groups. In the HC subjects, this type of clustering also made a distinction in cluster X (HC with high expression of inflammatory genes) and cluster Y (HC with reduced expression of inflammatory genes). Although the HC group also had higher cholesterol and LDL levels these did not reach statistical significance.

A (retinoid) metabolism and vitamin A is an important growth and differentiation factor for immune cells and their precursors [59-61]. PTPN7 (or HePTP) is a tyrosine phosphatase regulating the activity of p38 and ERK playing an important role in the differentiation of monocytes to progeny cells including macrophages, dendritic cells and pro-angiogenic cells. Moreover, CD9 and DHRS3 clustered in cluster analysis with the expression of miR-34c-5p in monocytes, underscoring a putative role of all these molecules in processes of cell differentiation and cell morphogenesis. Taking these data together, we therefore assume that our observation on up-regulated CD9 and DHRS3 and PTPN7 expression in T2D monocytes can (next to the over-expression of miR-34c-5p) be taken as a sign that the circulating monocytes in T2D patients have an altered potential for adhesion, migration and differentiation into progeny, such as macrophages, dendritic cells and vascular support cells.

This study does not support the view that monocytes are in general pro-inflammatory activated in T2D patients: The circulating monocytes of the T2D patients of the validation cohort failed to show a significant up-regulation of typical pro-inflammatory genes (such as IL-1B, IL-6, TNF, CCL4 and CCL20) as compared to monocytes of non-diabetic controls with a similar ethnic background, considering the T2D patient population in total. Some important pro-inflammatory genes were even down-regulated (DUSP2, ATF3) and down-regulation reached significance for PTGS2. Also miR-146a, which is a classical microRNA dampening inflammatory responses and earlier found as down-regulated in monocytes and macrophages of patients with (auto-)inflammatory conditions [38], was not reduced in the monocytes of our T2D Ecuadorian cases as compared to the non-diabetic controls (it was also not identified in the finding cohort). Our current data thus refute the earlier expressed views in literature [15], and by us [21], that circulating monocytes of T2D patients are in general characterized by a pro-inflammatory state.

However when we divided the Ecuadorian T2D and non-diabetic subjects on the basis of a subject cluster analysis using the expression data of the 24 mRNAs and 6 microRNAs, we identified two sets of subjects.

The diabetics in the first set were characterized by virtually normal serum lipid values. These patients did have a raised monocyte expression of pro-inflammatory cluster A genes, reaching significance for TNFAIP (A20), an important TNF-induced inflammatory gene.

The patients of the second set of subjects were characterized by raised cholesterol, LDL and triglycerides and a down-regulation of pro-inflammatory monocyte genes, reaching significance for DUSP2, ATF3 and PTGS2 (COX2). DUSP2 and ATF3 are important transcription regulators of inflammatory compounds, while PTGS2 is a well-known enzyme of the prostaglandin pathway.

The patient cluster data thus suggest that pro-inflammatory monocytes do circulate in T2D patients provided there is a normal serum lipid state. In case of dyslipidemia circulating monocytes have a significantly reduced expression of typical pro-inflammatory genes.

With regard to dyslipidemia being associated with reduced expression of pro-inflammatory genes, it is important to note that our Ecuadorian general population control group was atypical in also having many signs of dyslipidemia: Hypercholesterolemia was present in 68% and a raised LDL in 40% (BMIs were over 25 in 83% of the population). Considering the excessively high prevalence of dyslipidemia (and obesity) in the Quito general population control group, it is important to note that a recent healthcare report of the Ecuadorian government corroborates this high prevalence of dyslipidemia and obesity in urban Ecuadorian populations [62]. One can therefore ask the question whether, contrary to expectation, also in the Quito controls the high serum lipids might be associated with a down regulation of the inflammatory genes in circulating monocytes.

Our subject cluster analysis on the validation cohort delivered indeed two groups of non-diabetic Quito controls differing in pro-inflammatory state of monocytes; the non-diabetics of subject cluster Y had anti-inflammatory monocytes with various significantly down-regulated cluster A genes. Although their lipid values were higher, they did not reach statistical significance. To further investigate the concept of dyslipidemia being associated with an anti-inflammatory state of circulating monocytes, we thereafter compared in preliminary studies the monocytes of the Quito general population control group with those of a Dutch general population control group, which had been collected and analyzed at the same time as the Ecuadorian controls. The Dutch general population controls had normal lipid values (hypercholesterolemia none, raised LDL 14%), contrasting to the much higher values found in the Ecuadorian general population controls. We used for monocyte gene expression the same type of TLDA qPCR cards. When we compared outcomes of both groups (see S2 Table), we indeed found that the monocytes of the Ecuadorian non-diabetic general population controls with the high rate of abnormal serum lipids had significantly reduced expression levels of many of the classical inflammatory cluster A and B genes as compared to the Dutch general population controls. Cluster C genes were

largely unaltered in the monocytes of the group of dyslipidemia Ecuadorian non-diabetic general population controls. These data therefore strengthen the view that dyslipidemia is associated with a dampened inflammatory state of circulating monocytes. However a word of caution is here also in place; the usage of aspirin and NSAIDs is high in the general population of Ecuador [63,64], and drug effects may therefore also have played a role in the difference in monocyte inflammatory state between the Dutch and Ecuadorian controls.

It is further of interest to note that the found raised microRNA miR-34c-5p may have played a mechanistic role in the phenomenon of down regulated inflammatory gene expression in monocytes. In silico we found the inflammatory genes PTGS2 and PDE4B as direct targets of miR-34c-5p, and although functional studies are required to formally test such target interactions, these in silico data suggest that miR-34c-5p might be instrumental in the down regulation of the inflammatory state of the monocytes.

Remains the question how our abnormal monocyte gene expression relates to the genetic back ground of T2D subjects. Interestingly, genome-wide association studies for T2D have highlighted multiple genes implicated in adipo-cytokine pathways and cell cycle regulation [65,66]. Many of the genes of our monocyte gene cluster A are part of the adipo-cytokine network, further highlighting the etiological relevance of these pathways. However, it is unlikely that any genetic variation would be explaining the here observed gene expression differences in monocytes. All of the known common genetic variants for T2D have too small effect sizes that they would not discriminate between case/control status in this study [66]. Complex gene-environment (such as obesity and dyslipidemia) interactions most likely play a role in the here described aberrant monocyte gene expression. It must also be noted in this report on Ecuadorian patients that, although the T2D related GWAS polymorphisms have largely been detected in populations of white European ancestry, many are also prevalent in other ethnic groups [67].

LIMITATIONS

In retrospect our diabetic and non-diabetic control groups between the finding and validation studies differ considerably with regard to parameters that do influence the microRNA and mRNA profiles, such as lipid values (and use of statins that influence lipid values) and perhaps ethnicity (finding cohort comprised Germans and Ecuadorians, validation cohort only Ecuadorians). On the other hand our studies using these groups have unveiled here a hitherto unknown effect of abnormal lipids on circulating monocytes rendering them most likely better cells for vascular repair. In ongoing studies we make use of Dutch diabetic and non-diabetic subject to investigate whether this negative

association between lipid profile and monocyte inflammatory state can be confirmed in Caucasian diabetic populations (next reports).

Furthermore we used for the microRNA studies TaqMan qPCR assays for validation, after having used Exiqon arrays in the finding study. In a limited series we were able to also validate some of the microRNAs with Exiqon qPCR and it turned out that sensitivities for detection differed in some instances between the two methods for validation (Exiqon versus TaqMan qPCR), e.g. miR-155 was not detectable in the monocytes via TaqMan qPCR, while it was readily detected via Exiqon array and qPCR. On the other hand, miR-410 was better detectable by TaqMan qPCR. Therefore follow up studies should preferably include more extensive comparisons between the two detection methods to select for robust discriminating microRNAs.

CONCLUSION

Using microRNA and mRNA profiling and validation we found an over-expression of miR-34c-5p and of a set of three genes (CD9, DHRS3 and PTPN7) in the monocytes of Ecuadorian T2D patients suggesting an altered adhesion, differentiation potential and shape change potential of the circulating monocytes. We assume that this increased potential might be instrumental in vascular repair. With regard to inflammatory genes we only found a pro-inflammatory state of monocytes in T2D patients with normal serum lipids (who formed a minority within the diabetic group). Dyslipidemia coincided with a reduced expression of pro-inflammatory genes in circulating monocytes, which might be instrumental in strengthening the potential of monocytes for vascular repair.

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SUPPORTING INFORMATION

S1. Text. Ingenuity pathway analysis. Ingenuity systems was used to map the major pathways and processes in which miR-34c-5p is involved. The top molecular and cellular function of the miRNA predicted target genes was "cell morphology"; while the second top-associated network was "cell morphology/cellular assembly and organization/cellular development". We used miRecords as a resource for microRNA-target interactions.

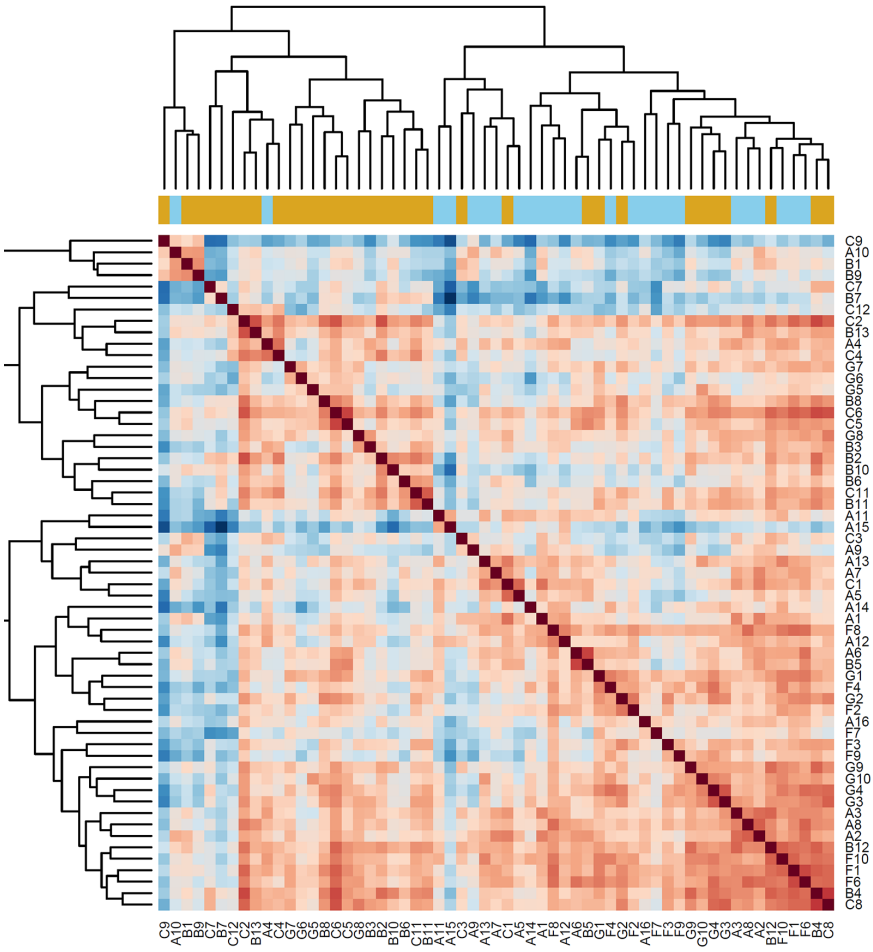

Figure S1. Dendrogram and heatmap of hierarchical clustering of T2D patients and non-diabetic controls of the finding cohort using microRNAs. This figure shows that partial separation can be made between T2D patients and healthy controls on the basis of the 15 microRNAs identified as optimal prediction signature. Two main subject clusters were identified. The first cluster contain 24 T2D patients (yellow) and only 2 healthy controls (blue), and the second mixed cluster contains 12 patients (yellow) and 23 healthy controls (blue).

Table S1. Differentially expressed monocyte microRNAs of T2D patients compared to non-diabetic controls of the finding cohort. This table shows the 142 microRNAs that were found to be differentially expressed in T2D patients compared to controls. 35% of the miRNAs were down-regulated and 65% were up-regulated.

Up-regulated microRNAs

	miRNA	p-value		miRNA	p-value		miRNA	p-value
1	hsa-let-7a-2*	0.004	32	hsa-miR-296-3p	0.046	63	hsa-miR-523	0.043
2	hsa-let-7e	0.016	33	hsa-miR-297	0.012	64	hsa-miR-541	0.002
3	hsa-miR-10a*	0.004	34	hsa-miR-298	0.014	65	hsa-miR-548b-5p	0.029
4	hsa-miR-122	0.010	35	hsa-miR-30b*	0.000	66	hsa-miR-550	0.012
5	hsa-miR-125a-3p	0.006	36	hsa-miR-30c-1*	0.006	67	hsa-miR-574-3p	0.029
6	hsa-miR-125a-5p	0.033	37	hsa-miR-32*	0.006	68	hsa-miR-574-5p	0.004
7	hsa-miR-125b-2*	0.004	38	hsa-miR-325	0.003	69	hsa-miR-576-3p	0.034
8	hsa-miR-1296	0.000	39	hsa-miR-328	0.041	70	hsa-miR-585	0.002
9	hsa-miR-130b	0.017	40	hsa-miR-329	0.023	71	hsa-miR-595	0.026
10	hsa-miR-135a*	0.033	41	hsa-miR-331-3p	0.017	72	hsa-miR-596	0.000
11	hsa-miR-138	0.007	42	hsa-miR-335	0.043	73	hsa-miR-601	0.043
12	hsa-miR-139-3p	0.004	43	hsa-miR-338-5p	0.006	74	hsa-miR-603	0.024
13	hsa-miR-143*	0.010	44	hsa-miR-34c-5p	0.002	75	hsa-miR-610	0.021
14	hsa-miR-184	0.015	45	hsa-miR-370	0.033	76	hsa-miR-617	0.022
15	hsa-miR-185	0.007	46	hsa-miR-371-3p	0.000	77	hsa-miR-625	0.005
16	hsa-miR-185*	0.002	47	hsa-miR-376a*	0.021	78	hsa-miR-629	0.009
17	hsa-miR-187*	0.004	48	hsa-miR-423-5p	0.024	79	hsa-miR-638	0.021
18	hsa-miR-1908	0.035	49	hsa-miR-432*	0.000	80	hsa-miR-642	0.012
19	hsa-miR-193a-5p	0.021	50	hsa-miR-433	0.036	81	hsa-miR-647	0.030
20	hsa-miR-193b*	0.004	51	hsa-miR-449a	0.002	82	hsa-miR-658	0.000
21	hsa-miR-194*	0.002	52	hsa-miR-483-5p	0.000	83	hsa-miR-659	0.003
22	hsa-miR-195	0.025	53	hsa-miR-488	0.021	84	hsa-miR-675	0.029
23	hsa-miR-198	0.002	54	hsa-miR-492	0.021	85	hsa-miR-760	0.035
24	hsa-miR-200b*	0.004	55	hsa-miR-497	0.008	86	hsa-miR-765	0.002
25	hsa-miR-202	0.022	56	hsa-miR-498	0.002	87	hsa-miR-877	0.002
26	hsa-miR-210	0.003	57	hsa-miR-501-3p	0.017	88	hsa-miR-885-3p	0.027
27	hsa-miR-215	0.017	58	hsa-miR-505*	0.012	89	hsa-miR-888*	0.032
28	hsa-miR-219-2-3p	0.044	59	hsa-miR-515-3p	0.012	90	hsa-miR-92b*	0.036
29	hsa-miR-22*	0.047	60	hsa-miR-516b	0.018	91	hsa-miR-936	0.000
30	hsa-miR-24-1*	0.046	61	hsa-miR-518c*	0.000	92	hsa-miR-96*	0.033
31	hsa-miR-25*	0.012	62	hsa-miR-519d	0.002	93	hsa-miR-99b*	0.001

Down-regulated microRNAs

	miRNA	p-value		miRNA	p-value		miRNA	p-value
1	hsa-let-7a	0.002	18	hsa-miR-302b*	0.018	34	hsa-miR-548c-3p	0.010
2	hsa-let-7c*	0.018	19	hsa-miR-302c*	0.043	35	hsa-miR-556-3p	0.019
3	hsa-let-7g	0.017	20	hsa-miR-30a	0.047	36	hsa-miR-586	0.002
4	hsa-miR-144*	0.032	21	hsa-miR-31*	0.013	37	hsa-miR-607	0.032
5	hsa-miR-148b	0.019	22	hsa-miR-326	0.012	38	hsa-miR-616*	0.049
6	hsa-miR-150	0.008	23	hsa-miR-374a	0.021	39	hsa-miR-625*	0.004
7	hsa-miR-154	0.029	24	hsa-miR-410	0.042	40	hsa-miR-628-3p	0.012
8	hsa-miR-191	0.006	25	hsa-miR-424	0.042	41	hsa-miR-649	0.040
9	hsa-miR-19a	0.035	26	hsa-miR-450b-5p	0.019	42	hsa-miR-651	0.029
10	hsa-miR-19a*	0.009	27	hsa-miR-486-5p	0.018	43	hsa-miR-652	0.000
11	hsa-miR-20b	0.008	28	hsa-miR-491-3p	0.009	44	hsa-miR-656	0.003
12	hsa-miR-216b	0.007	29	hsa-miR-494	0.010	45	hsa-miR-662	0.045
13	hsa-miR-223	0.000	30	hsa-miR-509-3-5p	0.001	46	hsa-miR-720	0.002
14	hsa-miR-223*	0.033	31	hsa-miR-520f	0.035	47	hsa-miR-9*	0.018
15	hsa-miR-26a	0.007	32	hsa-miR-532-5p	0.049	48	hsa-miR-921	0.023
16	hsa-miR-26b	0.046	33	hsa-miR-548a-3p	0.009	49	hsa-miR-943	0.002
17	hsa-miR-301b	0.023						

Table S2. Monocyte gene expression of the Ecuadorian non-diabetic general population controls vs the Dutch general population controls. Values represent the means and standard deviations of normalized Ct values (Ct gene/Ct reference gene ABL) by the ΔΔCt. Genes are given in the order of the cluster diagram given in this paper. The table shows significantly reduced expression levels of many of the classical inflammatory cluster A and B genes in the Ecuadorian non-diabetic controls compared to the Dutch controls. Cluster C genes were largely unaltered in the monocytes of the Ecuadorian group. Of note, these analyses were performed in the same time period to exclude technical variability.

Gene		Ecuadorian		Dutch		p-Value
		NDC		NDC		
		Mean	SD	Mean	SD	
CLUSTER A	IL6	***0.01***	0.6	0.30	0.7	**
	TNF	***2.14***	2.5	2.65	2.6	ns
	IL1B	***9.25***	85.8	70.95	107.1	***
	CCL2	0.40	0.8	0.71	1.1	*
	CCL20	***0.05***	1	0.49	1.1	**
	TNFAIP3	***1.91***	5.3	5.78	6.3	***
	PDE4B	***3.13***	6.4	7.81	8	***
	DUSP2	1.49	2.1	2.50	2.2	**
	PTGS2	3.22	3	2.57	2.2	ns
	ATF3	***1.62***	1.6	2.66	1.6	**
CLUSTER B	CDC42	1.12	0.7	1.13	1.4	ns
	PTX3	***0.52***	1.8	1.50	2	**
	CXCL2	***0.72***	7.6	5.55	8.5	***
	STX1A	***0.01***	0.1	0.04	0.1	**
	NAB2	0.29	0.5	0.41	0.5	ns
	EMP1	***0.49***	1.1	1.04	1.3	**
	BCL2A1	18.85	21	25.68	67.2	ns
	PTPN7	0.18	0.1	0.17	0.1	ns
CLUSTER C	FABP5	16.41	37.5	19.51	117.2	ns
	MAPK6	5.69	10.4	7.80	37.1	ns
	HSPA1A;HSPA1B	19.65	22.5	16.45	52.3	ns
	DHRS3	0.06	0	0.07	0	ns
	CD9	2.78	2.9	2.10	2.7	ns

Note. Statistical significance: *p< .05;**p< .01; ***p< .001

Analysis Name: Target prediction miR-34c-5p - 2014-04-15 05:38 PM
Analysis Creation Date: 2014-04-15
Build version: 302937
Content version: 18488943 (Release Date: 2014-03-23)

Analysis settings

View

Reference set: Ingenuity Knowledge Base (Genes Only)
Relationship to include: Direct and Indirect
Includes Endogenous Chemicals
Optional Analyses: My Pathways My List

Filter Summary:
Consider only relationships where
confidence = Experimentally Observed
Cutoff:

Summary of Analysis - Target prediction miR-34c-5p - 2014-04-15 05:38 PM

Top Canonical Pathways

Name	p-value	Ratio
Glioblastoma Multiforme Signaling	1,56E-08	43/168 (0,256)
Dopamine-DARPP32 Feedback in cAMP Signaling	1,43E-07	44/187 (0,235)
STAT3 Pathway	1,51E-07	26/80 (0,325)
Axonal Guidance Signaling	2,21E-07	89/487 (0,183)
Molecular Mechanisms of Cancer	2,91E-07	74/388 (0,191)

Top Upstream Regulators

Upstream Regulator	p-value of overlap	Predicted Activation State
mir-34	1,88E-23	
beta-estradiol	4,09E-12	
miR-34a-5p (and other miRNAs w/seed GGCAGUG)	2,25E-11	
TGFB1	1,15E-10	
AGT	1,82E-10	

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value	# Molecules
Cancer	4,04E-35 - 9,14E-06	1898
Developmental Disorder	4,32E-21 - 3,20E-06	414
Cardiovascular Disease	4,32E-17 - 5,45E-06	415
Neurological Disease	7,69E-12 - 4,12E-06	570
Gastrointestinal Disease	1,36E-11 - 5,38E-06	800

