The MacroScreen Platform

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Capturing Cardiovascular Disease Inflammation In Vitro

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Chapter 1

General introduction and outline of this thesis

Cardiovascular disease

Cardiovascular disease (CVD) is currently the number one cause of death worldwide[1]. Over the last decade, CVD-related death has increased by 12.5%[2] and in fact, one out of three deaths worldwide is now attributable to CVD[1,3]. Globally, most CVD deaths can be explained by ischemic heart disease (49.8%) and stroke (35.3%), followed by hypertensive heart disease, atrial fibrillation and peripheral vascular disease[1]. The major underlying cause for the development of CVD is atherosclerosis, the buildup of lipid-loaded plaques in the inner lining of an artery[4]. Atherosclerotic plaques can ultimately rupture and lead to the formation of a blood clot, resulting in the obstruction of blood flow in heart (ischemic heart disease), brain (stroke) or peripheral tissue (peripheral artery disease). CVD has become an obvious economic burden, as the European Heart Network has estimated the total CVD-related costs to be €210 billion per year[5]. Next to the costs generated by the healthcare of CVD patients (healthcare, medication, hospitalization) and indirect costs (patient work productivity loss), there are also costs associated with cardiovascular disease research[6]. Between 2010 and 2012, around €876 million was attributed to CVD research projects[6], all trying to better understand and predict the development of CVD. However, current prediction models often underestimate and overestimate CVD risk in high-risk profiles and low-risk profiles, respectively[7].

In the below paragraphs we will address on the main CVD risk factors and risk predictors/calculators. Next, we will discuss the ontogenesis of atherosclerosis, the main cause of CVD, with special emphasis on the role of the immune system in this disease. Lastly, we will address the use of a new high throughput analytical technique to study the immune system (mainly focusing on macrophages) in the development and prediction of CVD.

CVD risk factors

Before the 20th century, there was no clear way to predict the clinical consequences of plaque rupture and the only focus of CVD research was the treatment options. From 1950 onwards, several epidemiological studies have enabled a change in the research focus, aiming for better definition of accurate risk factors to use in risk prediction and disease prevention, rather than only treatment[1,8,9]. Nine main risk factors, including age, gender, dyslipidemia, blood pressure, smoking pattern, obesity, diabetes mellitus[10] and accounting for more than 90% of myocardial infarction (MI) and stroke, have been described by the INTERHEART[1,11] and the

INTERSTROKE[1,12] case-control studies, respectively. The Framingham heart study, which started in 1948, investigated the life-time risk of coronary heart disease (CHD) and established the Framingham Risk Score, reflecting the 10-year risk of developing CHD based on several risk factors, including age, sex, cholesterol levels, blood pressure and smoking pattern. In the INTERHEART study, dyslipidemia was the main individual predictor for the development of MI[11], whereas hypertension was the most important risk factor for stroke in the INTERSTROKE study[12]. Most individuals in the general population have at least one CHD risk factor, and CHD event risk score increases greatly in individuals having more than one risk factor[13–15]. Of all CVD risk factors, the five leading modifiable ones (dyslipidemia, hypertension, smoking pattern, obesity, and diabetes) account for around 50% of CVD-related death[16]. Below the latter risk factors will be discussed in more detail.

Dyslipidemia

Dyslipidemia is defined as an abnormal blood lipid concentration, including high triglycerides (TG) level, low high-density lipoproteins cholesterol (HDL) and elevated total (TC) or low-density lipoprotein (LDL) associated cholesterol[17]. To be transportable in blood, TG and LDL bind to apolipoproteins to form lipoproteins. Increased levels of TC, LDL and Apolipoprotein B have all been associated with high CVD risk and reducing LDL levels with statin treatment was repeatedly shown to reduce the risk for CVD development[1,18–20]. More recently, treatment with proprotein convertase subtilisin/kexin type 9 (PCSK9) antibodies showed a 60% reduction of LDL concentration, resulting in a 15% decrease in cardiovascular events on top of statins[19,20]. Furthermore, there is genetic evidence that elevated blood LDL concentration associates with CVD. Indeed, familial hypercholesterolemia (FH) is a genetic condition where LDL levels are abnormally high due to mutations in genes such as APOB, LDLR or PCSK9[21]. Patients suffering from FH have a higher risk for the development of CVD[21]. On the other hand, HDL is strongly and inversely correlated to CVD but clinical trials raising plasma HDL concentration so far failed to reduce CVD burden[22]. Next to the conventional aforementioned lipids measured to determine CVD risk, novel analytical techniques have allowed the identification and measurement of other lipid species that are potentially more specific for the prediction of CVD[23]. The lipidomics field has allowed the detection of other lipid classes independently linked to the development of CVD[24,25], as well as the total lipidome profile[26–29].

Hypertension

High blood pressure is widely accepted to be one of the strongest risk factor for CVD[2,30]. Hypertension accounts for 54% of stroke and 47% of ischemic heart disease worldwide[31]. Elevated systolic blood pressure is the best predictor of CVD in individuals over 50 years old[32], while both high systolic and diastolic blood pressure can be used as predictors in youngers individuals[33]. Furthermore, the benefit of antihypertensive therapies have been extensively acknowledged in several randomized controlled trials[34,35]. Indeed, reduction of 10 mm Hg by five different blood pressure lowering drug led to 20% risk reduction for major CVD events[36].

Smoking pattern

Smoking is a strong, modifiable risk factor for CVD. According to the WHO, 1.1 billion people worldwide were smoking in 2015[37]. In the INTERHEART study, current smoking was one of the strongest risk factors for the development of acute myocardial infarction (MI), accounting for 36% of the risk[11]. Moreover, there was a linear relationship between the number of cigarettes smoked per day and the odds of developing MI[11]. Duration of smoking is also an important determinant for CVD risk. Smoking fewer cigarettes/day for a longer time is associated with higher CVD risk than smoking more cigarettes/day for shorter time[38]. Smoking is a stronger risk predictor in women than in men, increasing CVD risk by six-fold versus three-fold, respectively when compared to never-smokers[39]. Moreover, cessation of smoking for one year already leads to a 50% risk reduction, which is further decreased after two years, to reach risk levels of non-smokers[40,41].

Obesity

Over the last 40 years, the prevalence of hypertension, smoking behavior and blood lipid levels have substantially reduced worldwide (31 to 15%, 39 to 26% and 34 to 17%, respectively)[42–44]. On the other hand, obesity prevalence almost tripled since 1975[45,46] and this increase was associated with a rise in (diagnosed) type 2 diabetes mellitus (T2DM) prevalence[42]. Obesity is defined as a Body Mass Index (BMI) ≥ 30 kg/m^2 and is subdivided in three classes, class I (BMI= 30-34.9, mild obesity), class II (BMI=35-39.9, moderate obesity) and class III (BMI≥ 40, severe obesity). According to the World Health Organization (WHO) in 2016, 39% of adults were overweight (BMI≥25), of which 13% were obese, reaching 650 million individuals[45]. Since 1975, obesity prevalence has nearly tripled and is expected to reach 21% by 2025 if this trend continues[45]. Obesity has been repeatedly shown to be an

independent risk factor for the development of CVD, as judged its correlation to CVD in absence of any other CVD risk factor[47].

Obesity has direct and indirect effects on CVD risk, mediated by a functional adaptation of the fat mass and the effects on other CVD risk factors. Regarding the former, obese individuals have excessive fat mass, also known as adipose tissue, and this accumulation of fat mass is directly linked to the development of atherosclerosis[48]. In lean individuals, a series of hormones, most commonly known as adipokines, are secreted by adipose tissue [47,49]. Adipokines are involved in glucose and lipid metabolism, inflammation and coagulation pathways and can either be pro- or anti-atherogenic[50]. During obesity, this adipose tissue secretion pattern is dysregulated and shifts toward a pro-atherogenic phenotype, where adipokines such as leptin, TNF-α, IL-6 and MCP-1 are overexpressed and contribute to the low-grade inflammatory state observed in obesity[49,51]. These pro-inflammatory adipokines have been positively associated to the development of CVD[52]. On the other hand, major cardioprotective adipokines including adiponectin, omentin and apelin, are downregulated during obesity and hence negatively correlated to the development of CVD[50,52]. Apart from this direct association, obesity is also indirectly linked to CVD due to its strong relationship with other CVD risk factors, such as dyslipidemia, hypertension and T2DM[53]. In 2017, more than 80% of T2DM cases were overweight or obese[54].

Diabetes

Diabetes Mellitus (DM) is a well-known risk factor for CVD, as it is independently associated with a two to four-fold increased risk for CVD[55]. DM prevalence keeps on increasing, affecting 382 million individuals in 2014[56]. The relationship between DM and CVD is complicated, as other CVD risk factors, such as obesity, hypertension and dyslipidemia, are often observed in patients with DM. Diabetes is closely linked to obesity, as one kilogram weight gain increases the risk to develop diabetes by 4,5 to 9%[57,58]. Furthermore, the prevalence of obese among T2DM patients ranges from 60% to 90% of all T2DM patients[54]. Glycemic control drugs have shown to help reduce CVD[59], however this effect was reported to be modest and mostly due the consequences on other risk factors[56,59,60].

Cardiovascular risk assessment

To assess the most effective treatment for high risk CVD patients, clinicians often use CVD risk calculators. To date, there are over 100 cardiovascular risk calculators

available[61] which are all based on the aforementioned traditional CVD risk factors. However, only 25 of these CVD risk prediction models have been externally validated in an independent cohort[61,62]. The most widely known CVD risk calculator is the Framingham risk score, derived from the Framingham study (FRS) in 1991. The FRS has been validated in 35 US cohorts, with area under the curve (AUC, or prediction power value) ranging from 0.60 to 0.83 and in 68 European populations with an AUC ranging from 0.53-0.88[62]. However, comparison between 25 different risk calculators in a similar population only revealed a 67% agreement between models[63]. This shows that the output of risk calculators markedly differs, depending on the population used to generate the score, but also on the clinical endpoints and the length of the calculated risk. Together, this emphasizes that CVD risk assessment for every individual patient should be done with a risk calculator, derived from a representative population, matching with the patient in question.

Atherosclerosis: definition

Atherosclerosis is the main underlying cause for CVD[8], and it is defined as a chronic, progressive disease typified by the accumulation of inflammatory cells, lipids and fibrous tissue in the inner wall of a medium to large sized artery. This so-called atherosclerotic plaque can ultimately rupture and lead to the formation of a blood clot, which in turn will restrict blood flow to a specific organ, such as the brain or the heart. Atherosclerosis is not a modern disease, as it has been observed in 4000 year-old mummies[64,65]. It was recognized for the first time in the beginning of the 19th century, by the French pathologist Jean Lobstein[66]. Initially, atherosclerosis was thought to be a lipid-driven disease, as cholesterol was observed in the plaques[67]. However, the presence of inflammatory cells in the lesion led to the hypothesis that atherosclerosis was not only a cholesterol storage disease[68]. Rudolf Virchow was the first one to explain the role of inflammatory cells in atherogenesis[66]. The implication of inflammation in the development of atherosclerosis was recently demonstrated in the Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS). The CANTOS trial is a double-blind trial including patients who already suffered a MI and with elevated levels of systemic inflammation (CRP> 2 mg/ml), despite statin therapy[69]. Patient groups who received 150mg of canakinumab, an interleukin-1 Beta (IL-1β) monoclonal antibody, on top of statin treatment and every three months had a significant lower risk for CVD[69]. However, these patients had higher incidence of infection and there was no significant difference in all-cause mortality[69].

Atherosclerosis development

The endothelium, the inner cellular layer of blood vessels, is an almost impermeable barrier between blood vessels and tissues. Upon stress (e.g. low-shear stress, hyperglycemia or inflammation), endothelial cells (ECs) become dysfunctional and the barrier integrity is weakened, leading to leakage, amongst others[70]. The process of atherosclerosis begins with excessive retention of low-density lipoprotein (LDL) in the artery's sub-endothelial space[8]. LDL is in turn biochemically modified within the arterial wall and activates the ECs. Activated ECs attract and capture blood monocytes via the expression of adhesion and chemoattractant molecules (such as vascular cell adhesion molecules (VCAMs) and chemokines) in attempt to clear the modified LDL. These monocytes enter the sub-endothelial space and differentiate into macrophages, which take up the modified LDL and enhance the inflammatory response[71]. These lipid-laden macrophages, called foam cells, accumulate in the lesion and undergo programmed cell-death, apoptosis, due to this lipid overload. One of the main functions of the macrophage is to clear dead cells under physiological conditions, a process called efferocytosis[72]. However, in more advanced lesions, the efferocytosis process is defective and the non-cleared apoptotic cells will become necrotic. Together this leads to the formation of the plaque necrotic core, which consists of apoptotic cell debris and lipids[73].

In addition, smooth muscle cells (SMCs), normally located in the medial layer of arteries, migrate to the intima and proliferate around the plaque in response to cytokines secreted by activated plaque macrophages. They form the fibrous cap by secreting extracellular matrix protein, such as collagen and proteoglycans[74]. The fibrous cap stabilizes the plaque and protects it from rupturing; however, as disease progresses, the fibrous cap becomes thinner and can ultimately rupture. Indeed, plaque macrophages secrete matrix metallo-proteases (MMPs) which degrade collagen, and subsequently the fibrous cap. Furthermore, SMCs undergo cell death due to the plaque inflammation and uptake of oxidized lipids and rupture-prone regions are often observed where there is a reduced number of SMCs and fibrosis, accompanied by an increased number of macrophages.

Immune cells in atherosclerosis

Next to macrophages, SMCs and ECs, plaques contain a broad range of immune cell types, which are involved in the initiation and progression of atherosclerosis, such

as neutrophils, dendritic cells and lymphocytes[75]. The onset of the disease is characterized by stimulation/activation of the innate immune system, which is subsequently followed by adaptive immune responses. The innate immune system is the first line of defense against pathogens such as viruses and bacteria. It acts rapidly and is non-specific. Micro-organisms express pathogen associated molecular pattern (PAMPs), which are recognized by surface receptors on innate immune cells (e.g. macrophages, neutrophils and dendritic cells) and engulfed. However, the innate immune system is not always able to eliminate the pathogens and, in that case, the adaptive immune response is activated. The adaptive immune response is slower but target-specific and involves antigen-presenting cells. These cells will activate several effector T-cell and B-cell types. In turn, the effector cells will produce specific "antigen receptors" or antigen specific antibodies, to clear the pathogens.

In atherosclerosis, both the innate and the adaptive immune responses play pivotal roles. Next to macrophages, other innate immune cell types are recruited to the atherosclerotic lesion, including neutrophils and dendritic cells[76]. Neutrophils accumulate in the lesions and their number positively correlates to plaque size[77,78]. They have been described to produce Neutrophil Extracellular Traps (NETs) to prime macrophages to produce inflammatory cytokines, resulting in increased atherogenesis[79]. Dendritic cells, on the other hand, act as bridge between the innate and the adaptive immune response by presenting antigens to adaptive immune cells. In healthy tissues, DCs are found in atherosclerotic-prone lesions and their number increases during the development and progression of the disease[80]. Furthermore, in advanced lesions, DCs localize to rupture-prone regions[81].

Similarly, T and B lymphocytes, as part of the adaptive immune response, have been observed in the plaque[82]. There are different kind of T-cell and B-cell subsets and this is reflected in their respective role during atherosclerosis. Indeed, the high heterogeneity of T- and B- cells allows them to have both pro- and anti-atherogenic phenotypes[83].

Furthermore, mast cells are also found in atherosclerotic plaques[84,85]. *In vivo* studies have shown that activation of mast cells led to an exacerbation of atherosclerosis formation and, inversely, inhibition of mast cells activation showed a reduction of atherosclerosis formation[85].

Lastly, Natural Killer (NK) and NK T cells have also been implicated in atherosclerosis. NK cells are mostly observed in unstable plaque, where they have been shown to induce apoptosis and hence associate with plaque progression and rupture[86]. In addition, NKT cells also show a pro-atherogenic function, as mice lacking NKT type I and type II cells have a reduction in atherosclerotic plaque formation[87].

Macrophages in acute myocardial infarction, and post-infarct repair

Aside from their important role in atherosclerosis, macrophages are also instrumental to another aspect of cardiovascular inflammation: post myocardial infarction repair. Following MI, cardiac remodelling takes place and can be divided into two phases, including an acute inflammatory reaction, followed by fibrosis and scar formation[88,89]. Both processes of the disease are tightly regulated by macrophages. In response to MI, monocytes infiltrate the myocardium, where they differentiate into macrophages and closely collaborate with cardiac resident macrophages to regulate cardiac repair[90]. In the early post-infarct phase, cardiac macrophages were observed to influence the pro-inflammatory response, by releasing a broad range of pro-inflammatory cytokines, such as IL-6 and TNF-α[91]. Contrarily, in the later post-infarct phases, cardiac macrophages were shown to enter a pro-repair phase, exhibiting increased phagocytic and proliferative capacity and this was further followed by a scar formation phenotype, where macrophages increased their expression of extracellular matrix remodelling genes[88].

Macrophages heterogeneity

As mentioned previously, macrophages are the most abundant cell type in atherosclerosis and play a central role in ischemia-reperfusion injury and healing in the heart. Currently, macrophages are divided into two main species: tissue resident macrophages, which are primarily of embryonic origin, and inflammatory macrophages, which are derived from blood monocytes and recruited to sites of inflammation/infection[92,93]. All macrophages share one important characteristic: they are extremely plastic and adapt extraordinarily well to their micro-environment. This high plasticity results in multiple macrophage phenotypes exerting a large variety of functions[94–96], such as controlling tissue homeostasis, as well as driving inflammatory processes, including phagocytosis, apoptosis, etc[94,97]. In addition, tissue-resident macrophages can exhibit tissue-maintenance functions

such as cellular debris clearance or immune surveillance, generally upon instruction by their microenvironments[92,94,96,98].

Over the last years, extensive research has been conducted to understand how macrophages respond to various stimuli. It was first believed that macrophages can polarize into two main phenotypes, M1 or the pro-inflammatory subtype and M2 or the anti-inflammatory subtype. However, recent evidence suggests that this M1/M2 paradigm is an oversimplification of macrophage polarization[99]. Indeed, the M1/M2 scheme is now seen as the two extremes of a multi-dimensional macrophage polarization spectrum[100]. Inflammatory stimuli, such as interferon-γ (IFNγ) or lipopolysaccharide (LPS), can induce a pro-inflammatory response in macrophages (classically activated/M1-macrophages), resulting in production and secretion of pro-inflammatory cytokines, such as IL-1β IL-6, IL-12 or tumour necrosis factor (TNF). On the other hand, cytokines such as interleukin-4 (IL-4) or IL-13 will polarize macrophages towards an anti-inflammatory phenotype (alternatively activated-/ M2-macrophages) and the macrophages will, in turn, produce anti-inflammatory molecules, such as IL-10[101].

Macrophage transcriptional plasticity has been widely studied; however, the functional implications of macrophage heterogeneity have been barely touched upon. Several key questions remain unanswered. To mention a few: (1) Do macrophages have distinct functions depending on the mode of activation? (2) Do they sense and respond differently to the surrounding micro-environment in disease and is this reflected by their functions? (3) Is the transcriptional plasticity of the macrophages mirrored by their functional phenotype? and (4) Can we build a functional map of macrophage plasticity that could serve as reference to interpret macrophages functional profile?

Using recent technological advances, it is now possible to study the macrophage heterogeneity at the functional level. The next section will discuss how high content analysis can help in understanding macrophage functions in atherosclerosis and CVD risk assessment.

High content analysis of cell function

The term high-content analysis (HCA) defines a technique used to image and measure complex cellular activity. It combines automated microscopy with advanced analytic tools to extract multi-parametric data of single cells. HCA is based

on fluorescent microscopy; generally, fluorescent probes are added to cells in culture and subsequent biological activity can be measured[102]. HCA technology provides multiple advantages over conventional functional screening. The latter were generally done in single tube format, and hence were labour intensive, slow and required large volumes of cell culture medium. Furthermore, the data analysis using HCA technologies allows rapid extraction of numerous features at the single-cell level. HCA makes it possible to simultaneously screen numerous variables in a semi-high throughput manner, at up to 384 (or even 1536) well plate format. Moreover, need for cell culture media, probes, cells and compounds/stimuli are therefore minimized, and thus costs. Also, all live measurements over time will not only give quantitative, but also spatiotemporal information, often not considered in functional analysis.

Over the last years, HCA technology has evolved, and imaging software solutions and microscopes nowadays enable automation and contain a robotic system. The development of easy-to-use image analysis tools have allowed scientists to extract a high number of features. HCA workflow is typically composed of the following standardized steps: (1) (Un)stimulated cells are stained for a special functional phenotype, the 96/384-well format plate is then placed in an automated fluorescent microscope that will measure in each well the exact same parameters, without changing the settings. (2) Once the acquiring is done, the data analysis starts. (3) Images are transferred in an image analysis tool, where each picture containing thousands of cells will be segmented. Digital segmentation algorithms detect and locate each cell or sub-cellular component and turn them into one single digital data point. (4) Following segmentation, interesting features are extracted. Features range from fluorescent signal intensity or percentage of positive cells, to shape/morphology of the signal.

HCA usually generates large data files of extracted features and the files are subsequently analysed using machine learning. Unsupervised and supervised machine learning help to extract phenotypic appreciations that were not necessarily referring to the first experimental question. Furthermore, machine learning algorithms help at integrating HCA data with other kind of data, such as phenotypic, proteomics or transcriptomics data, to build *in silico* models of cellular functions (**Figure 1**).