Molecular and Cellular Functions

Name	p-value	# Molecules
Cell Morphology	2,44E-19 - 9,35E-06	586
Cell Death and Survival	2,62E-19 - 9,44E-06	756
Molecular Transport	2,58E-18 - 3,81E-06	533
Cellular Movement	1,24E-17 - 5,92E-06	489
Cellular Development	2,24E-16 - 9,48E-06	717

Physiological System Development and Function

Name	p-value	# Molecules
Organismal Survival	2,32E-27 - 4,14E-06	588
Behavior	2,21E-23 - 9,31E-06	294
Nervous System Development and Function	2,80E-19 - 9,35E-06	541
Organismal Development	4,74E-18 - 7,90E-06	724
Tissue Morphology	7,25E-18 - 9,35E-06	586

Top Tox Functions

Assays: Clinical Chemistry and Hematology

Name	p-value	# Molecules
Increased Levels of Hematocrit	2,27E-03 - 2,27E-03	22
Increased Levels of Red Blood Cells	2,27E-03 - 7,34E-02	22
Increased Levels of Alkaline Phosphatase	6,81E-03 - 1,20E-01	18
Increased Levels of Albumin	6,10E-02 - 5,36E-01	6
Increased Levels of Blood Urea Nitrogen	7,77E-02 - 7,77E-02	4

Cardiotoxicity

Name	p-value	# Molecules
Heart Failure	4,01E-07 - 6,41E-01	61
Cardiac Arrythmia	1,17E-06 - 6,41E-01	50
Tachycardia	1,52E-06 - 1,20E-01	26
Cardiac Arteriopathy	5,44E-06 - 6,41E-01	63
Congenital Heart Anomaly	8,43E-05 - 1,00E00	38

Hepatotoxicity

Name	p-value	# Molecules
Liver Proliferation	9,72E-07 - 2,32E-01	49
Liver Hepatomegaly	2,48E-06 - 2,48E-06	25
Liver Inflammation/Hepatitis	9,62E-06 - 4,01E-01	60
Liver Steatosis	9,62E-06 - 4,01E-01	53
Liver Hypoplasia	3,31E-04 - 3,31E-04	14

Nephrotoxicity

Name	p-value	# Molecules
Renal Necrosis/Cell Death	3,37E-09 - 4,63E-01	96
Kidney Failure	2,40E-05 - 1,00E00	45
Renal Inflammation	2,50E-05 - 1,00E00	39
Renal Nephritis	2,50E-05 - 1,00E00	39
Glomerular Injury	6,18E-05 - 5,03E-01	33

Top Regulator Effect Networks

Top Networks

ID	Associated Network Functions	Score
1	Digestive System Development and Function, Infectious Disease, Organ Morphology	35
2	Cell Morphology, Cellular Assembly and Organization, Cellular Development	34
3	Cellular Development, Embryonic Development, Organ Development	34
4	Behavior, Molecular Transport, Cell Morphology	32
5	Connective Tissue Disorders, Dental Disease, Developmental Disorder	32

Top Tox Lists

Name	p-value	Ratio
Renal Necrosis/Cell Death	1,25E-07	96/472 (0,203)
Liver Proliferation	7,34E-06	49/216 (0,227)
Cardiac Fibrosis	1,8E-05	42/181 (0,232)
Cardiac Hypertrophy	2,94E-05	72/373 (0,193)
VDR/RXR Activation	2,59E-04	21/78 (0,269)

Summary of Analysis - Target prediction miR-34c-5p - 2014-04-15 05:38 PM

Top My Lists

Name	p-value	Ratio

Top My Pathways

Name	p-value	Ratio

Top Molecules

This analysis has no expression values.

Chapter 4

Decreased serum level of miR-146a as sign of chronic inflammation in type 2 diabetic patients

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ABSTRACT

Background. There is increasing evidence that chronic inflammation is an important determinant in insulin resistance and in the pathogenesis of type 2 diabetes (T2D). MicroRNAs constitute a newly discovered system of cell regulation and in particular two microRNAs (miR-146a and miR-155) have been described as regulators and biomarkers of inflammation.

Aim. To determine a putative association between the levels of miR-146a and miR-155 in serum of T2D patients, clinical parameters and serological indicators of inflammation.

Methods. We performed quantitative Real Time PCR (qPCR) of microRNAs from serum (56 Ecuadorian T2D ambulatory patients and 40 non-diabetic controls). In addition, we evaluated T2D-related serum cytokines, chemokines and growth factors using a commercially available multi-analyte cytometric bead array system. We correlated outcomes to clinical parameters, including BMI, HbA1c and lipid state.

Results.
- The Ecuadorian non-diabetic controls appeared as overweight (BMI >25: patients 85%, controls 82.5%) and as dyslipidemic (hypercholesterolemia: patients 60.7%, controls 67.5%) as the patients.
- The serum levels of miR-146a were significantly reduced in T2D patients as compared to these non-diabetic, but obese/dyslipidemic control group (mean patients 0.61, mean controls set at 1; $p= 0.042$), those of miR-155 were normal.
- The serum levels of both microRNAs correlated to each other ($r=0.478$; $p<0.001$) and to leptin levels. The microRNAs did not correlate to BMI, glycemia and dyslipidemia.
- From the tested cytokines, chemokines and growth factors, we found IL-8 and HGF significantly raised in T2D patients versus non-diabetic controls ($p= 0.011$ and 0.023 respectively).

Conclusions. This study shows decreased serum anti-inflammatory miR-146a, increased pro-inflammatory IL-8 and increased HGF (a vascular/insular repair factor) as discriminating markers of failure of glucose control occurring on the background of obesity and dyslipidemia.

Key words. Inflammation, T2D, miR-146a, miR-155, IL-8, HGF.

INTRODUCTION

It is well accepted that obesity and type 2 diabetes can be viewed as inflammatory disorders. Early, in the 1990s Hotamisligi et al. showed that TNF-α was present in obese individuals and animals in proportional levels to insulin resistance and they proposed a pathogenic role of inflammatory molecules, such as TNF-α, in the development of insulin resistance and diabetes [1]. To support this idea it was later shown that TNF-α was indeed capable to induce insulin resistance in lean animals [1-3] and that various pro-inflammatory cytokines trigger intracellular pathways such as Nuclear Factor for Kappa light chain in B-cells (NF- κB), IκB kinase-β (IKKβ) and Jun kinase (JNK) which are capable to inhibit the insulin signaling pathway [4-8].

Macrophages in adipose tissue as well as the adipocytes themselves are the prime source of the raised pro-inflammatory cytokines and adipokines, leading to a chronic pro-inflammatory state in obese subjects. In conjunction with these cellular responses in so-called "chronically inflamed" adipose tissue, a disturbed lipid metabolism is capable of inducing such a chronic pro-inflammatory state. High levels of Ox-LDL and low levels of HDL correlate to inflammatory activation and insulin resistance through a mechanism called lipotoxicity [4, 9-11]. Moreover, free fatty acids enhance the secretion of TNF-α, IL-6 and PAI-1, which stimulate macrophages to secrete more inflammatory cytokines and chemokines aggravating the feed-forward loop of inflammation [2, 11, 12]. All in all, there is a vast literature on increased levels of pro-inflammatory cytokines in the metabolic syndrome (MetS) and type 2 diabetes (T2D), and excellent reviews exist on this topic [13-17].

MicroRNAs represent a newly discovered level of cell regulation, functioning by inhibiting protein translation, and microRNAs have been suggested to be useful biomarkers in various pathological conditions, including diabetes [18, 19]. A substantial literature indicates that two microRNAs, i.e. miR-146a and miR-155, are key regulators of (auto)-inflammatory processes [20-31]. Dysregulation of these microRNAs in peripheral blood mononuclear cells (PBMC) has been implicated in diabetes [20, 32]. MiR-146a and miR-155 expression levels have been found to be significantly decreased in the PBMCs of patients with T2D as compared to control subjects and expression values correlated negatively to parameters of metabolic control (Hb1Ac, glucose) and signs of inflammation (NFκB mRNA levels in PBMC, circulatory levels of pro-inflammatory cytokines). MicroRNAs are, however, also detectable in serum and there are indications that microRNAs are very stable in this milieu [33-36], although they might be less stable in other milieus, such as the brain [37]. Measured in serum, they can serve as biomarkers and there is a study that has determined the level of miR-146a in the serum of T2D patients as one of the microRNAs of a set of 7 microRNAs considered to act as key regulators of the expression, production, secretion

or effectiveness of insulin [38]. This study found raised levels of these 7 microRNAs when evaluated in relatively small groups (n= 19 each) of newly diagnosed T2D patients as compared to pre-diabetic individuals and T2D-susceptible individuals [38].

In the current study we determined the levels of miR-146a and miR-155a in the serum of 56 Ecuadorian T2D patients and of 40 non-diabetic controls and associated the levels of these microRNAs to parameters of glucose control, dyslipidemia, obesity and the serum level of 12 T2D-related inflammatory mediators (TNFα, IL-1β, IL-6, NFG, HGF, PAI, Resistin, CCL2, Adiponectin, Leptin, IL-8, and CCL4) using a commercially available multi-analyte cytometric bead array system, especially developed for type 2 diabetes (Milliplex® Map, U.S.A.).

MATERIALS AND METHODS

Patients

A total of 56 patients positively diagnosed with type 2 diabetes, according to the criteria of The Expert Committee on the diagnosis and clasification of Diabetes Mellitus [39], were recruited in 4 medical centers of Quito-Ecuador (Eugenio Espejo Hospital, Club de Leones Sur, Fundación Oftalmológica del Valle and Fundación de la Psoriasis) from 2009 til 2012. Patients with immune disorders, serious medical illness, recent infections (last 2 weeks), obvious vascular complications, fever, pregnancy/postpartum, use of statins and LADA patients (positive GAD-65 Abs) were excluded. Forty non-diabetic controls taken from the same ethnic and societal background, not suffering from important medical disorders (including acute infection) served as controls. They were included at the same time as the patients and had to be over 30 years of age and preferably of the same gender as the patients. The Medical Ethical Review Committee of the Ecuadorian Corporation of Biotechnology Quito, Ecuador approved the study. Written informed consent was obtained from the patients and controls in the study. The Ethic Committee of the Central University also validated the ethical approval of the study. The Ecuadorian Ministry of Health (MSP) gave the respective permit to export and process the samples in Erasmus MC, Rotterdam, The Netherlands.

Serum cytokines and lipid profile

In the morning fasting venous peripheral blood (10 mL) was collected in a clotting tube and processed within 4 hours. Serum was frozen and stored at minus 800C for approximately 24 months before testing. The levels of TNFα, IL-1β, IL-6, NGF, HGF, PAI, Resistin, CCL2 (MCP-1), Adiponectin, Leptin, IL-8, and MIP1β (CCL4) were measured by flow cytometry (BD LSR II Biosciences, California, and EE.UU.) using a commercially available

multi-analyte cytometric bead array system (Milliplex® Map, U.S.A.). The data were analyzed using a 5-parameter logistic method for calculating analyte concentrations in samples (MAGPIX® with xPONENT software, Luminex, Austin, USA). Undetectable serum analyte levels were considered as 0 pg/ml and included in the statistical analysis. The lipid profile was performed according to standard lab procedures in Quito-Ecuador (AMCOR laboratory) and assays were validated in Erasmus MC.

MicroRNA quantitative real-time PCR (qPCR)

Total RNA was isolated from serum using the Qiagen miRNeasy kit (Qiagen, Hilden, Germany). In order to correct for variations in RNA isolation derived, we spiked-in a non-human (C. elegans) synthetic miRNA cel-miR-39 miRNA Mimic (MSY000010) into the sample before nucleic acid isolation. Subsequently, specific stem-looped reverse transcription primers were used to obtain cDNA for mature microRNAs. The RNA was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit from Applied Biosystems, The Netherlands (ABI). PCR was performed using pre-designed TaqMan® microRNA assays and TaqMan® Universal Master Mix, NoAmpErase®UNG, with an ABI 7900 HT real-time PCR machine. The PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and finally 1 min at 60°C. The spiked-in syn-cel-miR-39 goes through the entire RNA isolation process and serves as endogenous control for data normalization.

TaqMan assay data processing

SDS software (ABI) was used to collect the data and the RQ Manager Program (ABI) was used to assign, check and standardize CT values. Data Assist software was used to normalize the data to the syn-cel-miR-39. For threshold cycles below 40, the corresponding microRNA was considered detected, higher cycle numbers were not included in the calculations. The results were represented using the ddCT method (2 –ddCT, User Bulletin, ABI).

Data analysis

Statistical analysis was performed using SPSS 20 (IBM, Inc.) package for Windows. Data were tested for normal distribution using the Kolmogorov-Smirnov test. The Grubbs' test for outlier detection was applied (http://graphpad.com/support/faqid/1598/). Depending on the distribution pattern and the total number of subjects, parametric (normal distribution, independent t test) or nonparametric group comparison (Mann-Whitney U test) were applied. Correlations were determined by Spearman correlation. Level of significance were set at p= 0.05 (two tailed). A dendrogram visualizing associations was constructed in SPSS using hierarchical cluster analysis of the serum cytokines using the between-groups linkage method. Hierarchical regression analysis was used to test if means of miR-146a, IL-8 and HGF were significantly different between Non diabetic controls and T2D patients,

when controlling for BMI and lipids. Graphs were designed with GraphPad Prism 5.04 (GraphPad Software, Inc.) for Windows.

RESULTS

Patient and control characteristics

Table 1 shows the number of patients and non-diabetic controls used for this study and their ages, gender, HbA1c/hyperglycemia, BMI, lipid profile and medication. As expected, the T2D patients had a significantly higher fasting glucose and HbA1c level as the non-diabetic controls. 70% of the patients used oral anti-diabetic treatment and 30% used insulin. Of the patients 61% had a history of cardiovascular disease, while 48% had a family history (1st, 2nd degree) of diabetes (values were 29% and 29% respectively for the non-diabetic control group).

With regard to the non-diabetic control group, we selected the controls by asking hospital staff (60%) and accompanying care takers (40%) to volunteer to donate blood at the same time as the patients were investigated. Controls needed to be over 30 years of age, while we tried to match as much as possible for gender. Table 1 show that we did not completely succeed in matching for age, since our controls were on average 8 years younger than the T2D patients. Gender distribution was not significantly different with a slight over representation of women in the control group.

We found the collected non-diabetic controls to be as overweight as the patients with a normal BMI in only 17.5% of the 40 non-diabetic controls as compared to 14.8% of the 56 T2D patients. There were no differences in BMI between non-diabetic hospital staff and non-diabetic care-takers. The T2D patients and non-diabetic controls also appeared to have the same disturbed lipid profile; the non-diabetic controls had hypercholesterolemia in 67.5% of individuals, the T2D patients had a hypercholesterolemia in 60.7% of cases (for further details see Table 1). There were again no differences between non-diabetic hospital staff and non-diabetic care-takers with regard to dislipidemia.

MicroRNA's, cytokines, chemokines and growth factors in serum

Table 2 gives the mean and standard error of the mean (SEM) of the fold change values of the two tested microRNAs (146a and 155 versus the reference syn-cel-miR-39) in the serum of the T2D patients as compared to the non-diabetic controls. The serum levels of miR-146a were significantly reduced in T2D patients as compared to the non-diabetic controls (Figure 1), those of miR-155 were not. Nevertheless there existed a good correlation between the serum levels of both microRNAs ($r=0.478$; $p<0.001$).

Table 1. Patients and Non-diabetic controls characteristics. Table 1 shows the number of patients and controls used in this study and their ages, gender, comorbidities, HbA1c/hyperglycemia, BMI, lipid profile and medication use.

	Controls			T2D			Controls vs. T2D p-value
Group size n	40			56			
Age mean (range)	54 (32-87)			62 (38-85)			**0.002**
Gender							
Female n (%)	28 (70%)			34 (61%)			NA
Male n (%)	12 (30%)			22 (39%)			NA
Comorbidities							
Cardiovascular diseases n (%)	29%			61%			NA
Familiar antecedents of diabetes n (%)	29%			48%			NA
BMI mean (range) %	29.3 (23-42)	Normal	17,5%	29.2 (22-39)	Normal	14,8%	0,86
		Overweight	47,5%		Overweight	46,4%	
		Obese	35%		Obese	38,8%	
Glucose state							
Fasting Glucose mg/dL mean (range) %	86 (60.9- 180.5)	Normal	95%	144 (69 - 397)	Normal	51.8%	**0.00****
		High	5%		High	48.2%	
HbA1C mean (range) %	5.7 (3.9 - 6.7)	Normal	95%	7.1 (4.8 - 12.5)	Normal	35.7%	**0.00****
		High	5%		High	64.3%	
Lipid Profile							
Cholesterol mg/dL mean (range) %	235 (131-328)	Normal	32.5%	233 (143- 436)	Normal	39.3%	0,92
		High	67.5%		High	60.7%	
TG mean mg/dL mean (range) %	200 (92 -547)	Normal	62.5%	197 (76 - 411)	Normal	66.1%	0,88
		High	37.5%		High	33.9%	
HDL mean mg/dL mean (range) %	41.5 (25- 65)	Normal	45%	43 (18 -70)	Normal	58.9%	0,71
		Low	55%		Low	41.1%	
LDL mg/dL mean (range) %	155 (78 - 266)	Normal	57.5%	153 (77- 361)	Normal	62.5%	0,92
		High	42.5%		High	37.5%	
Medication							
Oral Anti-diabetic treatment	0%			70%			
Insulin treatment	0%			30%			
Aspirin	21%			30%			
Statins (%)	0%			0%			

Values in bold denote a significant difference between two groups.

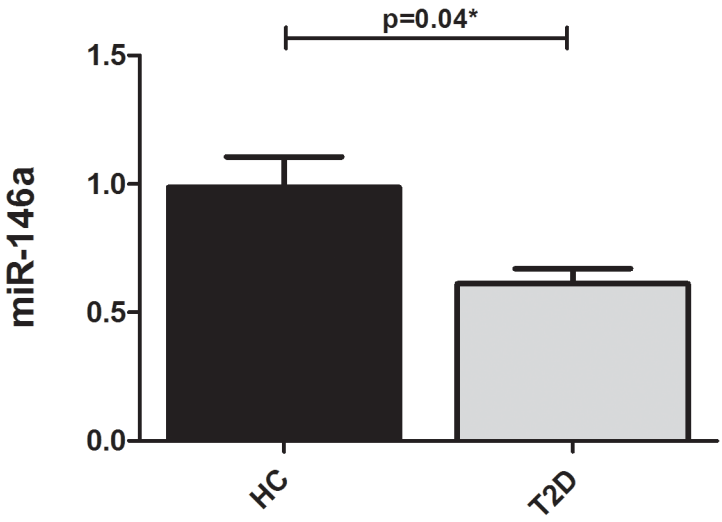

Figure 1. Expression level of serum miR-146a in Ecuadorian Non-diabetic controls and T2D patients. Figure 1 shows mean and standard deviation of the fold change values of miR-146a (reference microRNA sync-cel-mir-39) in the serum of the T2D patients as compared to Non-diabetic controls. Differences between groups were tested using independent T test. Levels of significance were set at p=0.05 (two-tailed).

Figure 2 shows the unsupervised cluster analysis of the levels of the microRNAs and the tested T2D-related cytokines, chemokines and growth factors in the serum of patients and non-diabetic controls. As can be seen from the diagram there was the expected strong clustering of both microRNAs, which also clustered to some extent with leptin. With regard to the other cytokines and chemokines, there existed a clustering of the pro-inflammatory mediators CCL4, IL-6, IL-1β and NGF, and between TNF-α, IL-8, HGF and resistin. To avoid inter-assay variation, serum levels were expressed in fold changes compared to controls for each mediator.

Table 2 gives the relative levels of the tested cytokines, chemokines and other mediators in the serum of the T2D patients and non-diabetic controls in the order of the cluster analysis. From the factors determined, only the levels of IL-8 and HGF appeared to be significantly different between T2D patients and the non-diabetic controls. Both IL-8 and HGF levels were higher in the serum of the T2D patients as compared to the non-diabetic controls. Resistin was also higher in the serum of the patients, but only approached the level of significance (p=0.09). All in all, the picture emerges of particularly the cluster of HGF, TNF-α, Resistin and IL-8 to be raised in the serum of the diabetic patients versus the non-diabetic controls.

The correlations of the level of the microRNAs with the cytokines/chemokines/ growth factors and clinical variables.

We performed correlation analyses between the different parameters measured and only took correlations with a level of $p < 0.01$ into consideration.

Since our patients and non-diabetic controls differed 8 years in age we took special notice of correlations with age. The microRNAs did not correlate with age. Of the cytokines HGF, resistin and adiponectin correlated positively to age. It is important to note that correction for age did not change the association of HGF with disease ($r = 0.258$, $p=0.008$). Of the clinical variables HbA1c levels correlated to age.

It is also of note that the levels of miR-146a and miR-155 correlated to each other, corroborating our findings in the cluster diagram. With regard to correlations of microRNAs with cytokines we found miR-146a to correlate significantly and positively to the serum PAI level ($r=0.259$; $p= 0.01$). There were no correlations of miR-146a and clinical variables. The serum miR-155 level correlated significant to the serum leptin level ($r= 0.326$, $p=0.001$) and IL-8 ($r=0.268$, $p=0.008$).

Serum IL-8 levels correlated to HbA1c levels ($r= 0.301$; $p=0.003$) and also positively to TNFα levels ($r=0.288$, $p=0.004$), which in turn correlated to HGF levels ($r= 0.367$; $p=0.000$), corroborating our findings in the cluster diagram (Figure 2). Positive correlations were also found between HGF and resistin levels and resistin and IL-6 levels, again corroborating the findings in the cluster diagram (Figure 2).

Expected significant correlations were between leptin and BMI and leptin and leptin and gender.