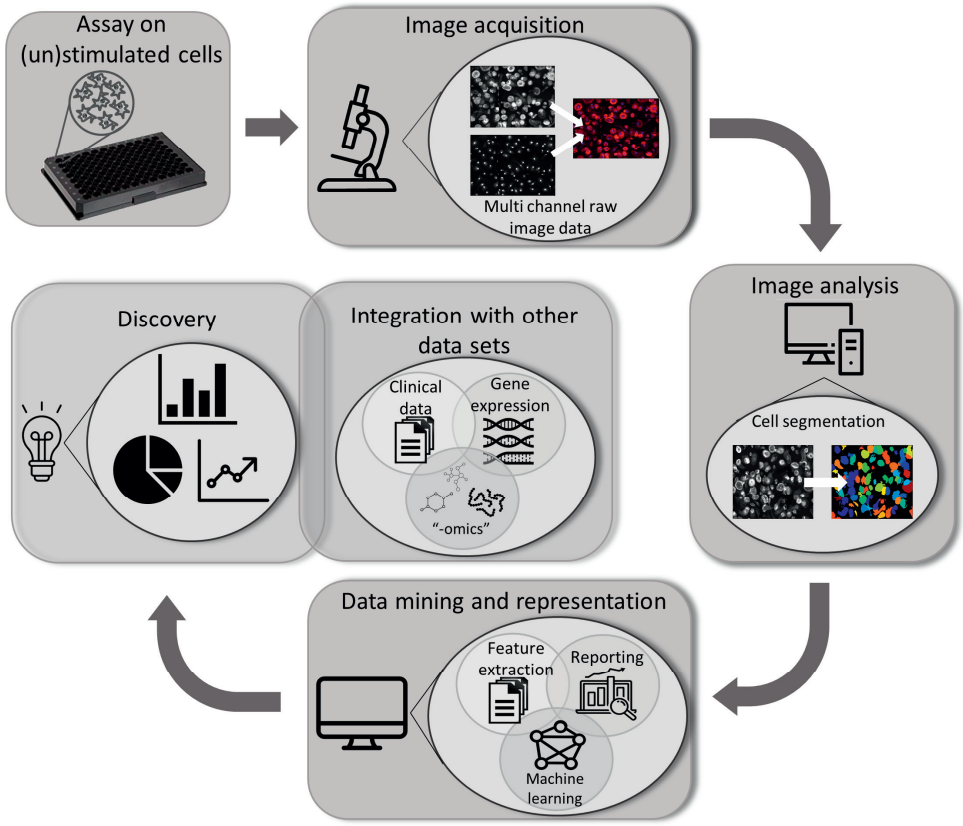

Figure 1. Standardized HCA pipeline
Fluorescent functional assays are performed on (un)stimulated cells, which are imaged with an automated microscope. Each cell will become an individual data point during the cell segmentation step. Data will be extracted using powerful computational software solutions. Relevant information will be analysed, represented and integrated to other types of data sets.

HCA was first developed in the 1980s by Pfizer for a drug screening purpose, generating new antibiotics[103]. Nowadays, it is not only used for high-throughput drug screening purpose, but also to image and analyse, in an unbiased way, general or specific cellular functions. In the past 15 years, biomedical research has increasingly embraced the use of HCA technologies and several genomic studies made use of HCA, where thousands of genes were knocked-out (KO) in a specific cell type and the subsequent morphological changes were analysed[104]. In 2005, Ohya *et al.* studied the yeast morphological phenotypes of 4,718 KO genes and were able to identify 2,378 candidate genes showing morphological differences in

at least one of the 254 parameters measured[105]. In the years that followed, HCA technologies were further applied to study the link between genes and functional profiles in several cell types and to build genomic atlases for a variety of functions, such as cell division[106], cell migration[107], or endocytosis[108]. More recently, technological advances in cell culture and imaging methods enabled us to study three-dimensional (3D) structures, also known as organoids, using HCA. Organoids are made of stem cells, organ progenitor cells or tissue derived cell suspensions grown with extracellular matrix material. By enabling cell-cell interaction, spatial organization of cells, organ-like functional diversity, they mimic the *in vivo* situation better than classical 2D cell monolayers[109] and have been commonly used in HCA cancer research[110–112]. Furthermore, HCA can be combined with confocal microscopy to provide a better resolution and image contrast during 2D/3D screening. Altogether, HCA technologies are great tools to accurately study cellular functions at single cell level. The next section will define the specific aim and hypothesis of this thesis and will explain how we plan to use HCA techniques to study macrophage functions in the context of atherosclerosis and AMI repair.

Aim and hypothesis of this thesis

Summarizing the above, macrophages are the most abundant cell type in atherosclerotic plaque and are implicated in its development at all stages of disease. Furthermore, they play a pivotal role in AMI repair. They are well known to be transcriptionally and phenotypically plastic and will respond profoundly to their (micro-)environment. In this thesis, we aim to build a functional map of macrophages by using a high content analysis system *in vitro* to study macrophage functions in CVD inflammation, including atherosclerosis, acute myocardial infarction and cardiometabolic disorders. This map could help to understand the role of macrophage in health and disease.

We hypothesize that macrophage transcriptional plasticity is reflected in their functional profile and that macrophages can sense their micro-environment and respond accordingly.

To test this, we will:

(1) Build a high content analyser platform (the MacroScreen platform) using human primary macrophages to define a macrophage functional profile map *in vitro* (**chapter 2**)

(2) Use the platform to analyse the response of macrophages to a cardiovascular disease environment, using two cohorts of acute myocardial infarction (**chapter 3**)
(3) Validate the platform in a mouse study focusing on apoptosis and atherosclerosis (**chapter 4**)
(4) Use the platform to study the effect of newly discovered biomarkers (bioactive lipids) for the development of obesity-related CVD (**chapter 5**).

Outline of the thesis

Macrophages are the most abundant cell type in atherosclerosis. Their transcriptional heterogeneity has been widely studied; however, a systematic study of their phenotypical plasticity is lacking. In **chapter 2**, we set out to build a "functionomics" platform (the MacroScreen), enabling us to detect several macrophage functions relevant for atherosclerosis and to generate a function-based atlas of human monocyte derived macrophage responses to a variety of established stimuli *in vitro*.

This functional map served as reference for a subsequent study, where we mapped the functional impact of the systemic status of patients that suffered an acute myocardial infarction (AMI), on human monocyte derived macrophages. Such study could be particularly relevant as macrophages are key players not only in atherosclerosis but also in AMI repair[90,113], and disease compromised functions may thus have direct implications for disease prognosis after infarct. **Chapter 3** investigates whether our functionomics platform can pick up any functional changes in macrophages exposed to the serum environment of AMI patients, whether these functional changes are reflected in their transcriptional profile, and if specific functional or transcriptional patterns are associated with a poor prognosis.

Chapter 4 describes the use of the MacroScreen platform for dissection of causal mechanisms in mouse studies of atherosclerosis. Macrophage apoptosis is an important process during atherosclerosis at all stages of the disease but this process can be beneficial or detrimental, depending on plaque stage[114,115]. In this HCA valorization study, we investigated the effects of a key regulator of myeloid apoptosis, the anti-apoptotic protein Mcl-1, on macrophage functions, including apoptosis, underpinning the benefits of the HCA platform for mechanistic *in vitro* studies.

In **chapter 5** the MacroScreen platform is deployed in a different setting. As described in the introduction, obesity is associated with CVD events, however not all obese individuals will develop CVD overtime. Conventional classifiers for CVD risk lack precision, and are generally not accurate at all within the obese population, thus more predictive biomarkers to stratify the obese individuals are eagerly awaited. In this chapter, we explored lipids for their potential to predict CVD risk in the obese population. After the discovery of novel biomarkers, we studied their causal relationship with macrophage function using our functionomics platform.

A general discussion of the main findings of this thesis is presented in **chapter 6**, also including future perspective and applications of our functionomics platform.

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Chapter 2

Towards a function-based model of macrophage plasticity: the MacroScreen platform

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In preparation

Chapter 3

The systemic acute myocardial infarction environment reprograms human macrophages

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Chapter 4

Low human and murine Mcl-1 expression leads to a pro-apoptotic plaque phenotype enriched in giant-cells.

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Abstract

The anti-apoptotic protein myeloid cell leukemia 1 (Mcl-1) plays an important role in survival and differentiation of leukocytes, more specifically of neutrophils. Here, we investigated the impact of myeloid Mcl-1 deletion in atherosclerosis. Western type diet fed LDL receptor-deficient mice were transplanted with either wild-type (WT) or LysMCre Mcl-$1^{fl/fl}$ (Mcl-$1^{-/-}$) bone marrow. Mcl-1 myeloid deletion resulted in enhanced apoptosis and lipid accumulation in atherosclerotic plaques. *In vitro*, Mcl-1 deficient macrophages also showed increased lipid accumulation, resulting in increased sensitivity to lipid-induced cell death. However, plaque size, necrotic core and macrophage content were similar in Mcl-$1^{-/-}$ compared to WT mice, most likely due to decreased circulating and plaque-residing neutrophils. Interestingly, Mcl$1^{-/-}$ peritoneal foam cells formed up to 45% more multinucleated giant cells (MGCs) *in vitro* compared to WT, which concurred with an increased MGC presence in atherosclerotic lesions of Mcl$1^{-/-}$ mice. Moreover, analysis of human unstable atherosclerotic lesions also revealed a significant inverse correlation between MGC lesion content and Mcl-1 gene expression, coinciding with the mouse data. Taken together, these findings suggest that myeloid Mcl-1 deletion leads to a more apoptotic, lipid and MGC-enriched phenotype. These potentially pro-atherogenic effects are however counteracted by neutropenia in circulation and plaque.

Introduction

Antiapoptotic Mcl-1 is a member of the apoptosis regulating Bcl-2 family[1]. It directly interacts with pro-apoptotic BH3-only proteins Bim and Bid and multidomain proapoptotic Bad[2–4], thereby inhibiting apoptosis. Mcl-1 is expressed in various tissues including hematopoietic cells[5], in which its overexpression delays cell death in response to various stimuli[6]. Indeed Mcl-1 is critical for neutrophil survival[7–10] at all differentiation stages[11]. Survival of macrophages lacking Mcl-1 does not seem to be impacted[9], albeit that Mcl-1 deficient macrophages were observed to be more sensitive to apoptosis induced either by phagocytosis[10], or infection[12].

While monocytes and macrophages have been the subject of extensive studies in the atherosclerosis field for years[13], neutrophils were only more recently investigated due to their scarce presence in atherosclerotic plaque. Nevertheless, it is now believed that neutrophils are instrumental in plaque development and later destabilization, as we and others have shown[14–16]. Warnatch *et al.* demonstrated that Neutrophil Extracellular Traps (NETs) prime macrophages to produce inflammatory cytokines, resulting in increased atherogenesis[17]. Dissection of the contribution of neutrophils to atherosclerotic plaque progression has however remained difficult, since other cell populations are also affected when neutrophils are experimentally manipulated. Deletion of Interferon Regulatory Factor 8 (IRF8) for example results in neutrophilia which exacerbates atherosclerosis in IRF8$^{-/-}$ ApoE$^{-/-}$ chimeric mice, but was at the same time accompanied by changes in monocytes and several dendritic cell populations[18,19]. In addition, neutrophils have thus far not been depleted during the complete course of atherosclerosis. Neutrophil depleting antibodies such as the anti–polymorphonuclear leukocyte (PMN) antibody cannot be sustained for more than 4 weeks in mice without affecting monocyte counts[16].

As Mcl-1 is vital for neutrophil survival, whilst presumably having mild effects on macrophage apoptosis, we hypothesized that myeloid Mcl-1 deletion could serve as an efficient neutropenia model during the full pathogenesis of atherosclerosis. Moreover, although several Bcl-2 family members have been investigated in the context of atherosclerosis[20–23], the role of Mcl-1 in disease progression has not been assessed thus far. To this end, we studied effects of specific deletion of Mcl-1 in the lysozyme M expressing myeloid subsets neutrophils and macrophages on early and advanced atherosclerosis using bone marrow transplantation of Mcl-1$^{fl/fl}$ LysMCre or wild-type (WT) bone marrow into low density lipoprotein receptor-null (LDLr$^{-/-}$)

mice. Our study shows that myeloid Mcl-1 deletion indeed has a profound impact on neutrophil survival. However, Mcl-1 deficient macrophages were seen to accumulate more lipids *in vitro* and *in vivo* and were subsequently more sensitive to apoptosis. Moreover, we show that Mcl-1 is implicated in the fusion of macrophages. These combined effects however counteracted each other affecting only viable cell plaque composition, but not plaque growth.

Material and methods

Animals

All animal work was approved by animal regulatory authority of Leiden University and performed in compliance with Dutch national guidelines. Low density lipoprotein receptor-null (LDLr$^{-/-}$) mice were obtained from the local animal breeding facility. Mcl-1$^{fl/fl}$ LysMcre mice were obtained from the Department of Immunology, Duke University Medical Center, USA and were backcrossed to C57BL/6J.

Mcl-1 gene expression during atherogenesis

Twenty male LDLr$^{-/-}$ mice were fed a Western-type diet (WTD) two weeks prior to surgery and throughout the experiment. Atherosclerotic carotid artery lesions were induced by perivascular collar placement[24]. Carotid Mcl-1 gene expression was analysed prior to and two, four, six and eight weeks after collar placement (n=4, per timepoint). The mice were anaesthetized and perfused with phosphate buffered saline (PBS) after which both common carotid arteries were isolated. After dissection of the adventitia, plaque containing segments were excised based on macroscopic examination, snapfrozen and stored at -80°c. Two to three atherosclerotic plaques were pooled per sample and total RNA was isolated using Trizol reagent (Invitrogen). Gene expression was analysed by real time PCR (qPCR) using ABI PRISM 7700 Sequence Detector (Applied Biosystems) with SYBR-Green technology. *Mcl-1* and housekeeping gene *Hprt* primers used are listed in supplementary table 1.

Mcl-1 gene expression in different cell types

Murine 3T3 fibroblasts, smooth muscle cells (SMCs), cardiac endothelial cells (MCECs), and BMDMs stimulated with either lipopolysaccharide (10 ng/ml) and Interferon-γ (IFN-γ, 100 U/ml) (M1 macrophage) or Interleukin-4 (IL-4, 20 ng/ml) (M2 macrophage) were cultured. Total RNA was extracted and gene expression was analysed by qPCR. *Mcl-1* and housekeeping gene *18S* primers used are listed in supplementary table 1.

Bone marrow transplantation and atherosclerosis induction

Male recipient LDLr$^{-/-}$ mice were housed in sterile ventilated cages with food (RM3, Special Diet Services) and water ad libitum. Antibiotics (83 mg/l ciprofloxacin, 67 mg/l Polymixin B and 5 g/l sugar) were supplied in the drinking water. Mice were

exposed to a single dose of 9 Gy total body irradiation (0.19Gy/min, 200 kV, 4 mA) using an Andrex Smart 225 Röntgen source (YXLON International) one day before transplantation. Bone marrow was extracted from femur and tibia of male Mcl-$1^{fl/fl}$ LysMcre (hereafter Mcl-$1^{-/-}$) donors and wild type (WT) littermates. Irradiated LDLr$^{-/-}$ mice received 2.5 x 10^6 BM cells of either Mcl-$1^{-/-}$ (n=17) or WT (n=15) via tail vein injection. After a recovery period of eight weeks mice were put on a WTD containing 0.25% cholesterol and 15% cacao butter (Diet W, Special Diet Services) for an additional five (plaque initiation) or ten weeks (advanced plaque).

Blood cell analysis and flow cytometry

Blood samples were taken by tail bleeding immediately before BMT, prior to (week 0) and after four weeks of WTD feeding (week 4) and at the time of sacrifice (week 5 or week 10). Peritoneal leukocytes were isolated at the time of sacrifice by peritoneal lavage with 10 ml PBS. Whole blood and peritoneal lavage samples were analysed using a Sysmex blood cell analyser (XT-2000i). For flow cytometry, white blood cells (WBC) and peritoneal leukocytes were stained with fluorescently labelled antibodies against F4/80, CD19, CD4, CD71 and CD11b (eBioscience) and Gr1, CD8 and CXCR4 (BD Pharmingen). Fluorescence-activated cell sorting (FACS) analysis was performed on a FACSCalibur with CellQuest software (BD Biosciences).

Tissue harvesting and analysis

Two hours before sacrifice, Mcl-$1^{-/-}$ or WT mice (n=5) received intraperitoneal injections of CXCL1 (200 ng/ml in 1 ml PBS) or PBS control. The mice were anesthetized and perfused with PBS. Cryosections of the aortic root tissue were stained with hematoxylin and eosin (HE) or Oil Red O (ORO). Lesion size was quantified using a Leica DMRE microscope with camera and Leica Qwin Imaging software (Leica Ltd). MGCs were defined as macrophages with two or more round nuclei on the HE slides and quantified by an animal pathologist. Immunohistochemical stainings were performed for macrophage (MOMA-2, Sigma) and vSMC (α-smooth muscle actin, Sigma) content. Apoptotic cell content was quantified using terminal deoxytransferase dUTP nick-end labeling (TUNEL) kit (Roche Diagnostics).

LDL and VLDL isolation and oxLDL preparation

LDL and very low-density lipoprotein (VLDL) were isolated from human plasma by density gradient ultracentrifugation for 20h at 4°C[25]. LDL concentration was adjusted to 0.5 mg/ml with PBS and oxidized by incubation with 0.32 mM $CuSO_4$

overnight at 37°C after which the oxidation reaction was terminated by addition of 50 µM EDTA. OxLDL was then dialyzed against PBS containing 10 µM EDTA for 24h. Final oxLDL and VLDL concentration were measured using the bicinchoninic acid protein kit (Thermo Fisher Scientific).

BMDMs and peritoneal macrophages

Bone marrow cells were isolated by flushing femurs and tibia with PBS and single cell suspensions were obtained by passing the suspension through a 70 µm nylon cell strainer (BD Falcon). Bone marrow cells were differentiated into macrophages by culturing in RPMI-1640 medium (GIBCO Invitrogen), supplemented with 10% (vol/vol) heat inactivated fetal calf serum (FCS) (GIBCO, Invitrogen), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 15% (vol/vol) L929-conditioned medium for seven days. After differentiation, BMDMs were harvested with lidocaine and replated at $0.5x10^5$ cells/well in a 96 well imaging plate (BD #353219, Corning Life Sciences) for apoptosis, lipid uptake and fusion assay. Peritoneal leukocytes were isolated from Mcl-$1^{-/-}$ and WT mice following an i.p. injection of PBS before sacrifice. Cells were pooled from 2-3 mice per genotype and plated at $0.25x10^6$ cells/well in 8 chamber culture slides or 24-well plates (BD Falcon) and non-adherent peritoneal macrophages were removed.

Lipid loading of BMDMs and peritoneal macrophages

Adherent peritoneal macrophages were stimulated with 20 µg/ml oxLDL or 50 µg/ml VLDL for 24h, after which slides were washed with PBS and stained with ORO. Lipid loading was quantified as the ratio between the ORO stained area and total cell surface. BMDMs were loaded with 20 µg/ml oxLDL or 50 µg/ml VLDL combined with 5 µg/ml and 12.5 µg/ml Topfluor cholesterol (Avanti Polar Lipids) respectively for 2.5h (D0) and 120h (D5). Cell nuclei were stained with Hoechst (Sigma Aldrich) at 15 µg/ml.

Apoptosis of peritoneal macrophages and BMDMs

Adherent peritoneal macrophages were stimulated with 40 µg/ml oxLDL for 24h. The macrophages were detached with Accutase (PAA Laboratories GmbH), stained with FITC-labeled Annexin-V (ImmunoTools) and propidium iodide (Sigma Aldrich) and subsequently analysed by flow cytometry (FACSCalibur, BD Biosciences). For the BMDM apoptosis assay, cells were loaded with 20 µg/ml oxLDL or 50 µg/ml VLDL for 6h (D0) or 120h (D5) and subsequently stained with Hoechst and Oregon-green or Alexa-647 labelled Annexin-V for 15 minutes[26].

Fusion assay

BMDMs were loaded with 20 µg/ml oxLDL or 50 µg/ml VLDL for either 24h (D1) or 120h (D5). Macrophage fusion was measured by co-staining with calcein-AM (ThermoFisher Scientific) at 1 µg/ml and Hoechst. MGCs were defined as having two or more nuclei per calcein-segmented cell.

Image analysis

For all 96-well plate immunofluorescence assays (apoptosis, lipid loading and cell fusion), nine images per well were taken using the high content analyser (HCA) BD pathway 855 (BD Biosciences) with 10x objective and further analysed using Attovision and DIVA software (BD Biosciences).

Human atherosclerotic plaque collection

Stable and unstable human carotid atherosclerotic plaques segments (classified according to Virmani *et al*.[27]) were collected from the same symptomatic patient (n=22/23) undergoing carotid endarterectomy in Maastricht University Medical Centre (MUMC, The Netherlands) and Zuyderland Medical Center (Sittard, the Netherlands). Collection, storage, and use in the Maastricht Pathology Tissue Collection (MPTC) were approved by medical ethical committee (16-4-181) and in accordance with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (http://www.fmwv.nl). Atherosclerotic plaque segments were alternatively snap-frozen for RNA and microarray analysis, or formalin-fixed for paraffin-embedding.

Human atherosclerotic plaque histology

The human plaque sections adjacent to the snapfrozen segment were classified to determine plaque type according to Virmani *et al*.[27]. HE staining was used to quantify plaque size, lipid core size and hemorrhage. Alizarin red staining was done to measure the percentage of calcification.