DISCUSSION

In this study we determined two inflammation-related microRNAs in the serum of Ecuadorian T2D patients. We observed a significantly reduced level of one of these microRNAs, i.e. of miR-146a, in the serum of T2D patients as compared to a non-diabetic control group. Reduced expression of miR-146a is classically considered a sign of a pro-inflammatory state. Boldin et al. described that miR-146a-null mice systematically overproduce pro-inflammatory cytokines (such as TNF-α IL-6 and IL-1β) in response to injection with a sub-lethal LPS dose. Tissue macrophages were the primary source of this enhanced pro-inflammatory cytokine production. This implicates miR-146a in attenuating macrophage inflammatory responses [40]. In agreement with these results, in vitro studies show that induction of miR-146a expression in monocyte/macrophage cell lines negatively regulates the inflammatory response [23, 41], while transfection with miR-146a inhibitors in both resting and LPS-stimulated macrophage-like cell lines had an opposite effect and

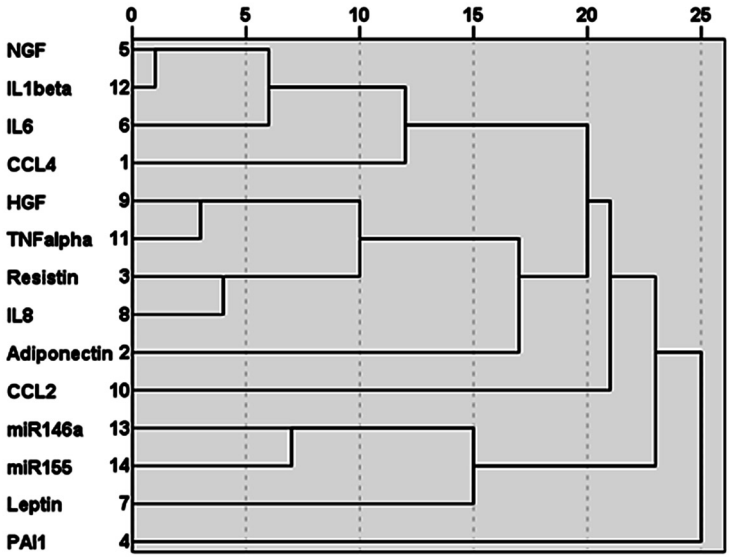

Figure 2. Dendrogram of unsupervised hierarchical cluster analysis of the tested serum levels of microRNAs, cytokines, chemokines and growth factors in T2D patients and Non-diabetic controls. The dendrogram shows the clustering of miR-146a and miR-155, and of the pro-inflammatory cytokines CCL4, IL-6, IL-1β and NGF and of TNF-α, IL-8, HGF and resistin.

resulted in an up-regulation of these inflammation-related genes. Collectively these data show that miR-146a is a strong down regulator of the production of classical inflammatory compounds in macrophages.

We also found the level of serum IL-8 significantly up regulated in the T2D patients as compared to the non-diabetic controls in agreement with previous findings of Herder et al [42]. IL-8 is considered a primary cytokine for M1 inflammatory macrophages. On the basis of these significant alterations in miR-146a and IL-8 levels we like to conclude that our study supports the concept of an activation of the inflammatory response system in T2D patients. The correlation of the IL-8 level with Hb1Ac supports the idea that chronic hyperglycemia plays at least a partial role in this activation.

A limitation of our study is that our non-diabetic control group was not matched for age to our diabetic patient group, and non-diabetic controls were on average 8 years younger than our patients; patients and non-diabetic controls did have similar readings for lipid profiles and BMI. In correlation analysis miR-146a levels and IL-8 levels appeared not to be dependent of age. When we performed hierarchical regression analysis for BMI and lipid profiles, it appeared that the disease state always was the determinant

Table 2. Cytokines, chemokines and growth factors in Non-diabetic controls and T2D patients. Group size, mean and SEM in the order of the cluster analysis. To avoid inter-assay variation, serum levels (pg/ml) were expressed in fold change compared to non-diabetic controls, the average of the Controls in each assay was set to one. Differences between groups were tested using independent T test. Levels of significance were set at p=0.05 (two-tailed).

	T2D			Controls vs. T2d *T test*
	N	**Mean**	**(SEM)**	**p-Value**
NGF	56	1,24	0,36	0,707
IL1beta	56	0,79	0,18	0,686
IL6	56	1,38	0,21	0,131
CCL4	56	0,92	0,1	0,775
HGF	56	1,34	0,09	**0.023***
TNFalpha	56	1,11	0,07	0,22
Resistin	56	1,19	0,08	0,097
IL8	56	2,19	0,36	**0.011***
Adiponectin	56	1,25	0,14	0,222
CCL2	56	1,00	0,07	0,883
miR146a	56	0,61	0,05	**0.042***
miR155	56	0,93	0,07	0,844
Leptin	56	0,86	0,11	0,338
PAI1	56	1,17	0,23	0,565

Values in bold denote a significant difference between two groups.

for abnormal miR-146a and IL-8 levels and that BMI and lipid profiles did virtually not determine these levels, except for IL-8 which was also determined by the cholesterol levels (see supplementary Table 1and 2). We are thus confident that indeed abnormal levels of miR-146a and IL-8 are determined by the T2D state in this study.

A reduced level of miR-155 has been described in the circulating leukocytes of T2D patients [32]. However we were not able to find a significant change of miR-155 in the serum of T2D patients as compared to our non-diabetic control group. We however did find a significant positive correlation between the serum levels of miR-155 and miR-146a and we found a clustered expression of both miR-146a and miR-155 with leptin in cluster analysis. Since leptin is primarily derived from adipose tissue, this might suggest that a

significant proportion of the circulating microRNAs miR-146a and miR-155 is produced by activated macrophages and adipocytes in adipose tissue.

Our T2D cases lacked a significant over-expression of several classical pro-inflammatory compounds in serum: similar levels of TNF-α, IL-1β and IL-6 were found in the serum of patients and non-diabetic controls. This contrasts to previous findings by others, such as Costantini et al., who observed increased levels of IL-1α, leptin, resistin and PAI-1 in T2D patients [43]. Our negative findings might be due to the fact that our non-diabetic controls appeared to have many signs of the metabolic syndrome: BMI values were over 25 in 82.5% (average BMI 29.3), while hypercholesterolemia was present in 67.5% with raised LDL in 42.5% of non-diabetic controls. These values were similar to the ones found in the T2D cases. The Ecuadorian non-diabetic control group was composed of care-takers (40%, friends and family), and hospital staff (60%) from the Quito area. Considering this excessively high prevalence of obesity and dyslipidemia in the Quito non-diabetic control group, it is important to note that a recent healthcare report of the Ecuadorian government corroborates this high prevalence of obesity and dyslipidemia in urban Ecuadorian populations [44].

In a parallel study we have collected Dutch T2D patients and Dutch non-diabetic controls that were tested at the same time with the same multi-analyte system for cytokines and growth factors. The Dutch healthy controls had on average a BMI of 23.8 and had normal lipid values (hypercholesterolemia none, raised LDL 14%). Interestingly our Ecuadorian "healthy" control group indeed had higher levels of CCL4 and IL-6 (see supplementary Table 4), suggesting that in particular obesity and dyslipidemia determine a higher level of these classical pro-inflammatory cytokines in serum, and not (only) the diabetes state and/or pathology per se.

Reduced levels of miR-146a have previously been found in T2D patients, be it in circulating leukocytes [20] . However, our report contrasts with another report that showed elevated levels of miR-146a in the serum of newly diagnosed T2D patients [38]. These elevated levels were found in comparison to the serum levels of pre-diabetic individuals with a disturbed OGT and T2D-susceptible individuals (family), who only had a moderate overweight (average BMI of 26) and moderate hypercholesterolemia (average 5.6 mmol/l), as had the newly T2D cases in that study. We therefore assume that the distinct status of the control population with regard to obesity and/or severe dyslipidemia might have played a role in the differences. In addition, our T2D patients had longstanding diabetes, and the stage of disease may have played a role as well.

Apart from the involvement of inflammatory miR-146a and IL-8, our study suggests an involvement of other molecular systems associated with the failure to control glucose homeostasis on the background of an already existing obesity and dyslipidemia.

First, significantly higher serum levels of HGF were found in the T2D patients as compared to the non-diabetic controls. HGF levels correlated to age, but correction for age left the association with T2D intact (similar results were obtained with corrections for BMI and dyslipidemia, see supplementary Table 3). HGF was first described as a hepatocyte factor involved in liver regeneration after partial hepatectomy [45]. Recent evidence shows that the factor is also produced by monocytes and macrophages and that it is involved in various regeneration processes, including vascular repair and β cell growth [46-48]. HGF can thus be viewed as a key factor in insulin resistance-associated compensatory mechanisms at the level of the pancreatic islet by stimulating its regeneration and at the level of the vasculature by stimulating repair of hyperglycemia-damaged vessels by inducing proliferation of endothelial cells. In marked contrast, however, HGF has also been implicated with a pathogenic role in macrovascular disease as HGF levels in type 2 diabetes patients correlated positively with carotid intimal-media thickness and plaque score [49].

In addition to a higher level of HGF there was also an over-expression (non-significant, p=0.09) of resistin in the serum of the Ecuadorian T2D cases as compared to the non-diabetic controls. Resistin was initially identified in adipocytes, but significant levels of resistin expression in humans are mainly found in immune cells, particularly monocytes [50, 51]. Resistin was first described as a factor contributing to the development of insulin resistance and diabetes in humans, but debate is still ongoing regarding its role in obesity, insulin sensitivity and the development of T2D. In addition also evidence for a pathogenic role of resistin in atherogenic vascular diseases is growing [52, 53].

In conclusion this study shows signs of chronic inflammation (decreased serum anti-inflammatory miR-146a/increased IL-8) and signs of islet and vascular repair (increased HGF) in patients with a failure to control glucose homeostasis when compared to non-diabetic controls with a similar prevalence of obesity and dyslipidemia. Our study also suggests that miR-146a can be considered as a serum biomarker of the inflammatory process linked to the failure of glucose control of the T2D state against a background of obesity and dyslipidemia.

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SUPPORTING INFORMATION

Table S1. Hierarchical Regression Model of miRNA-146a. Hierarchical regression analysis for BMI and lipid profiles shows that the disease state was the determinant for abnormal miR-146a.

	R	R^2	R^2	B	SE	β	t
			Change				
Model 1	0.213	.045	.000				
Disease				-.245	.118	-.212*	-2.070
BMI				-.003	.015	-.022	-.217
Model 2	0.211	.044	.001				
Disease				-.239	.117	-.208*	-2.051
Cholesterol				.000	.001	.031	.302
Model 3	0.214	.046	.003				
Disease				-.237	.117	-.206*	-2.032
HDL				-.003	.005	-.050	-.495
Model 4	0.21	.044	.001				
Disease				-.239	.117	-.208*	-2.050
LDL				.000	.001	.029	.285
Model 5	0.211	.044	.001				
Disease				-.240	.117	-.209*	-2.061
Tryglicerids				.000	.001	-.032	-.317

Note. Statistical significance: *p< .05;**p< .01; ***p< .001

Table S2. Hierarchical Regression Model of IL-8. Hierarchical regression analysis for BMI and lipid profiles shows that the disease state and cholesterol levels were the determinant for abnormal IL-8.

	R	R^2	R^2	B	SE	β	t
			Change				
Model 1	0.272	.074	.000				
Disease				1.207	.448	.272**	2.694
BMI				.008	.056	.014	.143
Model 2	0.324	.105	.038				
Disease				1.133	.434	.256**	2.614
Cholesterol				-.008	.004	-.195*	-1.983
Model 3	0.279	.078	.011				
Disease				1.168	.440	.264**	2.652
HDL				-.021	.020	-.104	-1.043
Model 4	0.299	.089	.022				
Disease				1.135	.437	.257**	2.595
LDL				-.006	.004	-.148	-1.498
Model 5	0.287	.083	.015				
Disease				1.138	.439	.258**	2.592
Tryglicerds				-.003	.003	-.123	-1.242

Note. Statistical significance: *p< .05;**p< .01; ***p< .001

Table S3. Hierarchical Regression Model of HGF. Hierarchical regression analysis for BMI and lipid profiles shows that the disease state was the determinant for abnormal HGF.

	R	R^2	R^2 Change	B	SE	β	t
Model 1	0.244	.060	.003				
Disease				.351	.151	.236*	2.320
BMI				-.011	.019	-.057	-.564
Model 2	0.232	.054	.000				
Disease				.342	.149	.231*	2.292
Cholesterol				.000	.001	-.009	-.089
Model 3	0.232	.054	.000				
Disease				.341	.149	.230*	2.283
HDL				.001	.007	.020	.199
Model 4	0.231	.054	.000				
Disease				.342	.149	.231*	2.294
LDL				1.704E-06	.001	.000	.001
Model 5	0.259	.067	.013				
Disease				.339	.148	.230*	2.292
Tryglicerids				-.001	.001	-.116	-1.160

Note. Statistical significance: *p< .05;**p< .01; ***p< .001

Data S1. Raw data points of the tested serum levels of microRNAs, cytokines, chemokines and growth factors of T2D patients and Non-diabetic controls. The levels of TNFα, IL-1β, IL-6, NGF, HGF, PAI, Resistin, CCL2 (MCP-1), Adiponectin, Leptin, IL-8, and MIP1β (CCL4) were measured by flow cytometry (BD LSR II Biosciences, California, and EE.UU.) using a commercially available multi-analyte cytometric bead array system (Milliplex® Map, U.S.A.).

MicroRNA quantitative real-time PCR (qPCR) was performed using pre-designed TaqMan® microRNA, with an ABI 7900 HT real-time PCR machine. SDS software (ABI) was used to collect the data.

Table S4. Cytokines, chemokines and growth factors of Ecuadorian non-diabetic controls and Dutch healthy controls. Group size, median, inter-quartile range (IQR) and p-values obtained by Mann—Whitney U-test is represented. Serum levels (pg/ml) are shown in the order of the cluster analysis. In a parallel study we have collected Dutch healthy controls that were tested at the same time with the same multi-analyte system for cytokines and growth factors. Ecuadorian non-diabetic controls showed higher levels of the classical pro-inflammatory cytokines (CCL4 and IL-6).

	Controls Ecuador			Controls Netherlands			
							(Ecu Cont vs. Dut Cont)
							Mann Whitney T
	N	**Median**	**(IQR)**	**N**	**Median**	**(IQR)**	**p-Value**
NGF	24	1.00	1.00	15	4.46	2.07	0.000**
IL-1beta	12	1.00	0.75	15	1.07	0.89	0.755
IL-6	34	4.50	3.00	15	0.98	1.73	0.000**
CCL4	34	95	101	15	52	77	0.065
HGF	34	733	414	15	829	693	0.374
TNFalpha	33	4.00	2.00	15	4.36	3.23	0.553
Resistin	34	36025	16895	15	53602	24980	0.002*
IL-8	34	6.00	3.25	15	5.90	2.48	0.420
Adi-ponectin	34	2325	1902	15	2855	1723	0.288
CCL2	34	304	177	15	232	149	0.083
Leptin	34	8599	8458	15	8834	10939	0.879
PAI-1	34	88898	31182	15	89170	39978	0.544

Values in bold denote a significant difference between two groups.

Chapter 5

Study on inflammation-related genes and microRNAs, with special emphasis on the vascular repair factor HGF and miR-574-3p, in monocytes and serum of patients with T2D

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ABSTRACT

Recently, we reported signs of inflammation (raised IL-8, reduced miR-146a) and signs of vascular repair (raised HGF) in the serum of Ecuadorian patients with type 2 diabetes (T2D). In contrast, we found that the circulating monocytes lacked up-regulation of classical inflammatory genes (IL-1B, IL-6, and TNF) and there was even significant down-regulation of PTGS2. Notably, genes and a microRNA involved in adhesion, cell differentiation and morphology (CD9, DHRS3, PTPN7 and miR-34c-5p) were up-regulated in the T2D monocytes, suggesting a role of the anti-inflammatory cells in adhesion, vascular repair and invasion.

Aim. To determine the gene expression of the vascular repair factor HGF in the circulating monocytes of patients with T2D and to investigate the relationship between HGF and the expression of the other previously tested monocyte genes and the contribution to the raised serum level of HGF. In addition, we tested the level of 6 microRNAs in the serum of the patients which were previously determined in the circulating monocytes.

Methods. A gene and microRNA expression study in the monocytes and serum of 64 Ecuadorian patients with T2D (37-85 years) and 44 non-diabetic controls (32-87 years).

Results. The gene expression of HGF was significantly raised in the monocytes of the patients with T2D and associated with the expression of genes involved in adhesion, cell differentiation and morphology. HGF gene expression did not correlate with the serum level of HGF. The monocyte expression of pro-inflammatory cytokine genes was also not associated with the serum levels of these cytokines. The level of miR-574-3p was significantly altered in the serum of the patients with T2D. The expression of miR-574-3p in serum clustered with the well-established inflammation regulating miR-146a. The level of the 6 tested microRNAs in serum did not correlate with their expression level in monocytes.

Conclusion. In T2D Ecuadorian patients, the microRNA and gene expression of important inflammatory factors and a vascular repair factor in T2D circulating monocytes differ from their expression in serum. While monocytes are set at an anti-inflammatory set point, the serum shows molecular signs of inflammation. Both compartments show molecular signs of vascular repair support, i.e. up-regulated HGF.

Key words. Type 2 diabetes, monocytes, serum, HGF, miR-574-3p.

INTRODUCTION

There is increasing evidence that monocytes, macrophages and related cells are closely involved in the pathogenesis of the metabolic syndrome (MetS) and type 2 diabetes (T2D). Importantly, in obesity the number of macrophages increases from 10-15% to 50-60% of total adipose tissue cells [1, 2]. The increase in macrophage number is accompanied by a hyper activation of the cells and leads to raised secretion of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, CCL-4, PAI-1) and chemokines (CCL2) causing a state of chronic low-grade of inflammation [1, 3-7] and insulin resistance.

Important circulating precursors for the macrophages in the adipose tissue [8-10] are the blood-borne monocytes. There is a relative paucity on the state of inflammatory activation of circulating monocytes in patients with the MetS [11, 12] and T2D [13], but increases in pattern recognition receptors, oxidative stress and the machinery for the production of pro-inflammatory cytokines have all been described [14-16].

Contrary to this view, we recently reported that monocytes of a group of 64 Ecuadorian patients with T2D were earlier characterized by an anti-inflammatory set point than a pro-inflammatory set point when compared to monocytes of a group of 44 non-diabetic controls. We found a decrease in expression of a cluster of 11 mutually correlated core inflammatory cytokine/compound genes (ILB, IL-6, TNF, TNFAIP3, PGS2, CCL20, CCL2, CCL4, PDE4B, DUSP2 and ATF3; reaching significance for PTGS) in the monocytes of patients with T2D [17]. However, the study on the monocytes of the patients with T2D also showed that there was up-regulated gene expression for genes occurring in a cluster of mutually correlating genes, many of which involved in adhesion, migration, cell differentiation and cell morphological change [17]. A significant up-regulation as compared to non-diabetic controls was reached for the genes CD9, DHRS3 and PTPN7. Other important genes in this gene cluster were MAPK6, NAB2, STX1A, EMP-1, CDC42, DHRS3, FABP5, BCL2A1, PTX3 and CXCL2. We interpreted these data as indicating that circulating monocytes in our group of patients with T2D were activated, but not towards an inflammatory state, but to a state of enhanced adhesion, migration and further differentiation into descendent cell types, most likely into monocyte-derived pro-angiogenic cells, instrumental in vascular repair. This view was further supported by our observation that the expression of miR-146a, a well-known inflammation down-regulating microRNA, was not changed in the T2D monocytes, while a microRNA targeting genes involved in processes of cell morphology and shape change, i.e. miR-34c-5p, was up-regulated as compared to the group of non-diabetic controls [17].

In the serum of the patients with T2D in whom we performed the monocyte studies, we found clear signs of inflammation [18]. Although there were no increases in the levels

of classical cytokines, such as of IL-1β, IL-6 and TNF-α, there was an increase in the level of serum IL-8, and also the level of miR-146a was significantly down regulated. HGF was increased in the serum of the cases with T2D too [18]. Since HGF is an important vascular repair factor [19-22] and an anti-inflammatory agent [23, 24], and monocyte-derived angiogenic cells are characterized by the expression of HGF [25], we hypothesized that there was an enhanced monocyte-linked endothelial repair mechanism going on in our patients with T2D.

In the present study, we therefore tested the hypothesis that HGF is increased in the circulating anti-inflammatory monocytes of patients with T2D and we determined the gene expression level of HGF (and the HGF-R, cMET) in the monocytes of our patients with T2D and investigated whether HGF belonged to the cluster of typical inflammatory compound genes or to the clusters of typical adhesion, migration and differentiation genes. In addition, we compared the monocyte gene expression levels of HGF to the serum HGF levels to investigate whether the circulating monocytes could be the main producers of this vascular repair factor in serum.

In addition, we measured the serum level of miR-34c-5p, the microRNA up regulated in the monocytes of patients with T2D and playing a role in cell shape processes, to see whether this microRNA was also raised in the serum of the patients. Finally, we determined the serum level of the other 5 microRNAs (miR-122, miR-138, miR-410, miR-574-3p and miR-92), which we had previously reported in a finding study as abnormally expressed in the monocytes of patients with T2D [17].

PATIENTS, MATERIALS AND METHODS

2.1. Subjects

A total of 64 subjects diagnosed with diabetes type 2, according to the criteria of The Expert Committee on the diagnosis and classification of Diabetes Mellitus [26]. Patients were recruited in 4 medical centers of Quito, Ecuador (Eugenio Espejo Hospital, Club de Leones Sur, Fundación Oftalmológica del Valle and Fundación de la Psoriasis) from 2009 until 2012. For demographic and clinical details see Table I. At the same time, 44 healthy controls with similar ethnical and social background, neither suffering from T2D nor other important medical disorders (including acute infection) served as controls. Controls had to be over 30 years of age (considering the age dependency of T2D) and preferably of the same gender as the patients.