Human atherosclerotic plaque immunohistochemistry

All stainings were performed on adjacent plaque sections for vascular endothelial marker CD31 (Dako), macrophage marker CD68 (Dako), T-cell marker CD3 (Dako) and MGC marker (cathepsin K[28]). MGCs were defined as cathepsin-K positive cells having two or more round nuclei. MGC were quantified in all sections and averaged per patient. Relative abundance of MGCs was calculated by dividing the number of MGCs by that of CD68 positive cells.

Human atherosclerotic plaque RNA extraction and transcriptomics

RNA was isolated by Guanidium Thiocyanate lysis followed by Cesium Chloride gradient centrifugation, and then purified using the Nucleospin RNAII kit. 750 ng of biotinylated cRNA per sample was hybridized to Illumina Human Sentrix-8 V2.0 BeadChip® and washed according to the Illumina standard procedure. Scanning was performed on the Illumina BeadStation 500. Raw expression data were extracted from the images using default settings and without normalization.

Statistics

Values are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using Prism (GraphPad Software). Statistically significant differences ($p<0.05$) were evaluated using the Student's t-test unless stated otherwise. Pearson correlation analysis was performed to assess the association between Mcl-1 gene expression or MGCs presence and clinical plaque traits. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Results

Mcl-1 deletion altered neutrophil levels and characteristics

We first quantified Mcl-1 gene expression during atherogenesis. Mcl-1 levels in collar-induced carotid artery lesions of $LDLr^{-/-}$ mice gradually increased during lesion development and in particular in advanced plaques, six weeks after collar induction (**Figure 1A**). Of note, this increase was not validated at the protein level. Mcl-1 was mostly expressed in activated macrophages (M1- or M2-macropahges), as compared to other cell types (**Figure 1B**). Mcl-1 was also detectable in human atherosclerotic plaques, and its expression did not differ between stable and unstable plaque (**Figure 1C**). However, Pearson correlation analysis revealed that Mcl-1 expression did correlate with pathogenic plaque traits, with more traits in unstable plaques, suggesting an involvement in the disease process (**Figure 1D**). To verify whether Mcl-1 deletion indeed resulted in an efficient neutropenia model in the context of atherosclerosis, $Ldlr^{-/-}$ recipient mice were transplanted with LysMCre $Mcl\text{-}1^{fl/fl}$ (hereafter $Mcl\text{-}1^{-/-}$) or wild type (WT) bone marrow (**Figure 1E**). $Mcl\text{-}1^{-/-}$ mice showed similar serum cholesterol and triglyceride levels, and body weight (**Supplementary Figure 1A-C**).

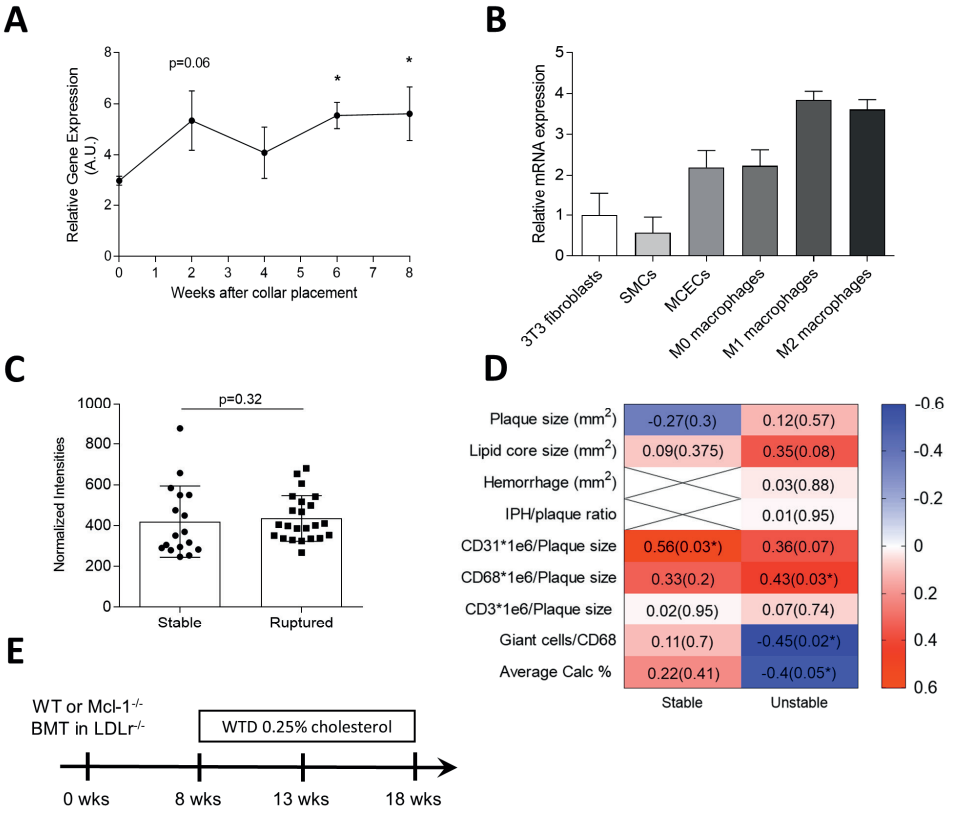

Figure 1. Regulation of Mcl-1 expression in atherosclerosis

A, B and **C.** Mcl-1 gene expression measured by RT-qPCR. **A.** Vascular Mcl-1 expression corrected for HPRT housekeeping gene in a model of collar induced carotid artery atherogenesis in LDLr$^{-/-}$ mice. **B**. Mcl-1 expression corrected for 18S housekeeping gene in different mouse cell types. SMCs: smooth muscle cells, MCECs: mouse cardiac endothelial cells. **C**. Mcl-1 gene expression in human atherosclerotic plaques, represented by microarray normalized intensities. **D.** Heatmap showing Pearson correlation coefficient (p-values) of Mcl-1 human plaque gene expression correlation with clinical plaque traits. N=22/23 (stable/unstable). **E**. Lethally irradiated LDLr$^{-/-}$ mice were reconstituted with WT or Mcl-1$^{-/-}$ bone marrow, and after 8 weeks of recovery, put on a Western Type Diet (WTD) containing 0.25% cholesterol for 5 weeks (n=17) or 10 weeks (n=19). All data is presented as mean ± SEM. *p<0.05

Compatible with the notion that Mcl-1 is essential for neutrophil survival[7], circulating and splenic neutrophil numbers were sharply reduced by 80% and 86%, respectively in Mcl-1$^{fl/fl}$ LysMcre mice[9]. Circulating neutrophils were depressed in Mcl-1$^{-/-}$ chimeras both at baseline (82% depletion) and even more so under hyperlipidemic conditions (91% depletion) (**Figure 2A**). Likewise, neutrophil

content in Mcl-1$^{-/-}$ atherosclerotic lesions was decreased, albeit to a lower extent than in blood (**Figure 2B and C**), hinting to an enhanced adhesive capacity or faster turnover of residual neutrophils in circulation. Considering that an elevated CXCR4/CXCR2 balance is associated with regress to the bone marrow and that CXCR4 is an established measure of neutrophil ageing[16], we examined neutrophil phenotype. CXCR4 expression on circulating and peritoneal residual neutrophils was increased (**Figure 2D and E** respectively), suggesting hyperactivation and increased SDF1 migratory capacity. Moreover, responsiveness of remaining neutrophils to the potent neutrophil chemokine CXCL1 was blunted, concordant with the reduced CXCR2 expression by pre-apoptotic neutrophils[16]. Peritoneal neutrophil influx 2h after i.p. injection of CXCL1 was prominent in WT transplanted mice, whereas Mcl-1$^{-/-}$ transplanted mice only showed a minor, non-significant, increase in peritoneal neutrophils (**Figure 2F and G**). Of note, neutrophil recruitment was paralleled by stromal egress of neutrophils into circulation in WT, but not Mcl1$^{-/-}$ mice (data not shown). Taken together, these results confirm Mcl-1 as a crucial neutrophil survival factor, also under hyperlipidemic conditions, and demonstrate that Mcl-1 myeloid deletion can be used as a genetic tool to induce a long-lasting, severe neutropenia in atherosclerosis.

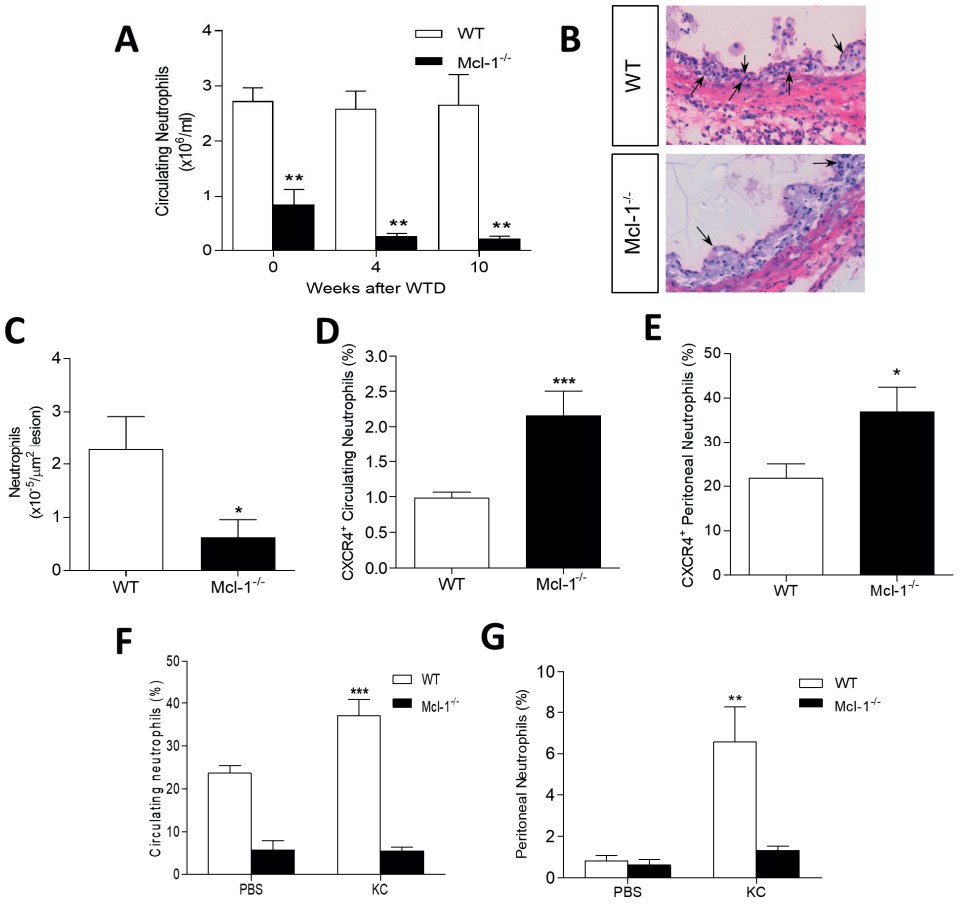

Figure 2. Mcl-1$^{-/-}$ chimeric mice have altered neutrophil levels and characteristics
A. Circulating neutrophils were defined as Gr1+ and measured by flow cytometry in blood samples obtained from tail vein of WT and Mcl-1$^{-/-}$ bone marrow chimeras. **B**. Representative H&E pictures of WT and Mcl-1$^{-/-}$ atherosclerotic plaques (n=19). Neutrophils are indicated by the arrow. **C**. in aortic root atherosclerotic lesions of BM transplanted LDLr$^{-/-}$ after 10 weeks of WTD (n=19). **D.** and **E.** CXCR4 expression in circulating and peritoneal neutrophils respectively assessed by flow cytometry (CXCR4 positive cells within the neutrophil gate of A). **F.** and **G.** Circulating and peritoneal neutrophil levels respectively 2h after CXCL1 injection measured by flow cytometry (CXCR4 positive cells within the neutrophil gate of A). Data is presented as mean ± SEM. ***p<0.001, **p<0.01 and *p<0.05.