Patients and controls with other immune disorders, other serious medical illnesses, recent infections (last 2 weeks), obvious vascular complications such as diabetic foot and

ulcers, fever, pregnancy/postpartum, use of statins and LADA patients (patients positive for GAD-65 Abs) were excluded. The Medical Ethical Review Committee of the Ecuadorian Corporation of Biotechnology Quito, Ecuador and the Ethic Committee of the Central University of Quito approved the study. Written informed consent was obtained of all subjects participating in the study. The Ecuadorian Ministry of Health (MSP) gave the permit to export and process the samples in Erasmus MC, Rotterdam, and The Netherlands.

2.2. Blood collection and preparation

In the morning fasting venous blood was collected. Ten mL were collected in a clotting tube and processed within 4 hours. Serum was frozen and stored at minus 80°C for approximately 24 months before testing. Thirty mL were collected in tubes containing sodium-heparin for immune cell preparation. From the heparinized blood, peripheral blood mononuclear cell (PBMC) suspensions were prepared in the afternoon by low-density gradient centrifugation, as previously described in detail [27] within 8 hours to avoid activation of the monocytes. PBMCs were frozen in 10% dimethylsulfoxide and stored in liquid nitrogen. This enabled us to test patient and control serum and immune cells in the same series of experiments later.

2.3. Isolation of monocytes

CD14-positive (CD14+) monocytes were isolated from frozen PBMCs by a magnetic cell sorting system (MACS; Miltenyi Biotec, Auburn, California). The purity of monocytes was >95% (determined by morphological screening after Trypan Blue staining and flow cytometric analysis). As previously reported; the positive versus negative selection of immune cells did not influence gene expression profiles [28].

2.4. Real time quantitative PCR (qRT PCR) for monocytes

2.4.1. mRNA expression in monocytes via TaqMan Array Cards

For the previous report we had determined the expression of 24 mRNAs in the monocytes of the T2D cases and the non-diabetic controls. RNA had been isolated from monocytes using RNeasy columns (Qiagen, Hilden, Germany), and both this method and quantitative RT-PCR has been described in detail elsewhere [30]. One µg of RNA was reverse-transcribed using the High Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed using custom TaqMan Arrays, format 48 (Applied Biosystems), according to the manufacturer's protocol and validated against the single RT-qPCR method. Per fill port, 400 ng of cDNA (converted from total RNA) was loaded. PCR amplification was performed using an Applied Biosystems Prism 7900HT sequence detection system with TaqMan Array block. Thermal cycler conditions were 2 min at 50°C, 10 min at 94.5°C, and then 30s at 97°C, and 1 min at 59.7°C for 40 cycles. Relative to the housekeeping gene

ABL1, the expressions of ATF3, BCL2A1, CCL20, CCL2, CCL7, CD9, CDC42, CXCL2, DHRS3, DUSP2, EMP1, FABP5, HSPA1A/HSPA1B, IL-1B, IL-6, MAPK6, NAB2, PDE4B, PTGS2, PTPN7, PTX3, STX1A, TNF, and TNFAIP3 were determined and values were calculated using the comparative threshold cycle (CT) method. ABL was chosen as a reference gene because it was previously shown that ABL was the most consistently expressed reference gene in hematopoietic cells [29]. The quantitative value obtained from q-PCR is a cycle threshold (Ct). The fold change values between different groups were determined from normalized Ct values (Ct gene – Ct housekeeping gene), by the ΔΔCt method.

2.4.2. Individual mRNA qRT–PCR assays for HGF, HGF-R, resistin.

For the current report we additionally determined the gene expression for HGF, the HGF-R (cMET), and resistin using the same cDNA used in the previous experiments (we measured resistin because resistin had also been found raised in the serum of the T2D cases, though just nor reaching significance, p=0.07) [18]. To obtain cDNA for q-PCR, 1 µg RNA was reversed-transcribed using the cDNA high-capacity cDNA Reverse Transcription kit (Applied Biosystems, USA).TaqMan probes and consensus primers for HGF, HGF-R and resistin were provided by Applied Biosystems. PCR amplification of the housekeeping gene ABL was performed for each sample to allow normalization between the samples. The quantitative value obtained from q-PCR is a cycle threshold (CT). The fold change values between different groups were determined from the normalized CT values (CT gene – CT housekeeping gene).

2.4.3. Individual microRNA qRT–PCR assays

Total RNA was isolated from purified monocytes using RNeasy columns (Qiagen, Hilden, Germany) as described by the manufacturer's manual. Purity and integrity of the RNA were assessed on the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip® reagent set (Agilent Technologies, Santa Clara, CA, USA) and the RNA was then stored at –80°C. Subsequently, specific stem-looped reverse transcription primers were used to obtain cDNA for mature microRNAs. The RNA was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit from Applied Biosystems, The Netherlands (ABI). PCR was performed using pre-designed TaqMan® microRNA assays and TaqMan® Universal Master Mix, NoAmpErase®UNG, with an ABI 7900 HT real-time PCR machine. The PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and finally 1 min at 60°C.

2.5. Serum cytokines

The levels of TNFα, IL-1β, IL-6, NGF, HGF, PAI, Resistin, CCL2 (MCP-1), Adiponectin, Leptin, IL-8, and MIP1β (CCL4) were measured by flow cytometry (BD LSR II Biosciences, California,

and EE.UU.) using a commercially available multi-analyte cytometric bead array system (Milliplex® Map, U.S.A.). The data were analyzed using a 5-parameter logistic method for calculating analyte concentrations in samples (MAGPIX® with xPONENT software, Luminex, Austin, USA). Undetectable serum analyte levels were considered as 0 pg/ml and included in the statistical analysis. Lipid and hepatic profile was performed according to standard lab procedures in Quito-Ecuador (AMCOR laboratory) and validated in Erasmus MC.

2.6 Serum microRNA quantitative real-time PCR (qPCR)

Total RNA was isolated from serum using the Qiagen miRNeasy kit (Qiagen, Hilden, Germany). In order to correct for variations in RNA isolation derived, we spiked-in a non-human (C. elegans) synthetic miRNA cel-miR-39 miRNA Mimic (MSY000010) into the sample before nucleic acid isolation. Subsequently, specific stem-looped reverse transcription primers were used to obtain cDNA for mature microRNAs. The RNA was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit from Applied Biosystems, The Netherlands (ABI). PCR was performed using pre-designed TaqMan® microRNA assays and TaqMan® Universal Master Mix, NoAmpErase®UNG, with an ABI 7900 HT real-time PCR machine. The PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and finally 1 min at 60°C. The spiked-in syn-cel-miR-39 goes through the entire RNA isolation process and serves as endogenous control for data normalization.

2.7. Data analysis

The SDS software (ABI) was used to collect the data and the RQ Manager Program (ABI) was used to assign, check, and standardize CT values. The Data Assist software (ABI) was used to normalize the data (ABL for mRNA expression, RNU44 for microRNA expression of cells and synCell-39 for serum microRNA expression). For threshold cycles below 40, the corresponding miRNA and mRNA were considered detected, higher cycle numbers were not included in calculations. The results were quantified using the ΔΔCT method (2−ΔΔCT, User Bulletin 2, ABI). Statistical analysis was performed using the SPSS (IBM, Inc.) package for Windows. Data were tested for normal distribution using the Kolmogorov-Smirnov test. The Grubbs' test for outlier detection was applied (http://graphpad.com/support/faqid/1598/). Depending on the distribution pattern and the total number of subjects, parametric (normal distribution, independent t test) or nonparametric group comparison (Mann-Whitney U test) were applied. Correlations were determined by Spearman's correlation coefficient. Levels of significance were set at $p \leq 0.05$ (two tailed). A dendrogram visualizing associations was constructed in SPSS using hierarchical cluster analysis of the serum cytokines, genes and microRNA expression using the between-groups linkage method. Graphs were designed with Illustrator CS6 for Windows.

RESULTS

HGF is over expressed in monocytes of patients with T2D

The HGF expression levels were significantly higher in the monocytes of the patients with T2D as compared to the non-diabetic controls (Fold Change (T2D vs non-diabetic controls) 1.17 ± SEM 0.62 p=0.03 n=59 Table 2A). The expression levels of the HGF-R (cMET, FC 1.34 ± 0.36 p=0.40, n=22) and of resistin (FC 0.47 ± 0.07, p=0,24, n=59) were not different between the groups (Table 2A).

Figure 1 shows the heat map and cluster diagram for HGF and resistin with the previously determined genes and the previously determined microRNAs. As can be seen HGF and resistin co-clustered positively with each other and with many genes of the cluster of adhesion/differentiation and shape change genes. Since HGF was significantly over expressed in the T2D monocytes, we focused on this compound. The association of HGF was significant at the p<0.001 level with DHRS3 (r=.498, p=0.004 n=32), CD9 (r=.490, p=0.004, n=32), BCL2A1 (r= .503, p=0.003, n=32), Resistin (r=.532, p=0.002, n=32), HSPA1 (r=.525, p=0.002, n=32), but existed also at a lower level for MAPK6 (r=.385, p=0.03, n=32) and STX1A (r=.419, p=0.024, n=32) . It is worthy to note that of these genes HGF, DHRS3 and CD9 were all three significantly higher expressed in the monocytes of cases with T2D as compared to the non-diabetic controls (see [17]).

It is also worthy to note that HGF expression did significantly negatively correlate with the expression of many genes of the inflammatory cluster in the monocytes (such as CCL4, IL-6, TNF, IL1-β, ATF3, CXCL2 and CCL20), reaching significance for TNFAIP3(r=-.350, p=0.05 n=32), supporting the concept that HGF is an anti-inflammatory agent. With regard to clinical parameters HGF expression in monocytes did positively correlate with the BMI (r=.327, p=0.011, n=59).

As reported previously, HGF was significantly raised in the serum of the patients with T2D as compared to the non-diabetic controls. Interestingly serum HGF correlated with monocyte DHRS3 gene expression (r=.326, p=0.008, n=64), but not with monocyte HGF gene expression (r=.189, p=0.152, n=59). Neither was there a positive or negative correlation for resistin expression in the monocytes and in the serum (data not shown), nor for any of the pro-inflammatory compounds, such as IL-1B, TNF, IL-6 and CCL2 (data not shown). This makes it unlikely that monocytes are the prime source of HGF, resistin or pro-inflammatory compounds in serum.

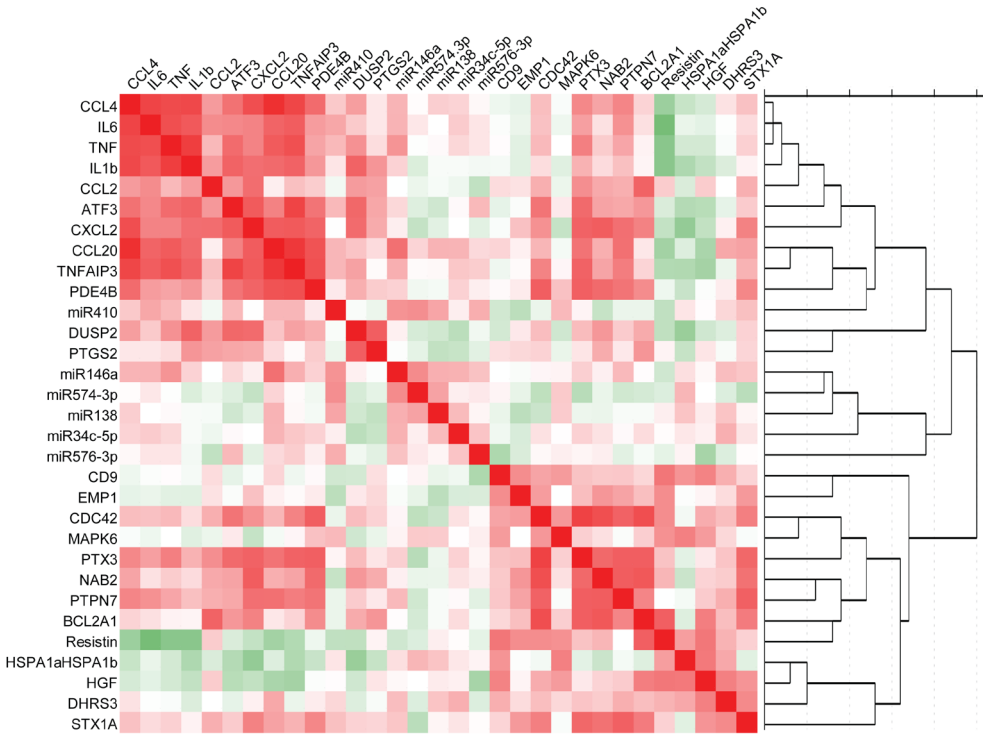

Figure 1. Hierarchical cluster analysis of the tested genes and microRNAs of the monocytes of type 2 diabetic patients and controls. The figure 1 show the heat map and cluster diagram for HGF and resistin with the previously determined genes and the previously determined microRNAs. HGF and resistin co-clustered positively with each other and with many genes of the cluster of adhesion/differentiation and shape change genes.

Mir-34c-5p is unaltered, but miR-574-3p is significantly reduced in the serum of patients with T2D

For the previous report on cytokines in T2D serum [18], we had determined the expression of various cytokines, growth factors, miR-146a and miR-155 in the serum of the cases with T2D and the non-diabetic controls. We found IL-8, HGF and resistin (the latter at a significance level of p=0.07) raised in the serum of the patients with T2D in comparison to the non-diabetic controls, while miR-146a was down-regulated (see also introduction).

For the current report, we determined the microRNAs miR-34c-5p, miR-122, miR-138, miR-410, miR-574-3p and miR-92 in the serum of the patients with T2D and the non-diabetic controls, since we had also measured these microRNAs in the monocytes of the patients in the previously reported study on gene and microRNA expression in the monocytes. In that

study, we reported that miR-34c-5p was significantly up-regulated in the monocytes of the patients with T2D (see also introduction).

Table 2B shows that in the current study the serum level of miR-34c-5p was not changed in the patients with T2D as compared to the non-diabetic controls. However, the serum level of microRNA miR-574-3p, was significantly reduced in the T2D serum as compared to the non-diabetic controls. Controlling for age, gender, BMI and dyslipidemia via hierarchical clustering showed that these factors did not contribute to the association of miR-574-3p with disease.

Figure 2 shows the heat map and cluster diagram of the measured microRNAs and cytokines/growth factors. Since miR-574-3p was found significantly reduced in the serum of the patients with T2D, we focused in particular on this microRNA. It is clear from Figure 2 that there is a strong clustering and association of miR-574-3p with miR-146a ($r=0.744$, $p<0.001$, $n=88$) and miR-410 ($r=0.324$, $p=0.03$, $n=80$), all microRNAs being decreased in the serum of the patients with T2D. The association of miR-574-3p with the other microRNAs and cytokines/growth factors in serum was not very strong, although there was an association with the serum CCL2 level ($r=0.337$, $p=0.001$, $n=89$).

It is also important to note that correlations between the serum levels of the tested microRNAs and the expression levels of the same microRNAs in monocytes were not present (data not shown).

Target prediction for miR-574-3p.

Since the expression of miR-574-3p was significantly down-regulated in the serum of patients with T2D, we asked if there were in silico indications linking miR-574-3p expression to processes of inflammation or cell adhesion/differentiation/shape change. We used miRecords as a resource for miRNA-target interactions integrating predicted miRNA targets produced by 11 established miRNA target prediction programs (DIANA-microT, MicroInspector, miRanda, MirTarget2, miTarget, NBmiRTar, PicTar, PITA, RNA22, RNAhybrid and TargetScan/TargetScanS, available at http://www.mirecords.bioled.org).

A minimal target gene prediction coverage of three algorithms was used to perform prediction analysis for miR-574-3p. Filtering to a minimum coverage of three algorithms resulted in 934 hits. Ingenuity pathway analysis (Ingenuity® Systems) was used for mapping of the predicted target genes to biological functions.

Interestingly, the top molecular and cellular function of the miR-574-3p predicted target genes was "cell morphology" and "cellular assembly and organization", while inflammation did not turn up in any of the predicted pathways (see supplementary data 1 Ingenuity analysis).

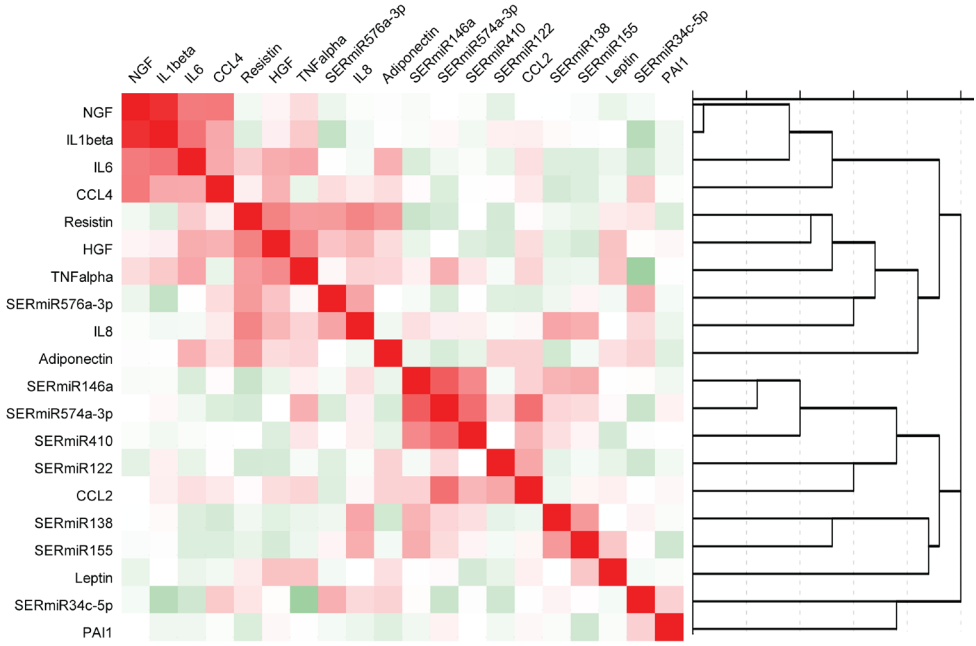

Figure 2. Hierarchical cluster analysis of the tested cytokines and microRNAs of the serum of type 2 diabetic patients and controls. Figure 2 shows that there is a strong clustering association of miR-574-3p with miR-146a and miR-410. The association of miR-574-3p with other microRNAs in serum was not strong. The unique association of miR-574-3p with cytokines/growth factors was with the serum CCL2 level.

DISCUSSION

This study showed that the gene expression of the vascular repair factor HGF was significantly raised in the monocytes of patients with T2D as compared to non-diabetic controls. HGF belonged to the cluster of adhesion, differentiation and shape change genes previously described as up-regulated in the T2D monocytes and correlated significantly to the expression of many genes in that cluster. The association of HGF with these differentiation, adhesion and shape change genes is in accordance with a view that the T2D monocytes are differentiating into the elongated vascular support pro-angiogenic cells (CACs) [25], HGF is a marker of such cells and suggests that the monocytes in our Ecuadorian patients with T2D are instrumental in repairing the vessel walls damaged by T2D related processes.

The up regulation of HGF in the T2D monocytes might have been instrumental too in the reported anti-inflammatory state of the monocytes which we reported at an earlier

occassion (see [17]). Indeed there is ample literature on the anti-inflammatory effects of HGF. It has been shown that monocytes treated with HGF produce high levels of IL-10, a potent immune suppressing cytokine. Mechanistically, HGF modulated IL-10 production in monocytes through the ERK1/2 pathway [23]. With regard to dendritic cells (DC), Molnarfi, et al. reported that DC differentiated in the presence of HGF adopt a pro-tolerogenic phenotype with increased ability to generate regulatory T cells [24], while with regard to endothelial cells Jeong-Ki Min, et al. showed that HGF suppresses Vascular Endothelial Growth Factor (VEGF)–induced inflammation by inhibiting the Nuclear Factor Kappa B (NFκB) pathway [30]. In support of this anti-inflammatory action of HGF, we found a negative correlation of intra-monocyte HGF expression with the cluster of inflammatory genes, reaching statistical significance for TNFAIP3 (A20, a molecule induced by TNFα signaling, [31]) expression.

We reported previously that HGF was raised in the serum of the Ecuadorian patients with T2D; however, we did not find a correlation of the serum HGF with the expression of the HGF gene in the monocytes. This suggests that the circulating HGF is not primarily produced by the circulating monocytes, but originates from other sources. This notion also applies to the other tested cytokines and growth factors in the serum of the patients with T2D, for which we could also not find a correlation with intra-monocyte gene expression. Within the serum, the level of HGF correlated with the levels of TNF-α, IL-8 and resistin. Since it is generally thought that these pro-inflammatory compounds and insulin-resistance inducing substances originate from the adipose tissue and the liver [2, 4, 32, 33], we assume that also HGF in the serum of the patients with T2D primarily stems from these sources. There is ample literature on the production of HGF by adipose tissue and the liver [32, 34-37].

Although HGF might originate from sources other than the circulating monocytes, it is nevertheless possible that HGF in the serum could have affected the function of the circulating monocytes in patients with T2D, since the current study shows that monocytes of patients with T2D do express the HGF receptor, though not differently from monocytes of the non-diabetic controls. Interestingly, the level of HGF in serum did not correlate with a reduced inflammatory gene expression in the monocytes, as we found for the intra-monocyte expressed HGF. This suggests that the monocyte-endogenously-produced HGF is more important in the down-regulation of the inflammatory state of the monocytes in the patients with T2D than the serum-borne HGF. The level of circulating HGF did correlate positively to the DHRS3 expression in the monocytes, suggesting that serum-borne HGF might influence the proliferation and differentiation potential of circulating monocytes to pro-angiogenic cells in patients with T2D.

Furthermore, we found that the level of miR-574-3p was reduced in the serum of patients with T2D, similar to miR-146a, of which we reported a down-regulation in the serum of the patients with T2D at an earlier occasion [18]. In the cluster analysis and in correlation studies there was a strong association between the serum level of miR-574-3p and miR-146a, miR-410 and miR-155. This suggests an association of serum miR-574-3p (and also serum miR-410) with inflammatory processes, since miR-146a and miR-155 are important inflammation-regulating microRNAs [38-41] . This notion is further supported by a positive correlation between the serum level of miR-574-3p and the level of CCL2 in serum.

However, when we studied in silico the putative targets of miR-574-3p, ingenuity analysis of the putative targets did not indicate inflammation as an important pathway, whereas cell morphology and cellular assembly and organization were clearly present. The literature on miR-574-3p is in accord with this notion and shows functions of miR-574-3p mainly in the regulation of tumor cell pathology: MiR-574-3p is anti-proliferative, anti-invasive and anti-migratory in gastric and prostate cancer cells [42-44]. In these studies it was found that cullin-1 might be a target of miR-574-3p, and interestingly cullin-1 regulates inflammation via NFκB, thus giving an opening to a relationship with inflammation in connection with cellular assembly and organization[44].

Our findings suggest a high pro-angiogenic potential of the circulating monocytes and the serum of Ecuadorian patients with T2D. It is important to note that Yang reported that down-regulation of miR-574-3p in pro-angiogenic cells appeared to be a marker of senescence; senescent pro-angiogenic cells have lost their proliferative capacity and are inflammatory (oxidative radicals) changed [45]. Hence, this might indicate that miR-574-3p is involved in the regulation of the anti-inflammatory and pro-angiogenic state of the circulating monocytes of patients with T2D.

Perhaps the most striking observation in the present study is the absence of correlation between the serum and intracellular monocyte levels of cytokines, growth factors and microRNAs. This suggests that the dynamics of the inflammation-related changes in the monocyte intracellular compartment differ substantially from the dynamics of inflammation-related changes in the serum compartment of patients with T2D. Collectively the data of the previous studies and the current study show that both the monocyte intracellular compartment and the serum compartment of our patients with T2D have undergone inflammation-related changes. However, the monocyte compartment shows in general a reduction in gene expression of typical pro-inflammatory genes, while genes and microRNAs involved in cell adhesion, cell differentiation, growth and vascular repair, such as HGF and miR-34c-5p, are up-regulated. The serum compartment, in contrast to the monocyte compartment, does show signs of high pro-inflammatory activity, e.g. high

levels of IL-8 and reduced levels of anti-inflammatory miR-146a and altered levels of miR-574-3p. The serum compartment also shows signs of higher activity of vascular repair and cellular growth induction, yet parameters do not correlate with the monocyte parameters of higher pro-angiogenic cell activity. Most likely different T2D related pathophysiological forces drive the activation and de-activation set points of the circulating monocyte and the serum compartment.

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AUTOR'S CONTRIBUTIONS

Lucy Baldeón R (LBR) Study design, data acquisition, carried out the experimental studies, analysis and interpretation of results. Manuscript preparation, and critically evaluate the manuscript.

Karin Weigelt (KW). Study design, analysis and interpretation of results, drafting of the manuscript and critically evaluated the manuscript.

Harm de Wit (HdW). Study design, data acquisition, analysis and interpretation of results, critically evaluated the manuscript, and carried out the experimental studies.

Behiye Ozcan (BO). Contributed to the Analysis and interpretation of results, manuscript preparation, and drafting of the manuscript and critically evaluated the manuscript.

Adri van Oudenaren (AvO). Data acquisition, analysis and interpretation of results, critically evaluated the manuscript, and carried out the experimental studies.

Fernando Sempértegui (FS). Helped in the design of the studies, analysis and interpretation of results, manuscript preparation, and drafting of the manuscript and critically evaluated the manuscript.

Eric Sijbrands (ES). Study design, analysis and interpretation of results, manuscript preparation, and drafting of the manuscript and critically evaluated the manuscript.

Laura Grosse (LG). Study design, data acquisition, analysis and interpretation of results, critically evaluated the manuscript, and carried out the experimental studies.

Anton Jan van Zooneveld (AJvZ). Study design, manuscript preparation, and drafting of the manuscript and critically evaluated the manuscript.

Hemmo A. Drexhage (HD). Study design, analysis and interpretation of results, manuscript preparation, drafting of the manuscript and critically evaluated the manuscript, supervised the experimental studies.

Pieter JM Leenen (PL). Study design, analysis and interpretation of results, manuscript preparation, drafting of the manuscript and critically evaluated the manuscript, supervised the experimental studies.

All authors approved the final manuscript

COMPETING INTERESTS

None declared.

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SUPPLEMENTARY DATA S1.

Ingenuity pathway analysis (Ingenuity® Systems) was used to map the major pathways and processes in which miR-574-3p is involved. The top molecular and cellular function of the miRNA predicted target genes was "cell morphology"; while the second top-associated network was "cell morphology" and "cellular assembly and organization". We used miRecords as a resource for microRNA-target interactions.

INGENUITY
PATHWAY ANALYSIS

Analysis Name: miRecords_target prediction miR-574-3p_Ingenuity - 2014-08-20 01:50 PM
Analysis Creation Date: 2014-08-20
Build version: 313398M
Content version: 18841524 (Release Date: 2014-06-24)

Analysis settings

View

Reference set: Ingenuity Knowledge Base (Genes Only)
Relationship to include: Direct and Indirect
Includes Endogenous Chemicals
Optional Analyses: My Pathways My List

Filter Summary:
Consider only molecules and/or relationships where
(species = Human) AND
(confidence = Experimentally Observed)
Cutoff:

Top Canonical Pathways

Name	p-value	Ratio
Huntington's Disease Signaling	9,98E-06	24/226 (0,106)
Molecular Mechanisms of Cancer	1,46E-05	32/359 (0,089)
Glioma Signaling	1,74E-05	14/94 (0,149)
Role of NFAT in Cardiac Hypertrophy	6,58E-05	19/176 (0,108)
GNRH Signaling	1,39E-04	15/127 (0,118)

Top Upstream Regulators

Upstream Regulator	p-value of overlap	Predicted Activation State
mir-145	7,98E-05	
miR-22-3p (miRNAs w/seed AGCUGCC)	3,21E-04	
Vegf	4,98E-04	
CDKN2C	5,15E-04	
AKT2	8,77E-04	

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value	# Molecules
Neurological Disease	2,65E-06 - 8,85E-03	143
Developmental Disorder	1,59E-05 - 9,99E-03	84
Hereditary Disorder	5,19E-05 - 9,99E-03	97
Psychological Disorders	5,19E-05 - 8,78E-03	94
Skeletal and Muscular Disorders	5,19E-05 - 9,70E-03	120

Molecular and Cellular Functions

Name	p-value	# Molecules
Cell Morphology	3,49E-09 - 8,80E-03	205
Cellular Assembly and Organization	3,49E-09 - 9,83E-03	173
Cellular Function and Maintenance	3,49E-09 - 9,83E-03	192
Cellular Development	4,56E-09 - 9,83E-03	242
Cellular Growth and Proliferation	2,52E-06 - 9,83E-03	239

Physiological System Development and Function

Name	p-value	# Molecules
Nervous System Development and Function	1,45E-08 - 9,83E-03	149
Tissue Development	1,45E-08 - 9,83E-03	200
Embryonic Development	1,40E-06 - 9,72E-03	165
Cardiovascular System Development and Function	1,41E-05 - 1,01E-02	92
Organismal Survival	2,53E-05 - 2,75E-03	150

Top Tox Functions

Assays: Clinical Chemistry and Hematology

Name	p-value	# Molecules
Decreased Levels of Albumin	7,70E-02 - 1,48E-01	1
Increased Levels of CRP	7,70E-02 - 7,70E-02	1
Increased Levels of Potassium	1,29E-01 - 1,29E-01	2
Increased Levels of Red Blood Cells	1,48E-01 - 3,18E-01	5
Increased Levels of ALT	2,14E-01 - 5,33E-01	1

Cardiotoxicity

Name	p-value	# Molecules
Cardiac Proliferation	2,53E-04 - 5,63E-02	11
Cardiac Hypertrophy	1,84E-03 - 8,61E-02	27
Cardiac Arteriopathy	4,51E-03 - 4,06E-01	18
Cardiac Dysfunction	8,39E-03 - 3,03E-01	9
Cardiac Necrosis/Cell Death	8,53E-03 - 3,46E-01	19

Hepatotoxicity

Name	p-value	# Molecules
Liver Dysplasia	1,11E-03 - 7,70E-02	3
Liver Proliferation	6,23E-03 - 4,30E-01	16
Liver Regeneration	8,78E-03 - 1,13E-01	7
Glutathione Depletion In Liver	1,59E-02 - 4,52E-01	4
Liver Fibrosis	3,93E-02 - 2,60E-01	9

Nephrotoxicity

Name	p-value	# Molecules
Renal Necrosis/Cell Death	1,99E-03 - 4,16E-01	31
Renal Proliferation	2,18E-02 - 1,48E-01	16
Nephrosis	2,78E-02 - 4,30E-01	6
Renal Degradation	3,93E-02 - 3,93E-02	1
Renal Inflammation	7,70E-02 - 4,30E-01	5

Top Regulator Effect Networks

Top Networks

ID	Associated Network Functions	Score
1	Cancer, Organismal Injury and Abnormalities, Reproductive System Disease	39
2	Cancer, Organismal Injury and Abnormalities, Reproductive System Disease	37
3	Cell Morphology, Cellular Compromise, Carbohydrate Metabolism	33
4	Cancer, Cellular Assembly and Organization, Gastrointestinal Disease	31
5	Cancer, Dermatological Diseases and Conditions, Hematological Disease	29

Top Tox Lists

Name	p-value	Ratio
RAR Activation	1,33E-03	16/173 (0,092)
Cardiac Hypertrophy	1,89E-03	27/375 (0,072)
PPARα/RXRα Activation	2,77E-03	15/169 (0,089)
Renal Necrosis/Cell Death	2,82E-03	31/463 (0,067)
Cardiac Necrosis/Cell Death	7,77E-03	19/262 (0,073)

Top My Lists

Name	p-value	Ratio

Top My Pathways

Name	p-value	Ratio

Top Molecules

This analysis has no expression values.

Chapter 6

General Conclusions

GENERAL CONCLUSIONS

Regarding our aim to identify a microRNA signature capable of distinguishing patients with T2D from individuals not suffering from T2D:

Using the expression of 15 microRNAs in monocytes in a finding study employing Exiqon array, a partial separation could be made between T2D and the non-diabetic state with a sensitivity of 66% and a specificity of 90%. However, this signature was not useful to validate as a microRNA signature of monocytes that can be used clinically as a discriminating parameter via qPCR, since the expression fold changes observed in Exiqon array were generally too low to allow reliable confirmation within the technical limitations of qPCR.

We thereafter used the microRNAs as biomarkers to test the biological function and inflammatory state of the circulating monocytes in patients with T2D, since there is a paucity of studies focusing on the inflammatory state of these circulating cells in T2D. Therefore, we selected from the differentially expressed microRNAs those with the highest fold changes (FC) between cases and controls with FC of >1.4 or <0.6. Another criterion for selection was that TaqMan probes and primers needed to be available. From the 142 differentially expressed microRNAs found in Exiqon, 5 microRNAs fulfilled the selection criteria: miR-138; miR-34c-5p; miR-410; miR-574-3p and miR-576-3p. Additionally, we tested microRNAs-146a and -155 in TaqMan analyses, since these microRNAs are well-known regulators of inflammation, and have been identified in T2D PBMC by others [38, 39]. We also correlated the microRNA expression and the expression of a set of genes previously found abnormally expressed in the monocytes of T2D patients. We tested for 25 selected genes forming two mutually correlating gene clusters. The first cluster comprised 12 pro-inflammatory cytokine/compound genes (IL-1B, IL-6, TNF, TNFAIP3, PGS2, CCL20, PTX3, PDE4B, DUSP2, ATF3, CXCL2 and BCL2A1); the second cluster comprised 12 chemotaxis, adhesion, motility, and metabolism genes (CCL2, CCL7, MAPK6, NAB2, CD9, STX1A, EMP-1, CDC42, PTPN7, DHRS3, FABP5, HSPA1A). In addition we also tested for HGF, being a marker of pro-angiogenic cells. We used the monocytes of a series of 64 Ecuadorian patients and 44 non-diabetic Ecuadorian controls as a validation cohort.

Monocytes

With regard to the circulating of the patients with T2D we found that

1. Patients had circulating monocytes showing an over-expression of a microRNA (i.e. miR-34c-5p) and a group of immune activation genes (CD9, DHRS3 and PTPN7, in this thesis called cluster C genes) involved in the adhesion of the cells, the shape change of the cells and the differentiation into mature cells. Since we found the vasculo-protective

cytokine HGF also over-expressed in these monocytes, we assume that these monocytes are instrumental in adhesion to the vessel wall and supporting angiogenesis (so-called monocyte-derived pro-angiogenic cells) (see Figure 1).

2. The monocytes of patients with high blood lipid values were characterized by an under-expression of a set of typical inflammatory genes (DUSP2,PTGS2 and ATF3). This is in contradiction to the general idea, and findings in previous studies, where monocytes in T2D patients are pro-inflammatory (see Figure 1).
3. Also in nondiabetic controls of the Quito area, which had more dyslipidemia than the non-diabetic controls of the Rotterdam area, the monocytes were characterized by this anti-inflammatory set point in comparison to the Rotterdam non-diabetic general population controls (see Table 1).
4. We did find pro-inflammatory monocytes (i.e. an over-expression of the set of typical inflammatory A and B genes) in patients with T2D and *normal* serum lipid values, which constituted a minority of the Ecuadorian patients (23%) (see Figure 1).

In summary, in our Ecuadorian T2D we found monocytes that show an increased expression of pro-angiogenic HGF and an anti-inflammatory, but adhesive/motile gene expression profile. MiR-34c-5p might be a biomarker of this monocyte process, other selected and tested microRNAs gave less clear results.

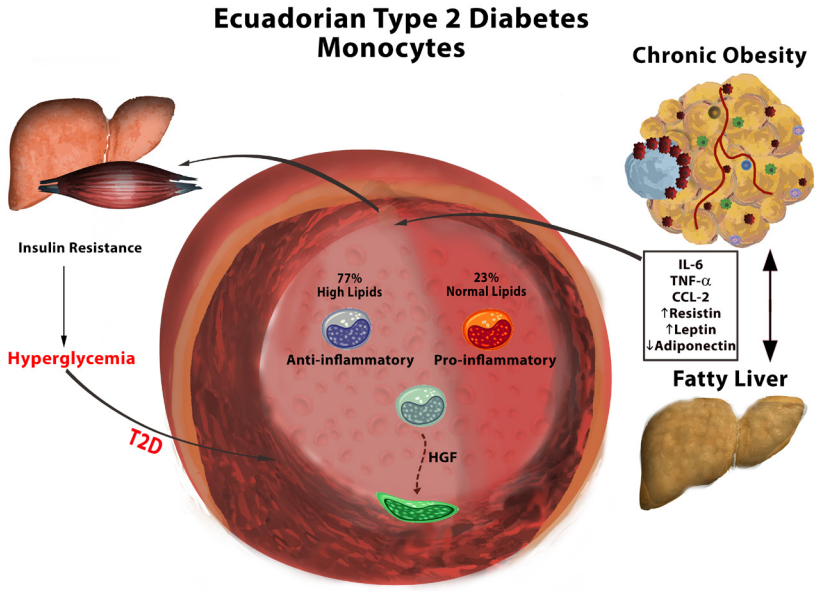

Figure 1. Hypothetical scheme for the pathogenesis of the immune abnormalities in monocytes of T2D as found in this body of work on Ecuadorian subjects.

Table 1. Monocyte gene expression of Ecuadorian and Dutch general population controls. The Quito controls have a higher prevalence of obesity and dyslipidemia, yet monocytes are less inflammatory (significantly lower expression of cluster A and B genes), but have higher production of the endothelial repair factor HGF.

Genes		HC Quito	HC Rdam	p- value
		FC to ABL	FC to ABL	Qto vs Rdam
CLUSTER A	CXCL2	0,72	5,55	*0,00*
	STX1A	**0,01**	**0,04**	*0,00*
	NAB2	0,29	0,41	0,11
	EMP1	**0,49**	**1,04**	*0,00*
	CDC42	1,12	1,13	0,97
	PTX3	**0,52**	**1,50**	*0,00*
	BCL2A1	18,85	25,68	0,45
	PTPN7	0,18	0,17	0,42
CLUSTER B	IL6	**0,01**	**0,30**	*0,00*
	TNF	2,14	2,65	0,29
	IL1B	**9,25**	**70,95**	*0,00*
	CCL2	**0,40**	**0,71**	*0,04*
	CCL20	**0,05**	**0,49**	*0,00*
	TNFAIP3	**1,91**	**5,78**	*0,00*
	PDE4B	**3,13**	**7,81**	*0,00*
	DUSP2	**1,49**	**2,50**	*0,01*
	PTGS2	3,22	2,57	0,36
	ATF3	**1,62**	**2,66**	*0,00*
CLUSTER C	FABP5	16,41	19,51	0,85
	MAPK6	5,69	7,80	0,67
	HSPA1	19,65	16,45	0,70
	DHRS3	0,06	0,07	0,52
	CD9	2,78	2,10	0,22
	HGF*	**0,95**	**0,55**	*0,00*
	Resistin*	3,55	0,64	0,08

We assume the following hypothetical mode of events:

1. In T2D patients in Quito and most likely due to a highly prevalent dyslipidemic state and vascular damage, the monocytes adopt a set point with a higher production of HGF and a gene expression profile suspicious for a capability to change shape and to differentiate further (most likely into pro-angiogenic cells).
3. These T2D monocytes show over- expression of miR-34c-5p, CD9, DHRS3 and PTPN7.
2. These monocytes are characterized by a reduced expression of inflammatory markers (ATF3, DUSP2, and PTGS2), unless there is nomolipidemia. Then the monocytes are pro-inflammatory.

Serum

We also tested microRNAs-146a and -155 and the above described microRNAs in the serum of the Ecuadorian patients and the non-diabetic Ecuadorian controls. We compared data to the level of a series of 12 pro-inflammatory and growth factors (TNFα, IL-1β, IL-6, NGF, HGF, PAI, Resistin, CCL2, Adiponectin, Leptin, IL-8, and CCL4), known to be differentially expressed in patients with T2D.

With regard to the serum of patients with T2D and the non-diabetic controls we found that

1. The levels of microRNAs and cytokines/growth factors in serum differed considerably from the expression of their respective microRNAs and genes in circulating monocytes. We therefore conclude that both compartments (serum and circulating monocytes) have their own dynamics. Most likely different (T2D-related pathophysiological) forces drive the activation and de-activation set points of the serum and circulating monocyte compartment.
2. In the serum of the Ecuadorian patients with T2D there were (only limited) signs of inflammation as compared to the non-diabetic Ecuadorian controls, namely a decrease of miR-146a and an increase of IL-8, while there were no statistically significant increases in classical inflammatory cytokines. There were clear signs of vascular repair, i.e. an increase of HGF.
3. The microRNA 574-3p was significantly down regulated in the serum of the T2D patients and correlated in expression to the down regulated miR-146a.
4. There was a high prevalence of obesity and dyslipidemia in the Ecuadorian non-diabetic general population control group (BMI > 25 in 82.5% average 29.3) [1]. These Ecuadorian non-diabetic controls had raised levels of various pro-inflammatory cytokines in their serum (IL-6 and CCL4) as compared to Dutch non-diabetic general population controls who appeared to have a low prevalence of obesity (BMI > 25 in 40% average 23, 8) and dyslipidemia [1].

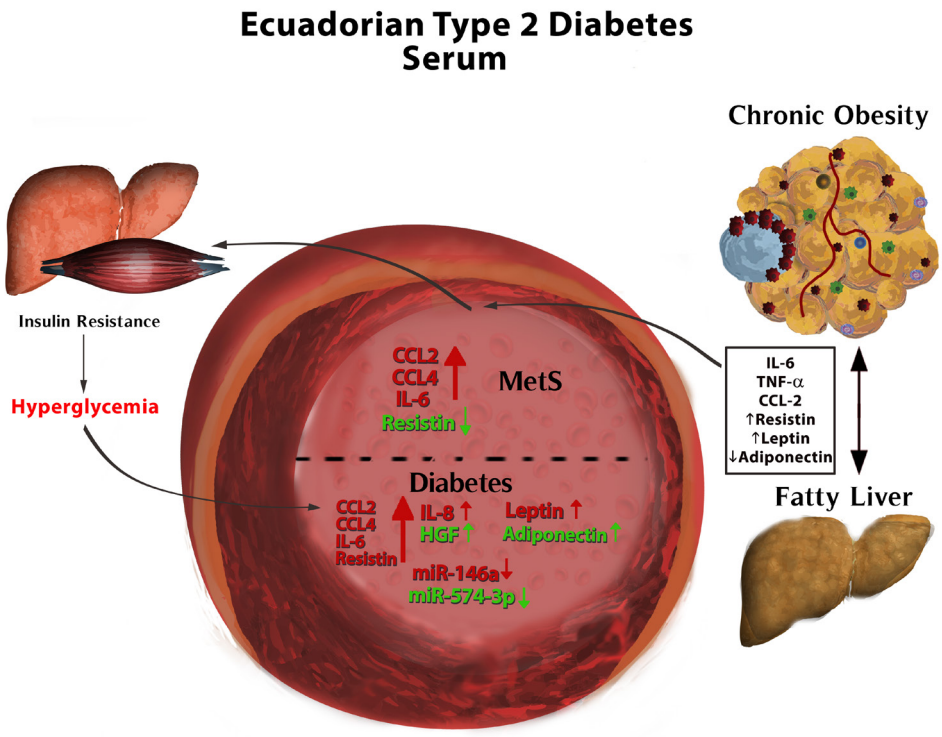

Figure 2. Hypothetical scheme for the pathogenesis of the immune abnormalities in serum of T2D as found in this body of work on Ecuadorian subjects.