Myeloid Mcl-1 deletion increased plaque apoptotic cell content but did not affect atherosclerotic lesion size

Despite its profound effects on circulating neutrophils, and on plaque neutrophil content, myeloid Mcl-1 deletion did neither alter early nor advanced plaque area, necrotic core size, or plaque macrophage content as compared to controls (**Figure 3A-D**). We did observe an increase in plaque apoptosis by 71% and 77% in atherosclerotic lesions of Mcl1$^{-/-}$ mice fed a WTD for 5 and 10 weeks, respectively, compared to WT mice (**Figure 3E**), suggesting that Mcl-1 not only plays an important role in the survival of neutrophils, but also of other myeloid plaque-resident cells, such as plaque macrophages and foam cells.

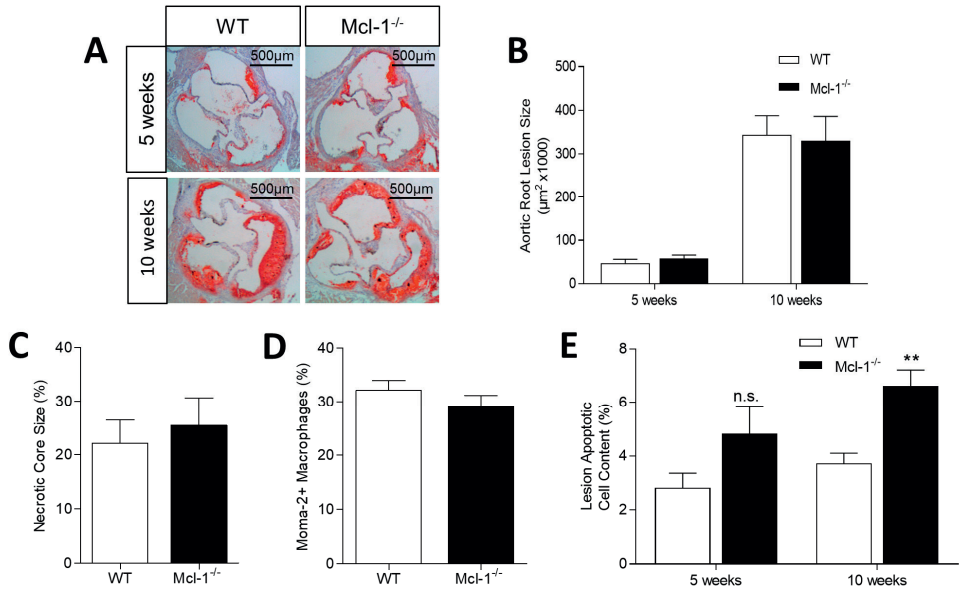

Figure 3. Effect of myeloid Mcl-1 deficiency on atherosclerotic lesion size and composition.

A. Representative micrographs of Oil Red O stained aortic root sections in WT and Mcl1$^{-/-}$ mice after 5 and 10 weeks of WTD. **B**. Atherosclerotic lesion size after 5 weeks or 10 weeks of WTD quantified on Oil Red O staining using Leica image analysis system. **C**. Plaque necrotic core size after 10 weeks of WTD. **D**. Plaque macrophage content after 10 weeks of WTD assessed on Moma-2 positive staining. **E**. Plaque apoptotic cell content after 5 weeks or 10 weeks of WTD, measured by TUNEL staining. Data is presented as mean ± SEM. **p<0.01.

Mcl-1 myeloid deletion enhanced lipid-induced apoptosis sensitivity and lipid loading capacity of macrophages

We next investigated the role of Mcl-1 in macrophages and foam cells in atherosclerosis, reasoning that hyperlipidemia could unleash a role for Mcl-1 in plaque macrophage biology, as has been described for macrophages undergoing infection[12]. Mcl-$1^{-/-}$ peritoneal macrophages displayed a higher level of apoptosis already at baseline, confirming that the hyperlipidemic environment by itself triggered the Mcl-1 survival program (**Figure 4A**). Moreover, Mcl-$1^{-/-}$ peritoneal macrophages, as well as Mcl-$1^{-/-}$ BMDMs (**Supplementary Figure 2A and B**), displayed an increased sensitivity towards oxLDL-induced cell death compared to WT macrophages (**Figure 4A and B** and **Supplementary Figure 3A-E online**). Interestingly, peritoneal foam cell numbers were increased by 2.5-fold in Mcl-$1^{-/-}$ compared to WT transplanted mice (**Figure 4C**), whereas total peritoneal macrophage numbers were unchanged (data not shown). This finding led us to examine the lipid loading capacity of peritoneal macrophages *in vitro*. In agreement with the elevated peritoneal foam cell counts *in vivo*, lipid accumulation in non-stimulated Mcl-$1^{-/-}$ BMDMs *in vitro* was markedly increased (**Figure 4D**). Lipid loading in WT macrophages remained unchanged after incubation with oxLDL for 24h but was substantially enhanced in Mcl-$1^{-/-}$ macrophages (**Figure 4D**). While incubation with VLDL increased lipid content in both WT and Mcl-$1^{-/-}$ macrophages, this increase was considerably higher in the latter cells (**Figure 4D**). In keeping with what is observed in peritoneal macrophages, Mcl-$1^{-/-}$ BMDMs also showed an increased lipid uptake capacity (**Figure 4E**). Thus, under conditions of hyperlipidemia Mcl-1 appears to be an active regulator of macrophage survival as well as of macrophage lipid loading.

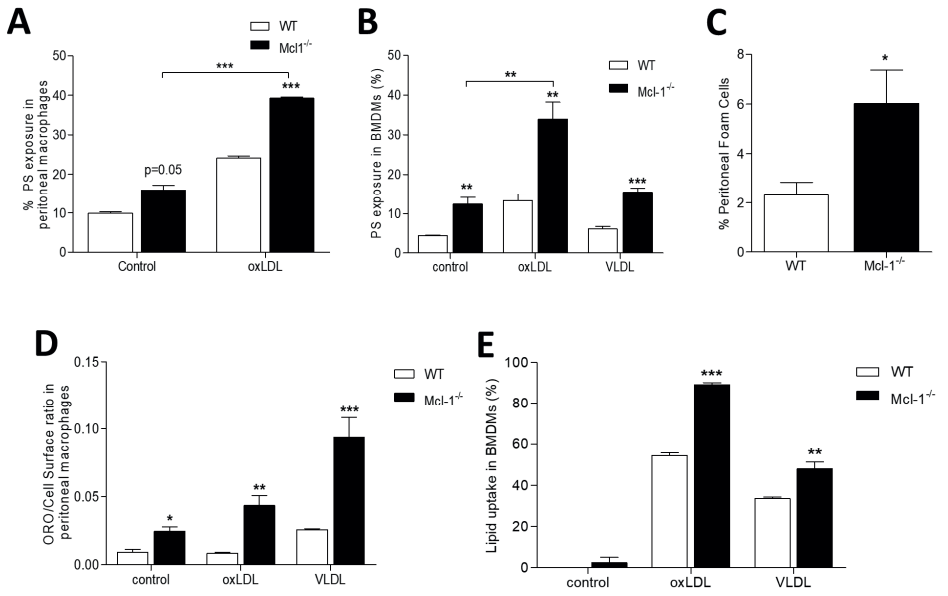

Figure 4. Effect of Mcl-1 myeloid deletion on macrophage apoptosis and lipid loading.
A. PS exposure of unstimulated or oxLDL (40 µg/ml) stimulated peritoneal macrophages measured by Annexin-V-OG staining. **B**. PS exposure of unstimulated or oxLDL/VLDL stimulated bone marrow derived macrophages (BMDMs) measured by Annexin-V-OG staining. **C**. Peritoneal foam cell presence of WT and Mcl-1$^{-/-}$ mice after 10 weeks of WTD assessed by Oil Red O staining *in vitro*. **D**. Lipid loading capacity of peritoneal macrophages after oxLDL (20 µg/ml) and vLDL (50 µg/ml) exposure. **E**. Lipid uptake capacity of BMDMs after oxLDL (20 µg/ml) and VLDL (50 µg/ml) incubation. Data is presented as mean ± SEM. ***p<0.001, **p<0.01 and *p<0.05.

Mcl1 deletion induces multinucleated giant cell formation

Much to our surprise, we noticed the abundant presence of multinucleated giant cells (MGCs) in plaques of Mcl-1$^{-/-}$ transplanted animals after 10 weeks on WTD. Indeed, MGC content per plaque was increased by 118% in the Mcl-1$^{-/-}$ transplanted animals as compared to WT (**Figure 5A and B**), suggesting that Mcl-1 myeloid deficiency leads to the formation of MGCs. In addition, Mcl-1 myeloid deficiency increased MGCs formation by peritoneal macrophages *in vitro* both in control conditions and after incubation with oxLDL and VLDL (**Figure 5C and D**). To further investigate the Mcl1-dependent changes underlying MGC formation, we incubated WT BMDMs with IL-4 and GM-CSF, two known cytokines to induce giant cells[29,30], and compared them to Mcl-1$^{-/-}$ BMDMs. While giant cell formation was unaffected after one week of culture (data not shown), it was significantly increased in Mcl-1^{-}

$^{/-}$ BMDMs compared to WT BMDMs at 13 days of culture (P value= 0.002, **Figure 5E**). Mechanistically, the effect was partially mediated by hyperlipidemia, as oxLDL or VLDL increased MGC formation in BMDM-derived cells to a larger extent (**Figure 5E**). Of note, after 13 days in culture, Mcl-1$^{-/-}$ BMDMs had partially lost their increased capacity of lipid uptake and subsequent lipid-induced apoptosis (**Figure 5F and G**, respectively).