Figure 2 depicts the following hypothetical mode of events;

1. Visceral obesity and a fatty liver lead to the production of inflammatory cytokines (in the reports of this thesis increases in CCL2, CCL4 and IL-6, and a reduction in serum resistin), which are systemically detectable in the serum of these individuals (immune signs of the MetS).
2. This inflammatory state leads to insulin resistance and the risk to develop T2D (in case the insulin resistance worsens and/or the production of insulin starts to lack behind the needs).

The developed T2D state (hyperinsulinemia as well as hyperglycemia) is characterized by a stronger expression of inflammatory cytokines in the serum: In addition to the aforementioned cytokines, now also an increase in IL-8, a switch from lower to higher resistin levels plus a decrease in miR-146a and miR-574-3p (both correlating to each other) were found.

In summary, the serum microRNAs and cytokines/growth factors follow different dynamics as compared to their genes and counterparts in circulating monocytes. In contrast to the circulating monocytes the serum of Ecuadorian T2D patients showed a mild pro-inflammatory profile as compared to Ecuadorian non-diabetic controls (with a decreased miR-146a). The Ecuadorian non-diabetic controls on their turn showed a mild pro-inflammatory profile as compared to non-diabetic Dutch general population controls (who are less obese and less dyslipidemic).

Chapter 7

Discussion

7.1 DISCUSSION

Comparison of the data collected in the Ecuadorian cohorts to preliminary findings in Dutch patients with T2D

At the same time as we collected serum and PBMC from the Ecuadorian patients with T2D and their respective non-diabetic controls, we collected in Rotterdam serum and PBMC from patients with T2D (n=28, mean age 47.7 years) and their respective non-diabetic controls (n=22, mean age 59.9 years). At more or less the same time we performed the same assays using the same laboratory conditions on the two sets of samples. Since we counterintuitively found a relatively low inflammatory serum state in Ecuadorian T2D patients and anti-inflammatory circulating monocytes (the literature poses the opposite, see introduction and general conclusion section), we have carried out a preliminary evaluation of the Dutch serum and circulating monocytes for comparison to the Ecuadorian data. We aimed to see whether we would find a similar low inflammatory state of the serum and an anti-inflammatory state of the monocytes in Dutch T2D patients. The data are given underneath.

Serum Cytokines

Table 1 shows that the Dutch patients with T2D were found to have a clearly stronger pro-inflammatory serum profile as compared to the Ecuadorian patients with T2D and high levels of the classical pro-inflammatory cytokines (IL-1β, IL-6, TNFα, IL-8 and CCL4) were detected, reaching statistical significance for TNFα in comparison to Ecuadorian T2D cases, and for all the cytokines in comparison to Dutch non-diabetic controls.

However most striking were the differences in the adipokines. We observed that pro-inflammatory leptin levels were almost three times higher in the Dutch patients with T2D, while anti-inflammatory adiponectin levels were two times higher in Ecuadorian T2D patients. Since adipose tissue plays a prominent role in the production of these adipokines, it is worthy to note that the BMI did differ between the Dutch and Ecuadorian patients with T2D, but only slightly (31.4 versus 28.4, p=0.01). Interestingly, Ecuadorian healthy controls were generally overweight, in contrast to the Dutch controls, but did not show altered adipokine levels compared to Dutch controls. Resistin levels, which are generally associated with obesity, were even lower in Ecuadorian controls.

Additionally, other known cytokines such as PAI-1 and resistin were also significantly lower in Ecuadorian T2D patients compared to Rotterdam T2D patients (see Table 1). HGF was higher in the Rotterdam T2D cases.

Table 1. Expression level of serum cytokines, chemokines and adipokines in Ecuadorian and Dutch non-diabetic controls (HC) and patients with T2D. Means and SDs are given. In the Quito HC group the red figures represent statistically higher significant values versus the HC Rotterdam group; the green value represents a statistically lower value. In the diabetic groups the red figures represent statistically higher significant values versus the respective HC control groups; the green value represents statistically lower value. P values in the table represent the p values between the HC groups and the diabetic groups of the two areas.

	CCL4	IL-8	IL-6	TNFα	IL-1β	CCL2	HGF	PAI-1		Leptin	Adiponectin	Resistin	
HC Quito	***121***	***7.95***	***4.88***	**4.1**	**0.87**	**311**	**771**	**92991**		**9973**	**26928123**	**39563**	**pg/ml**
SD	93	5.81	2.48	1.88	1.27	137	454	27767		9217	16417006	15520	
HC Rotterdam	**82**	**5.54**	**1.29**	**4.81**	**0.93**	**263**	**874**	**95452**		**7120**	**27851162**	**69406**	**pg/ml**
SD HC	59	1.86	1.29	1.84	0.48	113	461	21910		5922	18915934	55752	
p value	0.06	0.03	0.00	0.17	0.81	0.16	0.41	0.71		0.16	0.85	0.02	
T2D Quito	**91**	***15.32***	**7.91**	**5.19**	**0.97**	**332**	***1037***	**83576**		**8924**	**30831285**	**45479**	**pg/ml**
SD	94	18.47	9.59	2.51	1.2	174	652	27619		10393	28416829	25366	
T2D Rotterdam	111	***22.05***	***7.94***	***7.43***	**1.67**	**318**	***1437***	***116141***		***22851***	**19303343**	**62923**	**pg/ml**
SD	97	26.04	7.05	3.06	2.24	148	938	48311		35036	10528328	39096	
p value	0.42	0.26	0.99	***0.00***	0.15	0.73	0.06	***0.00***		0.05	***0.03***	0.05	

In summary, with regard to serum cytokines and adipokines Ecuadorian patients with T2D clearly show a lower inflammatory profile as compared to Dutch patients with T2D. The latter show the pro-inflammatory picture known from the literature.

Monocyte gene expression

We also analyzed the expression of the set of immune activation genes tested on the monocytes of the Ecuadorian T2D subjects on the monocytes of the Dutch T2D subjects. Data are given in Table 2, which shows that the gene expression of inflammatory compounds that belong to cluster A and B such as CXCL2, PTX3, IL-6, IL1β, CCL2, and CCL20 were found to be significantly overexpressed in Dutch T2D subjects as compared to Ecuadorian T2D subjects (and also to Dutch non-diabetic controls, data not shown). In contrast, in Ecuadorian T2D patients there was an overexpression in monocytes of cluster C genes (the genes with an adhesion, motility, and cell differentiation function), which reached statistical significance for CD9, DHRS3 and HGF.

Resistin was also over expressed in the monocytes of Dutch patients with T2D, as was a different microRNA, i.e. miR-410 (data not shown), as compared to what was found in the Ecuadorian cases of T2D (i.e. miR-34c-5p).

In summary, the monocytes of Dutch patients with T2D are clearly set at a high inflammatory set point (as is known from the literature, see introduction), while those of Ecuadorian patients with T2D are characterized by an overexpression of genes involved in adhesion, motility, cell differentiation and vascular repair, but not inflammation.

Figure 1 gives a cartoon of the alterations in the Rotterdam cases with T2D in the same lay-out as the cartoon given in Chapter 6 on the Ecuadorian cases with T2D to make easier comparison possible.

The differences found between the Ecuadorian and Dutch T2D cases also brings us to the question which factors (e.g. genetic, environmental) could be responsible for:

1. The decreased inflammatory state of the serum and the monocyte compartment of Ecuadorian patients with T2D?
2. The increased expression of HGF and genes/microRNAs involved in the adhesion, motility and shape change of the monocytes of Ecuadorian patients with T2D?

Table 2. Monocytes gene expression of Ecuadorian and Dutch T2D patients. Monocytes gene expression of Ecuadorian and Dutch T2D patients. Fold change and P-values are given. P-values in bold denote a significant difference between the diabetic group and the healthy control group of the respective area. The red figures represent a statistically higher significant FC values versus the respective HC control groups at the 0.05 level (the actual values of the HC are not shown in this figure), the orange values represent FC values versus the respective HC control groups at the 0.10 level; the green value represents statistically lower values at the 0.05 level.

Genes		*T2D Quito*		*T2D Rotterdam*	
		FC	P-Value	**FC**	p-Value
CLUSTER A	**CXCL2**	**1,19**	0,53	***2,07***	***0,05***
	STX1A	**1,49**	0,12	**1,59**	0,19
	NAB2	**1,04**	0,85	**0,43**	0,40
	EMP1	**1,21**	0,18	**1,48**	0,21
	CDC42	**1,11**	0,42	**1,14**	0,60
	PTX3	**0,92**	0,56	***2,19***	***0,04***
	PTPN7	***1,42***	***0,03***	**1,09**	0,65
CLUSTER B	**IL6**	**1,30**	0,39	**19,49**	***0,01***
	TNF	**1,08**	0,70	0,75	0,13
	IL1B	**0,70**	0,18	*1,99*	0,08
	CCL2	**0,96**	0,89	***4,91***	***0,01***
	CCL20	**1,07**	0,85	*2,56*	0,07
	TNFAIP3	**1,11**	0,54	0,88	0,50
	PDE4B	**0,96**	0,83	0,90	0,67
	DUSP2	**0,75**	0,15	0,76	0,17
	PTGS2	**0,60**	***0,05***	1,27	0,51
CLUSTER C	**FABP5**	**0,86**	0,48	1,08	0,64
	MAPK6	**0,87**	0,35	1,68	0,13
	HSPA1	**0,91**	0,46	1,08	0,50
	DHRS3	**1,39**	***0,03***	0,91	0,54
	CD9	**1,72**	***0,04***	0,65	0,37
	HGF	**1,17**	***0,03***	1,02	0,89
	Resistin	**0,47**	0,24	2,13	***0,03***

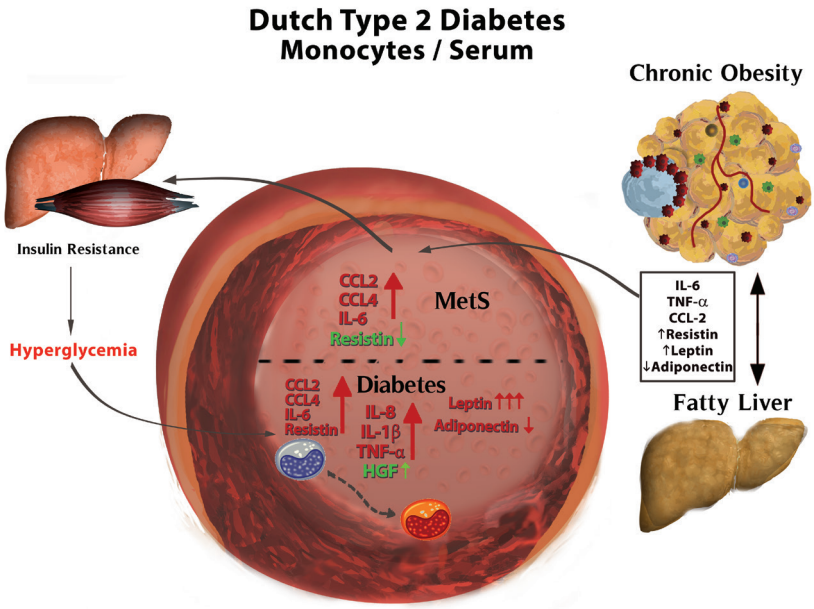

Figure 1. Serum and monocyte alterations of Dutch T2D patients.

Putative factors responsible for the reduced inflammatory state of the serum and monocyte compartment of Ecuadorian patients with T2D as compared to Dutch patients with T2D

Ethnic Differences in Leptin levels

It is well accepted that chronic obesity stimulates the production of the adipocyte-derived hormone leptin [1]. Leptin has direct effect on T cells, stimulating IFN-γ production, thereby promoting Th1 cell differentiation and accumulation of these cells in adipose tissue. IFN-γ also increases the MHC class II expression on adipocytes increasing in this way direct T cell stimulation [2]. Thus leptin is considered an important inflammatory compound. Notably, leptin-deficient (ob/ob) and leptin receptor–deficient (db/db) mice become obese, but the characteristic inflammation associated with obesity is absent [3]. Adiponectin, another adipokine, counteracts the effects of leptin. Adiponectin promotes M2 macrophage polarization [4], inhibits T cell activation [5] and modulate NK cell function [6]. Thus, in obesity a combination of increased leptin and reduced adiponectin production by adipocytes is well described [7-9].

Relevant to our question is that Bribiescas et al reported ethnic population differences in mean leptin levels in healthy men. They studied Ache Amerindians of Paraguay compared to US runners. Although Ache exhibited higher adiposity (fat %, Ache 17.9

± 1.8 SD; US runners 9.7 ± 3.2, $p < 0.0001$), leptin levels were nonetheless significantly higher in the runners (Ache 1.13 ng/ml ± 0.38 SD; runners 2.19 ± 1.15; $p < 0.007$)[10]. These results showed that there is an important ethnic variation in leptin levels independent of adiposity. Our data are similar in that our tested T2D populations were almost similar in BMI (Quito, 31.4, Rotterdam, 28.4), yet leptin levels were considerably higher in the Rotterdam group. Further support for an ethnic ground for difference in leptin levels comes from the studies of Mente et al [11], who investigated the ethnic differences in adiponectin and leptin concentration and their relationship with insulin resistance. Serum leptin was significantly higher in South Asians and Aboriginal people than in Europeans and Chinese. Adiponectin concentrations were significantly higher in Europeans and Aboriginal people than in South Asians and Chinese. This study clearly shows an important ethnic variation in the secretion level of leptin and adiponectin. Delgadillo et al studied the nutritional status and circulating leptin levels in the Amerindian Tepehuán compared with Mestizo populations of Durango City, Mexico. Both normal and overweigh Amerindian Tepehuán subjects showed lower leptin concentrations than the comparable Mestizo subjects [12].

In sum there is certainly ground for the idea that Amerindians have lower leptin production irrespective of obesity. It is tempting to speculate that genetic differences in the Ob gene that encodes leptin as well as in its receptor are the cause of this ethnic variation. For example among Taiwanese aboriginal populations, greater incidences of obesity were associated with the G- 2548A polymorphism in the promoter region of the leptin gene and the Gln223Arg (Q223R) polymorphism of the leptin receptor gene [13]. Similar findings are evident among Brazilian populations [14]. Variations in the proopiomelanocortin (POMC) gene have also been associated with variations in leptin levels [15]. Unfortunately, studies that investigate leptin and adiponectin profiles in relation to leptin, adiponectin or POMC gene polymorphisms in Ecuadorian populations have not been carried out.

Micronutrient Deficiencies in Ecuadorian patients

The micronutrient status may be another factor that contributes the observed differences between Ecuadorian and Rotterdam T2D patients. In Ecuadorian populations the nutritional deficiency for iron, vitamin A, vitamin D and zinc is well described [16-20].

Iron deficiency is described in around 38% of Andean populations [21]. A recent Ecuadorian survey reported 14,7% iron deficiency in women aged from 12 to 49 years old. In addition 25,7% of children under 5 years old had this deficiency [22]. Iron deficiency has detrimental effects on the immune system. Ekiz et al [23], reported that iron deficiency affects the cytokine activity (e.g. IL-6), diminishes the phagocytic activity, the oxidative burst activity of neutrophils and monocytes and the levels of immunoglobulins. In sum

iron deficiency can alter various mechanisms of innate and humoral immune defense resulting in an increased incidence and duration of infections.

Early in 1986 low vitamin A levels were reported in serum of 14.1% of 1600 preschool children of Ecuador [24]. In 1993, Rodriguez et al reported marginal vitamin A deficiency in around 17.6% of Ecuadorian children from 12 to 59 months [25]. This trend does not change over the time, recently a national Ecuadorian survey reported vitamin A deficiency in 17.1% of Ecuadorian children under 5 years [22]. Vitamin A deficiency is associated with an increased incidence of respiratory infections and intestinal diseases in children [17]. Vitamin A seems to be important in maintaining the homeostasis of both humoral and cellular immunity in the mucosa. In this way, vitamin A-deficient rats showed important alterations in mucosal dendritic cells, increased IL-12 production as well as TLR2 and MyD88 and decreased production of IFN-γ and IgA in intestinal mucosa [26]. Importantly, Garcia et al [27] reported that obese individuals have lower vitamin A levels as compared to lean controls. It seems that vitamin A has important effect in the chronic inflammatory effect in obese people since vitamin A deficiency increases a T-helper type 1 (Th1) response, elevates levels of pro-inflammatory cytokines, increases the expression of leptin, resistin and promotes adipogenesis. It is therefore unlikely that vitamin A deficiency plays an important role in the in general reduced inflammatory response in the obese Ecuadorian subjects.

In the Ecuadorian population zinc deficiency is described in approximately 43% of the elderly population [28]. In Ecuadorian women (12- 49 years old) the zinc deficiency is around 56,2% [22]. Zinc is an essential micronutrient required for neurological development and adequate immune function. Zn is a constructive component of many important enzymes and proteins. Nowadays there is a better understanding of the relationship between zinc deficiency and inflammation. For example, in the monocyte THP-1 cell line, zinc deficiency increased expression of several markers of inflammation such as ICAM-1, MHC class II, CD86 and an increased expression of cytokines such as IL-1β and IL-6 in response to LPS [29].

Hirano et al, showed that intracellular zinc participates in signaling events in immune cells. A decrease in intracellular free zinc induced surface expression of MHC class II molecules, which can activate CD4+ T cells [30-32]. Furthermore, intracellular zinc is capable of suppressing nuclear factor kappa B (NF-kB) and consequently the secretion of TNF-α, IL-1β, and other inflammatory cytokines [32, 33]. Additionally, zinc induces A-20 (TNFAIP3, one of our cluster 1 inflammatory genes, see chapter 3), which inhibits NF-kB signaling, resulting in a down regulation of the mRNAs for inflammatory cytokines [34, 35].

The association of Zn deficiency with the metabolic syndrome, T2D and diabetic complications is also well described [36]. Zn is a requirement for insulin storage and secretion; it has a direct or indirect antioxidant action, and an insulin-like action [36, 37].

However, Zn deficiency is associated with impaired Th-1 dependent adaptive immune response as evidenced by low IL-2 and IFNγ production by peripheral mononuclear cultured cells of zinc deficient older subjects [28]. Furthermore, cutaneous Delayed Type Hypersensitivity (DTH) to recall antigens is low in these older subjects [28]. The complex role of zinc in inflammation is discussed by Sempertegui in his thesis (ISBN 978-94-91811-08-1). Essentially, this role would depend on the duration and severity of deficiency which in turns is related to dietary income, uptake at intestinal level, transcellular transport of Zn ions, interaction with other micronutrients, and concurrent acute or chronic infections.

Therefore, there are not enough evidences to conclude on the association between chronic zinc deficiency with the decreased inflammatory state of serum and monocytes of the Ecuadorian subjects studied in this thesis. Nevertheless formal studies should be carried out on the relationship.

Chronic helminth infection in Ecuadorian patients

Currently, it is considered that diseases such as allergies and autoimmune diseases can be the consequence of an altered and diminished exposure to commensal microbes (the hygiene hypothesis) [38-41]. Several studies in human and animals indicate that helminth infection is associated with reduced allergic reactivity and enhanced suppressive activity of natural regulatory T cells production and IL-10 [39, 42-45]. In addition, helminths can stimulate the production of regulatory B cells [46, 47], natural killer T cells [48] and alternatively activated macrophages [49, 50], which all contribute to immune suppression.

In Ecuador the prevalence of helminth infection in the general population is not known in detail. However, Peplow examined in 1982 a total of 1568 people from 12 different regions of Ecuador. Remarkably, 96% of the samples were positive for parasites! On average 2.3 species per person were found [51]. In 2001 another study revealed that 90% of the children studied were positive for at least one pathogenic intestinal parasite; 51% had helminthic infection [52]. In 1985 the Ecuadorian Ministry of Health implemented an anti-parasite program (Pepin program) for massive parasite treatment. Recently, González et al reported in a study of 167 people with ages ranging from 5 to 82 years a helminth prevalence of 48.5% in rural areas, and 28.7% in an urban area [53]. Although strongly decreased compared to earlier years, these figures are still considerably higher than in Western Europe.

Based on the observation of a decreased frequency of autoimmune, allergic and inflammatory diseases in under-developed countries; helminth-related therapies are currently being studied as a promising treatment for these pathologies in developed

countries [54-56]. Specifically, chronic helminth infections might protect against insulin resistance by restricting the effective caloric intake by the host and indirectly via a Th2 polarization of the immune system [57]. Notably, Hays et al reported that aboriginal adults from Australia with previous S. stercoralis infection were 61% less prone to have T2D [58]. Additionally, a Dutch group is conducting a cluster-randomized placebo-controlled trial in Indonesian subjects which aims to determine whether helminth infections are associated with a better body insulin sensitivity and metabolic homeostasis [57].

Given the average age of patients and controls, is it such that the Ecuadorian subjects used in the studies presented in this thesis, had a high prevalence of past or present parasitic infection with a skewed Th2 system and M2-like macrophages? Or with other words: Is the decreased monocyte inflammatory profile observed in the Ecuadorian cohorts caused by chronic or past (childhood) exposure to helminthic parasites, which left an (epigenetic) imprint in MPS cells for M2 like reactions?

Andean High Altitude and Immune System

Chronic high altitude is without doubt one of the factors to be considered since the Ecuadorian subjects described in this thesis live at 2880 meters above sea level. It has been described that chronic high altitude exposure is able to induce immune suppression altering the number and function of immune cells [59-61]. The sympathico-adrenal axis plays a role in the immune adaptation occurring in high altitude areas [62]. Goel et al. reported that people living at high altitudes had significantly higher total leukocyte counts as compared to people living in lower areas. Importantly, monocytes appear to be the most vulnerable hematopoietic cell altered by high altitude. Lymphocytes in this study did not show a statistically significant difference [59]. Other studies show that chronic high altitude exposure activates innate immune responses and suppresses Th1 lymphocyte-mediated immunity [61, 63, 64]. However, decreased in vitro monocyte activation despite increases in the number of monocyte have also been reported, together with decreased activity of natural killer cells [59]. Needless to say that also further study on the effect of high altitude on the abnormal immune function in T2D as described in the introduction of this thesis is needed.