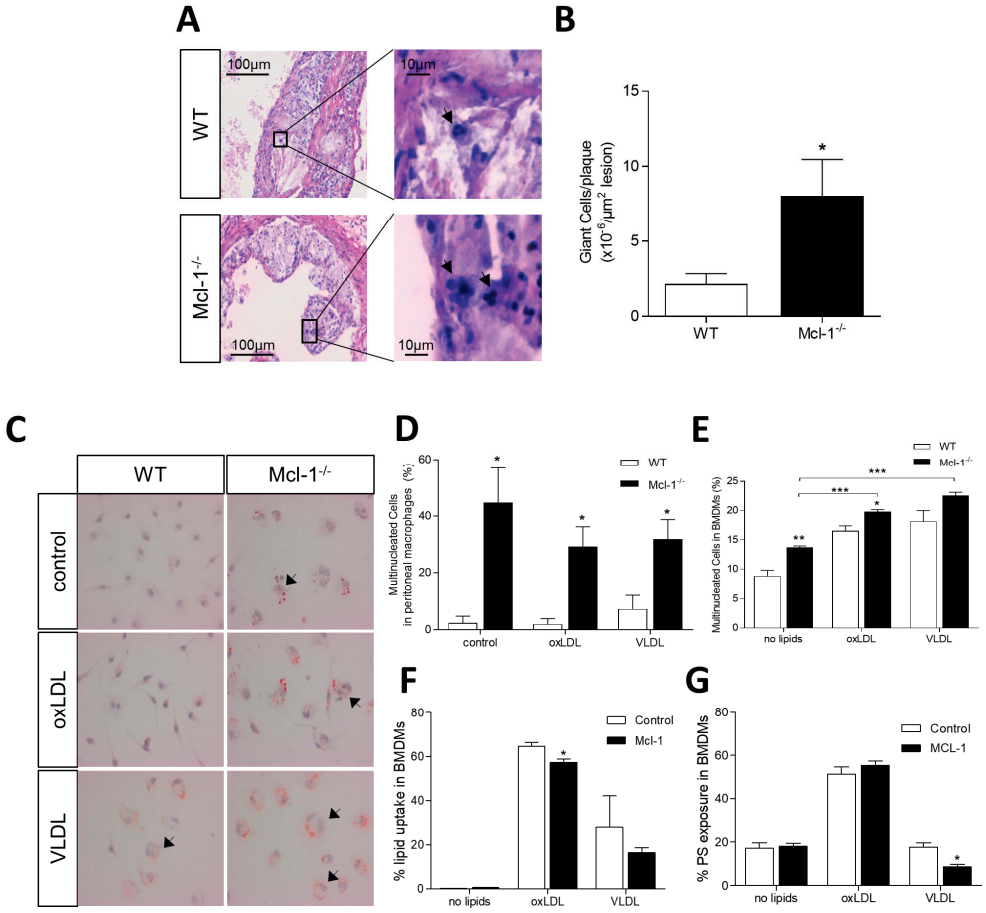

Figure 5. **Effect of Mcl-1 deletion on macrophage fusion**

A. Representative pictures of mouse aortic root sections stained with H&E. Multinucleated Giant Cells are indicated by the arrow. MGCs were quantified as cells containing two or more round nuclei. **B.** MGCs quantification in the atherosclerotic lesions of WT and Mcl1$^{-/-}$ chimeras after 10 weeks of WTD. **C**. Representative pictures of Oil Red O stained unstimulated, oxLDL or VLDL stimulated peritoneal macrophages. Multinucleated Giant Cells are indicated by the arrow **D.** Quantification of Giant Cell population in peritoneal

macrophages depicted in **C**. **E**. MGC in BMDMs after 6d of culture in the presence of either oxLDL, VLDL or nothing. **F.** Lipid loading capacity of MGCs after 13d in culture. **G**. PS exposure in MGC population after 13d in culture. Data is presented as mean ± SEM. ***p<0.001, **p<0.01 and *p<0.05

To assess the relevance of Mcl-1-dependent MGC formation in human atherosclerosis, we quantified MGCs in human plaques. Cathepsin K^+ multinucleated cells were frequently found in both stable and unstable plaques (**Figure 6A and B**). Interestingly, their presence inversely correlated with Mcl-1 gene expression in unstable plaque, and only in unstable lesions associated significantly with lesion hemorrhages and calcifications (P value= 0.044 and 0.055, respectively, **Figure 6C and D**). Thus, our results unveil a hitherto unknown link between Mcl-1 and MGC formation, which is influenced by hyperlipidemia, an association potentially preserved in human atherosclerotic lesions as well.

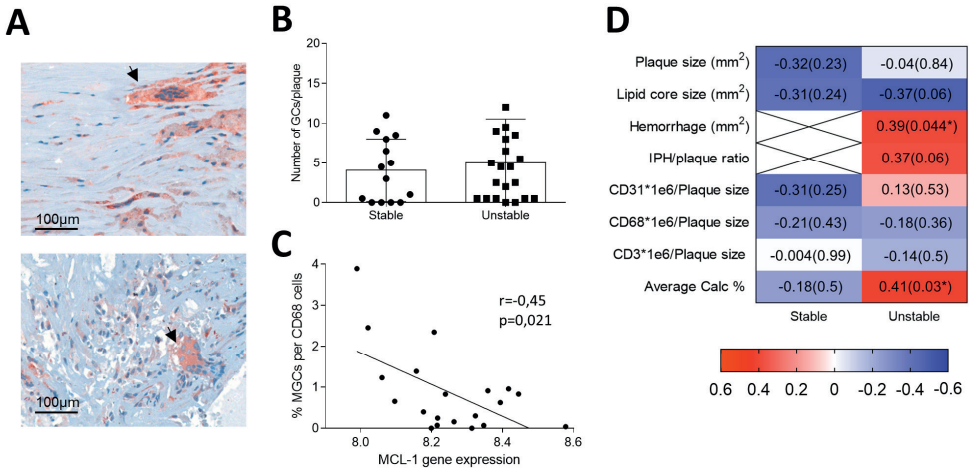

Figure 6. Presence of Multinucleated Giant Cells in human atherosclerotic plaques.
A. Representative pictures of cathepsin K stained human unstable plaques. Multinucleated Giant Cells are indicated by the arrow **B**. MGCs are quantified as cells positive for cathepsin K and containing two or more round nuclei. **C**. Pearson correlation analysis showing coefficient (p-values) between MGCs presence and MCL-1 gene expression levels in human unstable plaque segments (n=18). **D**. Heatmap showing Pearson's correlation coefficient/p-values between the presence of MGCs and other clinical plaque traits. N=22/23 (stable/unstable). * indicates significant correlation.

Discussion

In this study, we evaluated the effects of myeloid Mcl-1 deletion and its accompanying neutropenia on atherosclerosis progression. In addition to extreme neutropenia, Mcl-1 deficiency resulted in increased macrophage apoptosis and lipid handling, and triggered multinucleated giant cell formation. First, we found that myeloid Mcl-1 deletion dramatically reduced neutrophil numbers both in circulation and in atherosclerotic lesions. This is in keeping with extensive data on the vital role of Mcl-1 in neutrophil survival[9,10,31,32] which as we now demonstrated remains valid in a hyperlipidemic environment. On the other hand, Mcl-1 overexpression[33,34] and Bim deletion[23], a pro-apoptotic Bcl-2 family member and Mcl-1 antagonist, do not affect circulating myeloid cell numbers, suggesting that physiological Mcl-1 levels are sufficient for normal cell function. Our work thus provides a mouse model for continuous neutropenia, an important advantage to models used in earlier studies addressing neutrophil contribution to atherogenesis. Though Zernecke *et al*. demonstrated that CXCR4 blockade aggravated atherosclerosis due to increased neutrophil recruitment to the plaque, they were unable to extend the neutrophil depletion beyond a 4 week period[16]. They did show that plaques of neutrophil-depleted mice were smaller and had a lower neutrophil and macrophage content, but did not evaluate plaque apoptosis or necrotic core size. Similarly so, CCL3$^{-/-}$ LDLr$^{-/-}$ bone marrow chimeras with 50% less neutrophils, developed smaller atherosclerotic lesions[35]. Consequently, we were highly surprised by the unaffected atherosclerotic lesion burden in neutropenic LysMCre Mcl-1$^{-/-}$ mice.

Apparently the atheroprotective effects of Mcl-1 deletion in neutrophils in LDLr$^{-/-}$ mice are counteracted by potentially pro-atherogenic effects on other cell types targeted by the LysM conditional Mcl-1 deletion, we therefore examined in greater detail the role of Mcl-1 in atherosclerotic LysM^{+} monocytes and macrophages.

Although Mcl-1 loss was previously shown to have no effect on monocyte and macrophage development in wild type mice[9,10], Mcl-1$^{-/-}$ macrophages were more sensitive to apoptosis upon an infection[12] or phagocytic challenge[10]. We found that the apoptotic cell content in advanced aortic root lesions (10 weeks of WTD) was increased by 44% in mice with myeloid Mcl-1 deficiency. As neutrophils are only scarcely present in advanced lesions[14] and most apoptotic cells were located in the central atheroma (data not shown), the high apoptotic cell density is likely to reflect dying LysM^{+} plaque macrophages. Our work thus identifies Mcl-1 as a major survival

protein in atherosclerotic lesions. Atherosclerotic lesion burden was however unaltered in Mcl-1$^{-/-}$ BM recipients, as were necrotic core, macrophage and collagen content. Similar results were obtained when studying plaque initiation five weeks after WTD. Our results correspond with those from Thorp *et al.*[21], who showed increased macrophage apoptosis, but unchanged lesion burden in Bcl-2^{flox}-LysMCre ApoE$^{-/-}$ mice that are deficient in macrophage and neutrophil Bcl-2[21]. In turn, hematopoietic Bim deficiency, a pro-apoptotic Bcl-2-family member, had no impact on macrophage apoptosis and lesion burden in LDLr$^{-/-}$ mice[23]. Thus, Mcl-1$^{-/-}$ deletion in macrophages led to higher apoptosis level in advanced plaques, however it is clear from the above that this not always translates into a pro-atherogenic plaque progression.

In addition to an increased sensitivity to oxLDL induced cell death, Mcl-1$^{-/-}$ macrophages showed augmented lipid accumulation after incubation with oxLDL and VLDL. In keeping, we observed elevated foam cell levels *in vivo* in the peritoneal cavity of Mcl-1$^{-/-}$ BM compared to WT BM recipients. These findings seem to contrast with those of Halvorsen *et al.*[36], who reported reduced IL-10 induced oxLDL loading by THP-1 macrophages *in vitro* after siRNA mediated silencing of Mcl-1 and Bfl-1 expression. The authors did not assess effects of Mcl-1 inhibition alone, without IL-10 stimulation. Based on our data we hypothesize that the apoptosis-prone phenotype of Mcl-1$^{-/-}$ macrophages is at least partly caused by the increased uptake of lipids.