Putative factors responsible for the increased expression of HGF and genes/microRNAs involved in the adhesion, motility and shape change of the monocytes of Ecuadorian patients with T2D as compared to Dutch patients with T2D.

An important other difference between the Rotterdam and Quito cases with T2D is that the Rotterdam cases were treated with statins while the Quito cases were not. Therefore serum lipid values probably differ considerable between the Quito cases and the Rotterdam cases, the Quito cases mean cholesterol =237, triglycerides=205,

HDL=43 and LDL=158, while the Rotterdam cases probably had almost normal values (formal evaluation of the lipid values need to be carried out in the coming months for publication of the Rotterdam data). Chapter 3 shows that particularly in situations of high serum lipids the monocytes of the Quito T2D patients adopt a profile with relatively high expression of genes and microRNAs involved in shape change of the cell, but with a reduced inflammatory gene expression profile. A similar profile (high shape change gene expression, high HGF expression and reduced inflammatory gene expression) occurs in the Quito patients (high lipid values) as compared to the Rotterdam patients (normal lipid values). We therefore assume that high serum lipid values contrary to general belief, at least at the Ecuadorian genetic background, induce anti-inflammatory monocytes with a gene pattern compatible with well-functioning pro-angiogenic cells (PACs or CACs). Such PACs could be instrumental in the endothelial repair of vessel walls, damaged by the high serum lipids. However functional experiments on monocyte-derived PACs are needed to prove this assumption.

The development and well-functioning of PACs is highly sensitive to peripheral conditions. In situations of chronic low-grade inflammation, as induced by metabolic stress such as hyperglycemia, the availability and functionality of PACs has been described as significantly decreased [65, 66]. Many studies on MetS and T2D in human subjects and animals models show that monocytes/macrophages increase their pro-inflammatory characteristics under these circumstances and that the development of PACs is hampered under such circumstances[66].

Using experimentally induced diabetes (streptozotocin) in C57BL/6 and FVB mice [67], a 40% reduction of PACs and a 50% increase in the production of classical macrophages from bone marrow was observed in comparison to control mice. Since the conditions of culture of cells from the bone marrow of diabetic mice and control mice were identical, the findings indicate that an impaired glucose tolerance in vivo caused these alterations in the development of myeloid progenitors by promoting the development of pro-inflammatory macrophages at the expense of decreased PAC formation. In this study it was also reported that PACs and macrophages were derived from the same precursor fraction.

Another study showed that the number of PACs that was generated from precursors of peripheral blood in patients with diabetes, correlated inversely with the levels of HbA1c, reflecting the degree of hyperglycemia [67].

Taken together, all these findings have led to the classical concept that the MPS system in MetS and T2D is developmentally skewed towards catabolic tissue-destructive M1-like macrophages away from anabolic reparative M2-like monocyte-derived PACs.

Interestingly, in the Ecuadorian T2D patient cohort, we observed that such skewing towards catabolic M1 like macrophages away from anabolic M2 like monocyte-derived PACs indeed occurs in association with hyperglycemia, yet does not occur in situations of

hyperlipidemia. In contrast skewing towards M2 like monocyte-derived PACs away from catabolic M1 like macrophages occurred in the latter situations.

In some preliminary experiments we have tested PAC formation from monocytes in a series of Dutch T2D patients and non-diabetic controls and found that the generation of PACs was earlier related to gender (females > males), age (young > old) and BMI (lean > obese) than to disease. Further studies are obviously needed.

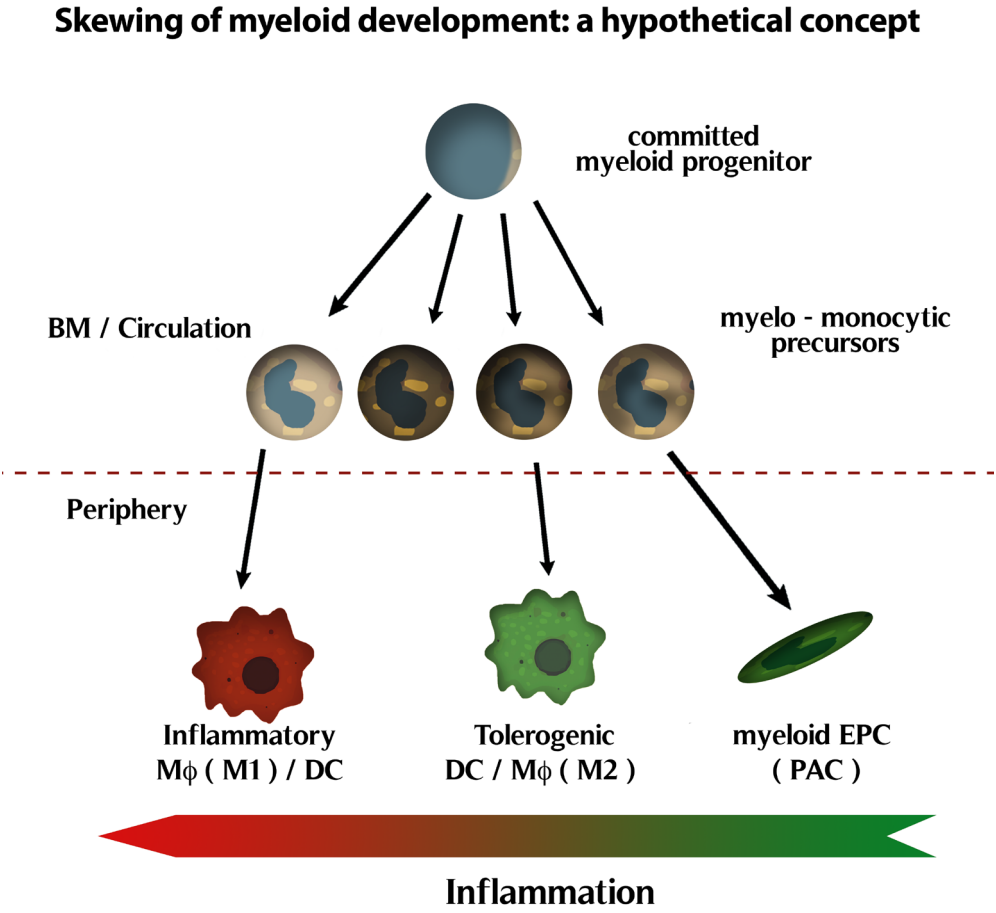

Figure 2. Skewing of myeloid development under inflammatory conditions: a hypothetical concept. The concept depicts the idea that due to inflammatory cytokines or other inflammatory mediators the normal steady state development of myeloid precursors into anabolic cells (vascular repair cells, M2 cells, tolerogenic dendritic cells) skews to catabolic M1 macrophages or dendritic cells capable of producing inflammatory Th1 and Th17 T cells.

7.2 LIMITATIONS OF OUR STUDIES

Patients and controls

In retrospect, we can see that a strong limitation of our series on Ecuadorian and Dutch/German T2D patients is their heterogeneous ethnic and environmental background. At the start of our studies we did not make any ethnic separation for the Exiqon microRNA expression arrays on monocyte material. Post hoc we did analyze these groups separately, but unfortunately studies became underpowered. It is also clear that we should have stratified for other determinants than hyperglycemia as a marker for T2D. Other conditions of the MetS, such as serum lipid state and body weight, play an equally important role for the inflammatory state of serum and monocytes. Also age and gender, smoking, oral contraceptive use and other drug use have been found by us and others in flanking studies as confounders of the inflammatory state. Needless to say that future studies need large numbers of subjects and should carefully control for all these determinants. In the series of Ecuadorian subjects studied for this thesis, our non-diabetic control group was not matched for age to our diabetic patient group, and non-diabetic controls were on average 8 years younger than our patients. In addition, the non-diabetic control group has a high prevalence of obesity and dyslipidemia. We have in our analysis controlled for these confounders, but an a priori matching should have been better on large numbers of patients and non-diabetic general population controls.

Laboratory techniques and analysis

To determine the microRNA profiles in monocytes we used the Exiqon array system, which allowed us to find a considerable number of significantly different microRNAs between controls and T2D patients when combining the Ecuadorian and Dutch/German patient cohorts. To validate the microRNAs with greatest expression differences in these finding cohorts we used TaqMan qPCR on RNA isolated from monocytes from a distinct Ecuadorian T2D patient population. Only a single microRNA, miR-34c-5p, showed significantly different expression between patients and controls. At least part of the inability to reproduce most results obtained in the finding cohort might be ascribed to the profound technical differences between the Exiqon array technique and TaqMan qPCR. Both techniques have strengths and weaknesses regarding the ability to identify selectively particular microRNAs (e.g. monocyte microRNA-155 is more difficult to detect in qPCR than in Exiqon array). In hindsight, using the same technique for finding and validation studies would have been preferable and might have revealed more microRNAs, since technical variation would have been eliminated. On the other hand our approach probably identifies very robust biomarker microRNAs.

7.3 FUTURE STUDIES

Our studies described in this thesis and in the literature support the view that T2D is a not a clear separate and distinct pathological entity, but rather a phase in a continuum of processes partly marked by dynamic changes in pro-inflammatory and anti-inflammatory states of the MPS and other immune cells, such as the T cells. In flanking research on another (auto-)inflammatory conditions, such as mood disorders (which show a higher incidence of T2D) members of our team have found dynamic changes in the pro-inflammatory and anti-inflammatory state of circulating monocytes depending on inheritance, age and activity and duration of the mood disorder [68, 69]. With regard to T cells our department at ErasmusMC described that the peripheral blood T-cell compartment of diabetes-free "relatively healthy" morbidly obese subjects is characterized by an increased homeostatic proliferation of both CD4+ and CD8+T cells [70]. This increased homeostatic proliferation was associated with an increase in peripheral blood CD4+ T cell numbers, with a skewing toward a Treg- and Th2- dominated phenotype, suggesting an anti-inflammatory set point of the peripheral blood CD4+ T-cell compartment. On the basis of literature data which show a pro-inflammatory set point of the T cell system in atherosclerotic plaques and T2D, we speculated that changes away from the T regulatory and Th2-dominated phenotype toward a more pro-inflammatory Th1- or Th17-dominated set point may prove an important indicator, or even mediator, for the development of atherosclerosis or diabetes in diabetes-free "relatively healthy" morbidly obese subjects. Longitudinal studies in obese subjects will be important to address these issues further.

Indeed further detailed research is necessary to unravel pro-inflammatory and anti-inflammatory states of different immune compartments (adipose tissue, circulating monocytes, serum/plasma, vessel walls and pancreatic islets) in the different phases of obesity/dyslipidemia developing into insulin resistance/diabetes and atherosclerosis. Also attention should be paid to the ethnic back ground of the studied subjects, as well as various environmental factors (high altitude, micronutrient state) and comorbidities (such as chronic helminthic infections, and probably the microbiome in general).

Only when we have unraveled the pathogenic influence of these factors and states, we will be able to detect biomarkers, such as microRNAs, for these states and transitions between these states.

At present we have embarked on a large study in the Quito population (two cohorts of the general population of two socio-economic backgrounds) to clarify some of the issues. The following research issues should be addressed to make further progress:

1. Determining the prevalence of obesity, dyslipidemia, hyperglycemia and another inflammatory condition (major depression) in these populations and to determine their relationship with nutritional state and chronic parasite infection.

2. Confirming the anti-inflammatory monocyte and serum profile in the T2D patients of these cohorts, paying in particular attention to leptin and HGF levels and their expression in circulating monocytes.
3. Studying possible environmental (e.g. parasite, micronutrients, altitude) and genetic (e.g. leptin polymorphism) factors that are related to altered leptin levels and pro-inflammatory and anti-inflammatory gene expression profiles in subjects of the cohorts.
4. Studying the same parameters in cohorts of different ethnic backgrounds, such as cohorts sampled in the Netherlands.

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Addendum

ABBREVIATIONS

Abs	Antibodies
A20	Tumor necrosis factor a-induced protein (TNFAIP) 3
ABL1	Abelson murine leukemia viral oncogene homolog 1
ATF3	Cyclic AMP-dependent transcription factor ATF-3
BCL2A1	Bcl-2-related protein A1
CACs	Circulating pro-angiogenic cells
CCL2	Chemokine (C-C motif) ligand 2. Monocyte chemotactic protein 1 (MCP1)
CCL20	Chemokine (C-C motif) ligand 20
CCL4	Chemokine (C-C motif) ligand 4
CCL7	Chemokine (C-C motif) ligand 7
CD9	Cell division 9
CDC42	Cell division control protein 42 homolog
cDNA	Complementary Deoxyribonucleic acid
cMET	Hepatocyte Growth Factor receptor
Ct	Cycle threshold
CXCL2	Chemokine (C-X-C motif) ligand 2
DC	Dendritic cell
DHRS3	Short-chain dehydrogenase/reductase 3
DUSP2	Dual specificity protein phosphatase 2
EGF	Epidermal growth factor
EMP1	Epithelial membrane protein 1
FABP5	Fatty acid-binding protein, epidermal
GAD-65	Glutamic acid decarboxylase 65
HGF	Hepatocyte growth factor
HGF-R	Hepatocyte growth factor receptor
HSPA1A	Heat shock 70 kDa protein 1A
HSPA1B	Heat shock 70kDa protein 1B
IL-10	Interleukin 10
IL-6	Interleukin 6
ILB	Interleukin 1 beta
LADA	Latent Autoimmune Diabetes in Adults
MAPK6	Mitogen-activated protein kinase 6
MCP-1	Monocyte chemotactic protein 1 (CCL2)
MetS	Metabolic Syndrome
MIP1β	Macrophage inflammatory protein 1 beta (CCL4)
miR-122	MicroRNA-122

miR-138	MicroRNA-138
miR-146a	MicroRNA-146a
miR-410	MicroRNA-410
miR-574-3p	MicroRNA-574-3p
miR-92	MicroRNA-92
mRNA	Messenger Ribonucleic acid
NAB2	NGFI-A-binding protein 2
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PDE4B	cAMP-specific 3',5'-cyclic phosphodiesterase 4B
PDFG	Platelet-derived growth factor
PGS2	Prostaglandin synthase-2
PTGS2	Prostaglandin-endoperoxide synthase 2
PTPN7	Protein tyrosine phosphatase non-receptor type 7
PTX3	Pentraxin-related protein 3
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
STX1A	Syntaxin-1A
TNF	Tumor necrosis factors
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TGFβ	Transforming growth factor β
T2D	Type 2 diabetes
VEGF	Vascular endothelial growth factor

ENGLISH SUMMARY

The immune system is composed of a complex group of molecules, cells and tissues with various beneficial functions such as defense against pathogens and tumors via an inflammatory reaction and tissue repair. However, excessive inflammatory immune responses are the cause of allergies, auto-inflammation and autoimmune diseases.

In this thesis we focus in particular on a special group of cells of the immune system, namely the mononuclear phagocytes, and more specifically on the monocytes of patients with type 2 diabetes (T2D). Human monocytes constitute 2-10% of all leukocytes in the peripheral blood; typically they express the cell surface marker CD14. The monocytes are the precursors of macrophages which are recognized as important pathophysiologic agents in chronic inflammatory processes such as obesity, which can lead to the Metabolic Syndrome (MetS) and T2D. Depending on the triggering factor macrophages can skew towards two polar phenotypes (M1 or M2 macrophages). The M1 macrophages (also called classically activated macrophages) express numerous pro-inflammatory mediators including TNF-α, IL-1, IL-6, reactive nitrogen and oxygen intermediates, which have a strong microbicidal and tumoricidal activity, while M2 macrophages (also called alternatively activated macrophages) express molecules and growth factors associated with anti-inflammatory reactions, tissue repair and remodeling.

A pathologic increase of visceral adipose tissue causes activation of local innate and adaptive immune cells which skew towards a production of pro-inflammatory cytokines/chemokines. These cytokines/chemokines spill over in the circulation and are capable of inactivating the insulin receptor in multiple cell types, including muscle and liver cells. This causes insulin resistance and T2D. The adipose tissue dysfunction is manifest not only for the secretion of cytokines (e.g. of TNF, IL-6, IL-1β, PAI1) and chemokines (CCL2, CCL4, CCL20, CXCL14), but also for the secretion of proinflammatory adipokines (e.g. leptin). This chronic proinflammatory secretion pattern in adipose tissue can be seen as a systemically low grade of inflammation which perpetuates the positive feedback loop of inflammation.

Similar to what is happening worldwide, the prevalence of obesity, the MetS and T2D is increasing exponentially in Ecuador. The social factors seem to be the most important inducing factors for the development of this disease. The prevalence of disease increases with age and is particularly high in the older population, averaging 10% in groups of 50-59 years. Overall, the prevalence of diabetes in urban areas ranges between 7 and 8%, while in rural areas it is only 1 to 2%.

MicroRNAs are important regulators of translation and stability of messenger RNA (mRNA). They negatively regulate gene expression at post-transcriptional level by mediating translational repression or degradation of the mRNA targets. The microRNAs have been highly conserved during evolution; around almost 2000 mature microRNAs have been identified in the human. The dysregulation of the microRNA network has been implicated in the development of MetS and diabetes. Various inflammatory triggers appear to induce the selective expression of microRNAs in monocytes/macrophages, which in turn functionally affect the expression of proteins involved in the inflammatory cascade. Additionally, circulating microRNA expression patterns have been suggested to have a predictive value as potential biomarkers in diabetes.

The aim of the studies described in this thesis is firstly to establish a putative dysregulated pattern of microRNAs in monocytes of T2D patients which is able to distinguish patients from non-diabetic controls (biomarker study), and secondly to correlate the dysregulated microRNA expression to inflammatory markers in monocytes and serum to understand better the molecular pathogenic processes playing a role in T2D.

In the first part of Chapter 3 we describe that, in an Exiqon array study on the monocytes of 34 T2D patients recruited in the German Diabetes Center, Düsseldorf, Germany (n=10) and from three medical centers in Quito, Ecuador (n=24) in 2009; and a total of 25 healthy controls (n =9 from Germany and n =16 from Ecuador), we were able to identify 142 significantly differentially expressed microRNAs, 15 having the strongest power to discriminate T2D patients from controls. However, using this approach only partial separation could be made between T2D cases and non-diabetic controls (sensitivity 66%, specificity 90%). We thereafter continued to use the microRNAs as markers to test the inflammatory function and state of the circulating monocytes in patients with T2D, since there is a paucity of studies focusing on the inflammatory state of these circulating cells in T2D. Therefore, we chose to select from the differentially expressed microRNAs those with the highest fold changes (FC) between cases and controls with FC of >1.4 or <0.6 to have solid microRNA markers for testing in TaqMan analyses. Another criterion for selection was that TaqMan probes and primers were available. The microRNAs that fulfilled the selection criteria were miR-138; miR-34c-5p; miR-410; miR-574-3p and miR-576-3p. Additionally, we tested microRNAs-146a and -155, since these microRNAs are well known regulators of inflammation, and have been identified in T2D PBMC by others before. In the TaqMan studies on a new "validation" cohort of 64 T2D patients (mea age=61 years; BMI= 29.5; hypercholesterolemia= 63% and a raised LDL in 44%) recruited from four medical centers in Quito, Ecuador from 2009 till 2012 and a total of 44 healthy controls (mean age=53 years; BMI=28.7; hypercholesterolemia=68% and raised LDL in 50%); one microRNA (miR-34c-5p)

was validated as significantly over expressed in the T2D monocytes. This microRNA is known to have a function in altering the expression of c-Met, the receptor for HGF. In addition, this microRNA participates in diverse cellular processes, such as inflammatory responses; growth, apoptosis and invasiveness of tumor cells. Mir-34c-5p correlated to the expression of miR-138, miR-146a and miR-574-3p, although these latter microRNAs were not significantly differently expressed.

Furthermore, we tested for 25 selected genes in the T2D monocytes, these 24 selected genes form two mutually correlating gene clusters. The first cluster comprises 12 pro-inflammatory cytokine/compound genes (IL-1B, IL-6, TNF, TNFAIP3, PGS2, CCL20, PTX3, PDE4B, DUSP2, ATF3, CXCL2 and BCL2A1); the second cluster comprises 12 chemotaxis, adhesion, motility, and metabolism genes (CCL2, CCL7, MAPK6, NAB2, CD9, STX1A, EMP-1, CDC42, PTPN7, DHRS3, FABP5, HSPA1A). We found over expression of 3 mRNAs of the second cluster (CD9, DHRS3 and PTPN7) in the validation cohort of the 64 Ecuadorian patients and 44 area controls. Mir-34c-5p correlated to FABP5, MAPK6, HSPA1, DHRS3 and CD9. Since the abnormally expressed mRNAs and microRNA-34c-5p are important for cell morphology, adhesion, shape change, and cell differentiation, we assume that the T2D monocytes of the Ecuadorian patients are involved in such processes. We hypothesize that the monocytes in the T2D patients might be pro-angiogenic cells.

In general there did not exist an over expression of classical inflammatory genes in the monocytes of the Ecuadorian T2D patients. Classical inflammatory genes (e.g. TNFAIP3) were only over-expressed in monocytes of patients with normal serum lipid values. Remarkably, in dyslipidemia, there was a significant reduction in the expression of inflammatory genes (e.g. ATF3, DUSP2 and PTGS2). Therefore we assume that in Ecuadorian patients with dyslipidemia the circulating monocytes are pro-angiogenic cells with an anti-inflammatory profile.

Since HGF is a well-known T2D related molecule with angiogenic and islet repair properties and since HGF is used as a marker for pro-angiogenic cells, we also tested HGF in the monocytes of the validation series of the 64 Ecuadorian patients and the 44 non-diabetic Ecuadorian controls and related the expression of HGF to the other genes and the microRNAs in the monocytes. These experiments are described in the first part of Chapter 4. HGF was indeed over expressed in the monocytes and primarily related to the cluster 2 genes.

We also tested the above referred "monocyte" microRNAs in the serum of the validation cohort of the Ecuadorian patients and the non-diabetic controls (Chapter 4 and 5). We

compared data to the serum level of a commercially available series of 12 T2D related compounds of inflammatory or growth factor character (TNFα, IL-1β, IL-6, NGF, HGF, PAI, Resistin, CCL2, Adiponectin, Leptin, IL-8, and CCL4). This study showed a decreased serum level of the anti-inflammatory microRNA 146a, an increased level of pro-inflammatory IL-8 and an increased HGF level as discriminating markers between Ecuadorian T2D patients and their non-diabetic controls. Since the non-diabetic controls had the same high frequency of obesity and dyslipidemia these serum markers can be seen as discriminating markers of the process of failure of glucose control occurring on the background of obesity and dyslipidemia.

In Chapter 5, we also compared the serum level of the microRNAs and the inflammatory/immune activation compounds to the expression levels found of the same compounds in the circulating monocytes of T2D patients, to see if there are indications for the idea that the circulating monocytes are the source of the compounds in serum.

Although HGF expression was significantly raised in the serum and the monocytes of the T2D patients (see before),gene expression in the monocytes did not correlate with the serum level of HGF. Neither did the expression of pro-inflammatory cytokine genes in monocytes to the serum levels of these cytokines, nor the level of the 6 tested microRNAs

We concluded that in T2D Ecuadorian patients, the microRNA and gene expression of important inflammatory and repair genes in T2D circulating monocytes differ from their expression in serum.

In conclusion the monocyte compartment of Ecuadorian T2D patients shows in general a reduction in gene expression of typical pro-inflammatory genes (predominantly in dyslipidemia), while genes and microRNAs involved in cell adhesion, cell differentiation, growth and vascular repair, such as HGF and miR-34c-5p, are up regulated. The serum compartment, in contrast to the monocyte compartment, does show signs of pro-inflammatory activity, e.g. high levels of IL-8 and reduced levels of anti-inflammatory miR-146a and altered levels of miR-574-3p. Moreover the serum compartment has higher levels of HGF.

Collectively the findings in the studies for this thesis in addition suggest that the dynamics of the diabetes-related changes in the monocyte intracellular compartment differ substantially from the dynamics of the diabetes-related changes in the serum compartment of patients with T2D. Most likely different T2D related pathophysiological forces drive the activation and de-activation set points of the circulating monocyte and the serum compartment.

In the general conclusion section we compare the findings collected in the Ecuadorian cohort to preliminary findings in Dutch patients from the Rotterdam area with T2D (n=28) and their respective non-diabetic controls (n=22). We aimed to see whether we would find a similar low inflammatory state of the serum and an anti-inflammatory state of the monocytes in Dutch T2D patients.

The serum cytokine tests showed that the Dutch patients with T2D have high levels of the classical pro-inflammatory cytokines (IL-1β, IL-6, TNFα, IL-8 and CCL4) as compared to the Ecuadorian patients with T2D. Importantly, we observed that pro-inflammatory leptin levels were almost three times higher in the Dutch patients with T2D, while anti-inflammatory adiponectin levels were two times higher in Ecuadorian T2D patients.

We also analyzed the expression of the set of immune genes in the monocytes of the Dutch T2D subjects. The gene expression of inflammatory compounds that belong to the inflammatory clusters A and B such as CXCL2, PTX3, IL-6, IL1β, CCL2, and CCL20 were found to be significantly over expressed in Dutch T2D subjects. In contrast, in Ecuadorian T2D patients there was an over expression in monocytes of cluster C genes (the genes with an adhesion, motility, and cell differentiation function), which reached statistical significance for CD9, DHRS3 and HGF (see before). Resistin was also over expressed in the monocytes of Dutch patients with T2D, as was a different microRNA, i.e. miR-410, as compared to what was found in the Ecuadorian cases of T2D (i.e. miR-34c-5p).

In the discussion section we mainly discuss which factors could be responsible for the differences between the Ecuadorian and Dutch individuals and patients, i.e. the reduced inflammatory state of the serum and monocyte compartment of Ecuadorian patients with T2D as compared to Dutch patients with T2D. Several factors could be in theory responsible, we discuss ethnic differences in leptin levels, micronutrient deficiencies in Ecuadorian patients (vitamin A, iron, and zinc), chronic helminth infection in Ecuadorian subjects (the hygiene hypothesis), and the effect of Andean high altitude on the immune system as possible causes of the differences found between Rotterdam and Quito subjects. A role for medication is also possible since many patients in Rotterdam are on statins, while those in Quito are not.

SPANISH SUMMARY

El sistema inmune está compuesto por un complejo grupo de moléculas, células y tejidos con diversas funciones beneficiosas tales como la defensa contra patógenos y tumores a través de reacciones inflamatorias y reparación de tejidos. Sin embargo, las respuestas inmunes inflamatorias excesivas son la causa de alergias, auto-inflamación y enfermedades-autoinmunes.

En esta tesis nos centramos sobre todo en un grupo especial de células del sistema inmune; los fagocitos mononucleares, y más específicamente en los monocitos de pacientes con diabetes mellitus tipo 2 (DM2). Los monocitos humanos constituyen el 2-10% de todos los leucocitos en la sangre periférica; normalmente expresan el marcador de superficie celular CD14. Los monocitos son los precursores de los macrófagos quienes son reconocidos como agentes fisiopatológicos en los procesos inflamatorios crónicos tales como la obesidad, el síndrome metabólico (SM) y DM2. Dependiendo del estímulo, los macrófagos pueden diferenciase en dos fenotipos distintos (macrófagos M1 y M2). Los macrófagos M1 (también llamados macrófagos clásicamente activados) expresan numerosos mediadores pro-inflamatorios como TNF-α, IL-1, IL-6, especies reactivas del nitrógeno y del oxígeno. Estas substancias poseen una fuerte actividad microbicida y tumoricida. Por el contrario, los macrófagos M2 (también llamados macrófagos activados alternativamente) expresan moléculas y factores de crecimiento asociados con reacciones anti-inflamatorias, de reparación de tejidos y de remodelación.

Un incremento patológico de tejido adiposo, especialmente el tejido visceral provoca la activación de células inmunitarias innatas y adaptativas locales que inducen la producción de quimiocinas y citoquinas pro-inflamatorias. Estas quimiocinas/citocinas se vierten en la circulación y son capaces de inactivar los receptores de insulina en múltiples tipos de células, incluyendo las células musculares y hepáticas. Como consecuencia se induce una resistencia a la insulina e inevitablemente DM2. La disfunción del tejido adiposo se manifiesta no sólo para la secreción de citoquinas (ej. TNF-α, IL-6, IL-1β, PAI 1) y quimiocinas (CCL2, CCL4, CCL20, CXCL14), sino también por la secreción de adipoquinas proinflamatorias (ej. leptina). Este patrón de secreción proinflamatorio en el tejido adiposo se perpetúa crónicamente, provocando una retroalimentación positiva de inflamación.

La prevalencia de obesidad, SM y DM2 está aumentando de manera exponencial en Ecuador al igual que en todo el mundo. Los factores sociales parecen ser los factores inductores más importantes para el desarrollo de esta enfermedad. La prevalencia de la enfermedad aumenta con la edad y es particularmente alta en la población más vieja,

alcanzando una prevalencia del 10% en los grupos de 50-59 años. En las zonas urbanas la prevalencia oscila entre el 7 y el 8%, mientras que en las zonas rurales es sólo de 1 a 2%.

Los microARNs son importantes reguladores de la traducción y estabilidad del ARN mensajero (ARNm). Estas moléculas regulan negativamente la expresión génica a nivel post-transcripcional reprimiendo la traducción o degradando el ARNm. Los microARNs han sido altamente conservados durante la evolución, actualmente casi 2.000 microARNs maduros se han identificado en el ser humano. La desregulación de los microARNs ha sido implicada en el desarrollo de SM y DM2. Varios factores inflamatorios parecen inducir la expresión selectiva de microARNs en monocitos/ macrófagos, que a su vez afectan funcionalmente la expresión de varias proteínas implicadas en la cascada inflamatoria. Además, se han sugerido que los patrones de expresión de microARNs circulantes en suero/ plasma podría tener un valor predictivo como biomarcadores de diabetes.

El primer objetivo de los estudios descritos en esta tesis fue establecer un patrón de microARNs en monocitos de pacientes con DM2 que sea capaz de distinguir a los pacientes de los controles no diabéticos (estudio de biomarcadores); en segundo lugar nuestro objetivo fue correlacionar la expresión alterada de microARNs, genes y moléculas pro-inflamatorias de los monocitos y del suero de los pacientes entre sí, y con las características clínicas para entender mejor los procesos patogénicos de esta enfermedad.

En la primera parte del capítulo 3 se describe un estudio de microarray (Exiqon) en monocitos de 34 pacientes con DM2 reclutados en el Centro de Diabetes en Alemania, Düsseldorf (n = 10) y en tres centros médicos en Quito, Ecuador (n = 24) en el año 2009. En este estudio, identificamos 142 microARNs significativamente expresados; de los cuales 15 fueron capaces de discriminar entre pacientes y controles. Sin embargo, utilizando este método sólo pudimos hacer una separación parcial entre casos y controles (sensibilidad 66% y especificidad 90%). A partir de entonces continuamos utilizando los microARNs como bio-marcadores para determinar la función y el estado inflamatorio de los monocitos circulantes en pacientes diabéticos. Optamos por seleccionar los microARNs con diferencias más significativas (Fold Change de> 1,4 o <0,6). Los microARNs que cumplieron con este criterio de selección fueron miR-138; miR-34c-5p; miR-410; miR-574-3p y miR-576-3p. Adicionalmente, determinamos la expresión del miR-146a y miR-155, ya que estos microARNs son conocidos reguladores de inflamación, y han sido identificados en células mononucleares periféricas de pacientes diabéticos. Utilizando PCR en tiempo real (TaqMan) en un nuevo grupo de "validación"; 64 pacientes con DM2 (edad media=61; IMC=29,5; hipercolesterolemia=63% y LDL elevada 44%) y 44 controles sanos (edad media=53; IMC=28,7; hipercolesterolemia = 68% y LDL elevada 50%) fueron

reclutados en cuatro centros médicos en Quito, Ecuador desde 2009 hasta 2012. El microARN-34c-5p fue validado como significativamente sobreexpresado en los monocitos de pacientes con DM2. Una de las funciones de este microARN es regular la expresión de c-Met, el receptor del factor de crecimiento hepático (HGF). Además, este microARN participa en diversos procesos celulares, tales como crecimiento, apoptosis e invasión de células tumorales. Adicionalmente, en estos estudios determinamos la expresión de 24 genes en los monocitos de pacientes con DM2. Seleccionamos un primer grupo de 13 genes que codifican para la expresión de citoquinas y moléculas pro-inflamatorias (IL-1β, IL-6, TNF-α, TNFAIP3, PTGS2, CCL20, PTX3, PDE4B, DUSP2, ATF3, CXCL2 y BCL2A1); y un segundo grupo que comprende 12 genes con funciones de quimiotaxis, adhesión y motilidad (CCL2, CCL7, MAPK6, NAB2, CD9, STX1A, EMP-1, CDC42, PTPN7, DHRS3, FABP5, HSPA1A). Observamos la sobreexpresión de 3 ARNm (CD9, DHRS3 y PTPN7) pertenecientes al segundo grupo. Dado que los ARNm anormalmente expresados y el microARN-34c-5p cumplen importantes funciones en el cambio de morfología celular, adhesión y diferenciación celular; suponemos que los monocitos de los pacientes ecuatorianos con DM2 están involucrados en tales procesos. Creemos que los monocitos de estos pacientes podrían ser células pro-angiogénicas.

En general en los monocitos de los pacientes diabéticos ecuatorianos no encontramos una sobreexpresión de genes inflamatorios clásicos. Uno de los genes inflamatorios clásicos (ej. TNFAIP3) sólo fue expresado en los monocitos de pacientes con valores normales de lípidos. Sorprendentemente, en casos de dislipidemia, encontramos una reducción significativa de la expresión de genes inflamatorios (ej. ATF3, DUSP2 y PTGS2). Por lo tanto suponemos que los monocitos circulantes de pacientes ecuatorianos con dislipidemia son capaces de diferenciarse en células pro-angiogénicas con un perfil anti-inflamatorio.

El factor de crecimiento hepático (HGF) es una molécula que también ha sido relacionada con DM2. Esta molécula tiene propiedades angiogénicas y se la utiliza como un marcador de células pro-angiogénicas. Debido a los hallazgos antes descritos, también determinamos la expresión de HGF en los monocitos de los pacientes diabéticos (capítulo 3). Encontramos una importante sobreexpresión de HGF en los monocitos que correlacionó con el segundo grupo de genes.

Adicionalmente, en nuestros estudios también determinamos los niveles séricos de 12 compuestos relacionados con DM2 comercialmente disponibles (TNFα, IL-1β, IL-6, NGF, HGF, PAI, resistin, CCL2, adiponectin, leptin, IL- 8 y CCL4) y la expresión de los microARNs antes mencionados en el suero de los pacientes ecuatorianos del grupo de validación (Capítulo 4 y 5). En este estudio observamos una disminución del nivel sérico del microARN anti-inflamatorio 146a; un incremento del nivel de la citoquina pro-inflamatoria IL-8

y un incremento sérico del factor de crecimiento HGF. Notablemente, debido a que los controles no diabéticos tuvieron un índice de obesidad y dislipidemia comparable con los pacientes, estas moléculas séricas pueden servir como marcadores discriminantes de la falta de control de la glucosa en pacientes diabéticos.

En el capítulo 5, describimos un estudio en el cual comparamos los niveles séricos de los microARNs y los compuestos de activación/inflamatoria inmune con los niveles de expresión de microARNs y genes en monocitos circulantes de los pacientes con DM2. El objetivo de este estudio fue comprobar si los monocitos circulantes son la fuente de secreción de esas moléculas que son vertidas en el suero. Encontramos la expresión de HGF significativamente elevado en el suero y en los monocitos de los pacientes. Sin embargo no hubo una correlación positiva de expresión entre estos dos compartimentos. Tampoco encontramos correlación entre los niveles de citoquinas pro-inflamatorias y los 6 microARNs del suero y los monocitos. Los monocitos de pacientes ecuatorianos con DM2 mostraron en general una reducción de la expresión de los genes pro-inflamatorios típicos, mientras que los genes y microARN implicados en adhesión celular, diferenciación celular, crecimiento y reparación vascular (ej. HGF y miR-34c-5p) estuvieron sobre expresados. En el suero de estos pacientes, en contraste, encontramos signos de actividad pro-inflamatoria (ej. incremento de IL-8 y disminución de miR-146a). Estos hallazgos sugieren que la dinámica de los cambios relacionados con la inflamación en el compartimento intracelular (monocitos) y el suero tiene su propia dinámica. Al parecer diferentes mecanismos fisiopatológicos inducen la activación o desactivación de los procesos inflamatorios.

En la sección de discusión presentamos y comparamos los datos de los pacientes ecuatorianos con algunos hallazgos preliminares de pacientes holandeses con DM2 (n = 28) y sus respectivos controles no diabéticos (n = 22). El objetivo de esta comparación fue comprobar si también existía una disminución del estado inflamatorio en los monocitos y en el suero de los pacientes holandeses. Observamos, que en el suero de los pacientes diabéticos holandeses hubo una alta expresión de las citoquinas pro-inflamatorias clásicas (IL-1β, IL-6, TNFα, IL-8 y CCL4) en comparación con los pacientes ecuatorianos. Además es importante destacar que los niveles de leptina (molécula proinflamatoria) fueron casi tres veces mayor en los pacientes holandeses; por el contrario, los niveles de adiponectin (molécula antiinflamatoria) fueron dos veces más altos en los pacientes diabéticos ecuatorianos.

También analizamos la expresión de los genes seleccionados en los monocitos de los pacientes holandeses. Encontramos que la expresión de los compuestos inflamatorios que pertenecen al grupo A y B tales como CXCL2, PTX3, IL-6, IL1β, CCL2, y CCL20 estuvieron

significativamente sobreexpresado en pacientes holandeses. Por el contrario, en pacientes ecuatorianos hubo una sobreexpresión de los genes del grupo C (genes de adhesión, motilidad y diferenciación celular), que fueron estadísticamente significativos para CD9, DHRS3 y HGF. En los monocitos de los pacientes holandeses además encontramos una elevada expresión de resistin así como el microARN-410, los cuales difirieron en los pacientes ecuatorianos.

Al fin de esta sección exploramos las posibles causas de las diferencias encontradas en los pacientes ecuatorianos en comparación con pacientes holandeses; hacemos referencia a las diferencias étnicas en los niveles de secreción de leptina, la deficiencia de micronutrientes en pacientes ecuatorianos (vitamina A, hierro y zinc), la infección crónica por helmintos en pacientes ecuatorianos (hipótesis de la higiene), y la afectación del Sistema Inmune en poblaciones que viven en grandes alturas (Andes). El efecto de la medicación también pudo haber causado las diferencias encontradas ya que todos los pacientes holandeses estuvieron tratados con estatinas, y los pacientes ecuatorianos no.

CURRICULUM VITAE

Lucy Baldeón Rojas was born on July 18th, 1977 in Quito, Ecuador. She grew up with her parents Gerardo and Lidia, a sister (Alicia) and a brother (Coqui). She attended secondary school at Manuela Cañizares High School, Quito, Ecuador.

She started her study in medicine in August 1995 at the Central University of Ecuador (UCE), Quito, where she was an immunology assistant for 5 years. She obtained the degree of Doctor in Medicine and Surgery from the UCE in 2002, and graduated with honors (cum laude). After that she did a "social medical year" (obligatory in Ecuador) working as general physician in a rural area in a small town (El Carmen) in the cost region of Ecuador. This involved a research project entitled "Dose-response trial of prophylactic zinc supplements, with or without copper, in young Ecuadorian children at risk of zinc deficiency".

From 2004 until 2006 she studied English at the Fulbright Commission to obtain the English proficiency diploma.

In 2006 she won a scholarship to follow Internal Medicine specialization at UCE; however, in the same year she also won a SENESCYT scholarship to follow a Biomedical Master Program at the Free University (Vrije University, VU), Amsterdam, The Netherlands. At the VU she graduated as a Master in Immunology and Infectious Diseases.

In September 2008 she started her PhD under the supervision of Dr. Pieter Leenen and Prof. dr. Hemmo A. Drexhage at the Department of Immunology, Erasmus University Medical Center, Rotterdam, The Netherlands. The research project she worked on was entitled "Defective endothelial progenitor cell development in diabetes: microRNA profile as biomarker for skewed myeloid differentiation" and was financed by the Dutch Diabetic Foundation. Part of the work on patients has now been published and reported in this thesis. The animal research will be published in the coming years.

She returned to Ecuador in 2013. In March 2014, she obtained a position as docent-researcher at the Central University of Ecuador (Universidad Central de Ecuador), teaching Immunology. Together with prof dr. Hemmo A. Drexhage (PROMETEO, SENESCYT, 2014-2016) she holds a grant to conduct a research project in Quito entitled "Immune Inflammatory Alterations in MetS and T2D and its Mental and Cardiovascular complications".

She happily lives with her son Gabriel (who was born in the Netherlands) in Quito, Ecuador.

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A

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PHD PORTFOLIO

Name PhD student:	Lucy Baldeón Rojas
ErasmusMC departments:	Immunology
Research school:	Molecular Medicine
PhD period:	2008-2013
Promotors:	Prof. dr. H.A. Drexhage
Co-promotors:	Dr. P.J.M. Leenen

1. PHD TRAINING

		Workload	
General courses		***Hours***	***ECTS***
2008	Course on Neuro-Immuno-Endocrinology	40	1.4
2009	Molecular Immunology	64	2.3
2009	Couse on Laboratory Animal Science	120	4.3
2009	Biostatistics for clinicians	120	4.3
2010	The course Molecular Diagnostics V	32	1.1
2010	The Photoshop CS3 Workshop for PhD-students and other researchers	16	0.6
2010	The Basic Introduction Course on SPSS	16	0.6
2011	The Workshop Writing Succesfull Grant Proposals	16	0.6
		8	0.3
Specific courses			
2010	The Workshop on Basic Data analysis on gene expression arrays V (BAGE)	16	0.6
2010	Analysis of microarray gene expression data	32	1.1

		Workload	
Seminars and workshops		***Hours***	***ECTS***
2008-2013	Department journal club	120	4.3
2013	JDRF Scientists Meeting. T1D Scientsist meeting	8	0.3
International conferences			
2012	48th Annual Meeting Diabetes EASD, Berlin (poster presentation)	40	1.4
2013	II Foro de Estudiantes Ecuatorianos en Europa, Milan (oral presentation)	16	0.6
2013	IL-1 mediated Inflammation and Diabetes Meeting (oral presentation)	16	0.6

2. TEACHING

2009-2013	Supervisor histology and immunology workshops for 1st and 2nd year medical students	130	4.6
2010-2012	Supervisor of master-student internship	80	2.9
TOTAL		**874**	**32**

PUBLISHED ARTICLES

1. **Lucy Baldeón Rojas**, Karin Weigelt, Harm de Wit, Behiye Ozcan, Adri van Oudenaren, Fernando Sempértegui, Eric Sijbrands, Laura Grosse, Anton-Jan van Zonneveld, Hemmo A. Drexhage, Pieter J.M. Leenen. Type 2 Diabetes Monocyte MicroRNA and mRNA Expression: Dyslipidemia Associates with Increased Differentiation-Related Genes but Not Inflammatory Activation . PLoS ONE. 2015 10(6): e0129421. doi:10.1371/journal.pone. 0129421.
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ARTICLE IN SUBMISSION

1. **Lucy Baldeón Rojas**, Karin Weigelt, Harm de Wit, Behiye Ozcan, Adri van Oudenaren, Fernando Sempértegui, Eric Sijbrands, Laura Grosse, Anton-Jan van Zonneveld, Hemmo A. Drexhage, Pieter J.M. Leenen. A study on inflammation-related genes and microRNAs, with special emphasis on the vascular repair factor HGF and miR-754-3p, in monocytes and serum of patients with T2D Diabetology & Metabolic Syndrome. June 2015.