Another remarkable characteristic was the high propensity of Mcl-1$^{-/-}$ cells to form multinucleated giant cells (MGCs). MGCs, a hallmark of several chronic inflammatory diseases[37,38], originate from monocyte-macrophage lineage and result from cell fusion[37,39]. Giao *et al.* have illustrated the presence of TRAP-positive (osteoclast like giant) cells in close relation to calcified regions and TRAP-negative MGCs in advanced human atherosclerotic plaques[40]. In addition, Samokhin *et al*.[41] showed that mice fed a Paigen diet displayed a 4-fold increase in MGC number in atherosclerotic lesions [41]. In our study, we observed a higher macrophage fusion capacity in both Mcl-1$^{-/-}$ peritoneal and BMDMs, which increased even more upon oxLDL or VLDL stimulation. Furthermore, Mcl-1$^{-/-}$ deficiency in BMDMs seemed to promote MGC formation, independently of lipid uptake. In line with our *in vitro* findings, Mcl-1$^{-/-}$ atherosclerotic plaques had a 4-fold increase in MGC presence as compared to WT lesions. Additionally, the presence of MGCs in human unstable plaques correlated negatively with Mcl-1 gene expression and significantly

associated with hemorrhages and calcifications in the lesion. The exact role of MGCs in atherosclerosis is not yet fully understood, however it was previously shown that MGCs facilitate vascular smooth muscle cell migration in the context of atherosclerosis by producing cathepsin K and destroying the elastin fibers[41]. Although not providing conclusive evidence, our findings support such pro-atherogenic role of MGCs. To our knowledge, we are the first to implicate Mcl-1 in the fusion of macrophages. Possibly, this is related to increased oxidative phosphorylation capacity for energy production in Mcl-$1^{-/-}$ deficient BMDMs (data not shown), however the exact mechanism by which Mcl-1 induces MGC formation remains to be investigated.

In summary, myeloid Mcl-1 deficiency led to a profound and sustained neutropenia in hyperlipidemic $LDLr^{-/-}$ mice accompanied by enhanced oxLDL induced macrophage death *in vitro*, as well as increased atherosclerotic lesion apoptosis. Furthermore, Mcl-1 deficiency was shown to enhance lipid uptake and induce the formation of MGCs *in vitro* and *in vivo.* In line with the observation in mice, Mcl-1 gene expression negatively correlated with the presence of MGCs in human unstable plaque. Taken together, the markedly lower neutrophil numbers mask the combined effects of a more lipid rich and apoptotic plaque in myeloid Mcl-1 deficient animals, and result in an unchanged lesion development and progression. Our results clearly identify Mcl-1 as a macrophage survival protein under hyperlipidemia and uncover a hitherto unknown role for Mcl-1 in MGC formation.

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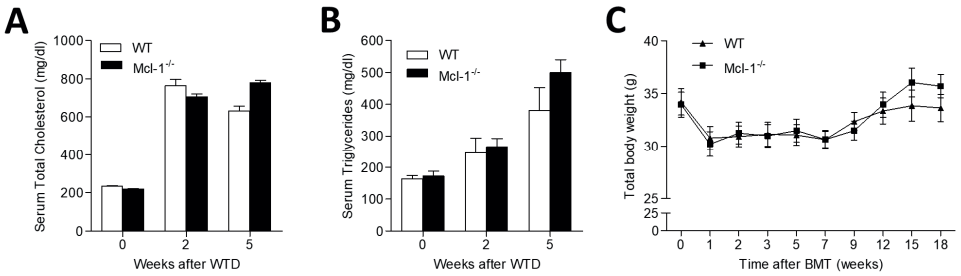
Supplemental data

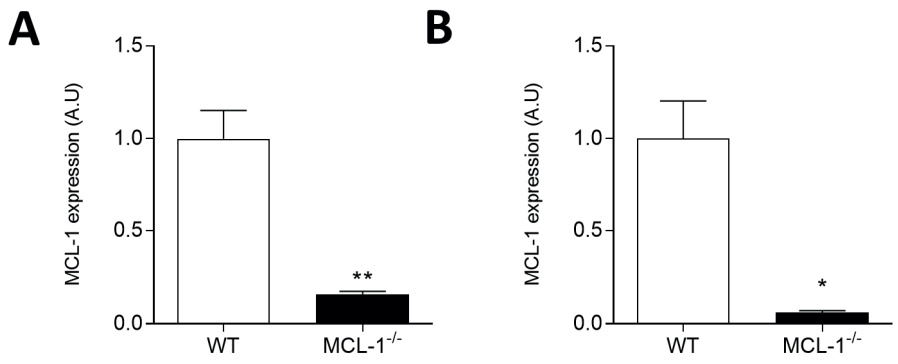
Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Mcl-1	AAGAGGCTGGGATGGGTTTGT	AGTCCCCTATTGCACTCACAAG
HPRT	TTGCTCGAGATGTCATGAAGGA	AGCAGGTCAGCAAAGAACTTATAG
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Supplementary Table 1: Primer sequences

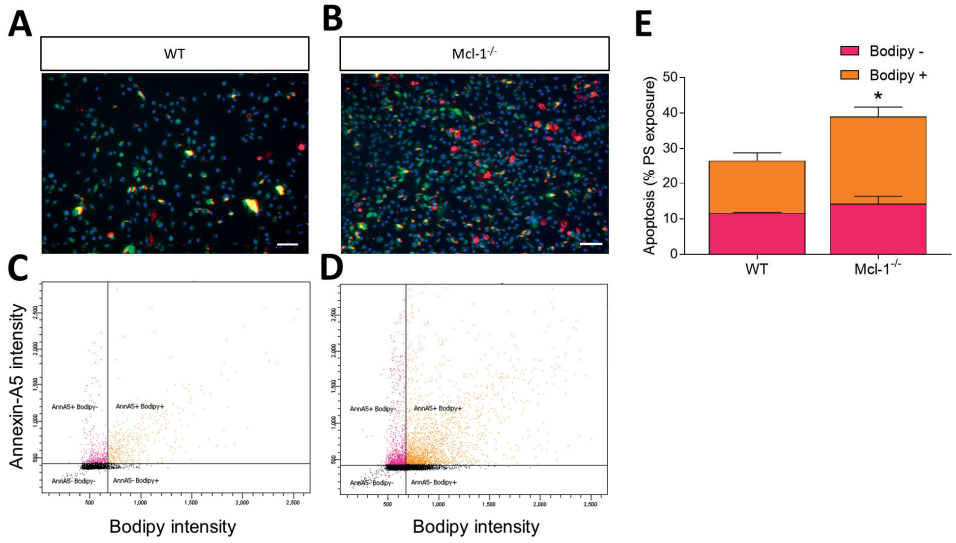


Supplementary Figure 1. **Lipid levels and total body weight of Mcl-1$^{-/-}$ chimeras are unchanged.**

A. Serum total cholesterol levels at 0, 2 and 5 weeks after WTD. **B**. Serum triglyceride levels at 0, 2 and 5 weeks after WTD. Total body weight of WT and Mcl-1$^{-/-}$ chimeras. Data is presented as mean ± SEM. *p<0.05



Supplementary Figure 2: **Mcl-1 gene knock-out confirmation by qPCR.**
Mcl-1 gene expression in BMDMs from Mcl-$1^{-/-}$ mice is almost blunted as compared to that of WT BMDM at day 7d (**A)** or 12d (**B**) of culturing. Data is presented as mean ± SEM. *p<0.05; **p<0.01.



Supplementary Figure 3: Lipid induced-apoptosis in Mcl-1$^{-/-}$ BMDMs.
A. WT and **B**. Mcl-1$^{-/-}$ BMDMs were incubated for 2,5h with fluorescently-labeled oxLDL (bodipy, 20 µg/ml) and subsequently stained for PS exposure using Alexa-647 labelled Annexin-A5 and cell associated fluorescence was measured by using a fluorescent microscope (BD pathway 855; representative image at 10X magnification). **C**. and **D**. show representative analysis performed with the DIVA software and **E**. represents the quantification. Data is presented as mean ± SEM. *p<0.05

Chapter 5

Cardiovascular event prediction by machine learning: identification of a blood-based lipid signature to stratify obese subjects.

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Submitted

Chapter 6

General discussion

Main findings of this thesis

Macrophages are the most abundant immune cell type in atherosclerosis and their transcriptional plasticity has been widely demonstrated[1]. However, a clear link between transcriptional heterogeneity and the corresponding functional activity was lacking. Therefore, in this thesis we aimed at building a macrophage functional map using a high content analysis system *in vitro* to study macrophage functions in CVD inflammation, including atherosclerosis, acute myocardial infarction repair and cardiometabolic disorders, and link it to specific transcriptional profiles.

In this chapter, we will discuss the following main findings and deliverables of this thesis:

(1) The successful development of a ready-to-use "functionomics" platform (called the MacroScreen). This platform is able to measure 19 distinct functions of human and mouse macrophages in a semi-automated manner and at microscale (**chapter 2 to 5**).

(2) The identification of a macrophage functional activation map. Macrophages transcriptional plasticity, which cannot be fully captured by the M1/M2 dichotomy, has direct repercussion on their functional profile (**chapter 2**).

(3) The functional responses of macrophages to their environment. Systemic changes following acute myocardial infarction (AMI) reprograms human macrophages by dysregulating their functional and transcriptional profile. Furthermore, these transcriptional and functional changes correlate with clinical progression of the patients (**chapter 3**).

(4) The discovery of a highly predictive lipid-signature for the development of obesity-related CVD. These lipids not only predict future CVD events but also seem causally implicated in the pathophysiology of atherosclerosis and related CVD, as assessed by our functionomics platform. (**chapter 5**).

In the following, integrative discussion, we will (1) address the use of macrophages as sensors for CVD inflammation and discuss relevant macrophage functions in CVD, macrophage plasticity and the impact of macrophage source that can be used

in the MacroScreen; (2) compare the MacroScreen to other currently available humanized platforms; and (3) focus on patient systemic environment and its relation to CVD.

The MacroScreen: a novel tool to study innate immune responses in cardiovascular diseases

The use of macrophages as sensors in the MacroScreen: Macrophage functions in CVD and their measurement in the MacroScreen

In **chapters 2-5**, we used human macrophages to assess changes in cellular function associated with cardiovascular inflammation, including atherosclerosis (**chapter 4**), repair after acute myocardial infarction (AMI, **chapter 4**), and cardiometabolic diseases (**chapter 5**). Macrophages are innate immune cells implicated in atherosclerosis and AMI repair. First, in atherosclerosis, macrophages are the most abundant immune cells and are involved at all stages of the disease. They derive from blood monocytes that are recruited to the vascular wall where they differentiate into macrophages and ingest oxidized low-density lipoprotein (oxLDL) to become lipid-laden foam cells, forming the atherosclerotic plaque. Second, after an AMI event, macrophages are recruited to the ischemic heart and contribute to cardiac repair by regulating, amongst others, inflammation, apoptosis and extracellular matrix degradation and formation, and angiogenesis[2]. In **chapter 2**, we describe the development of a high content analysis platform able to measure 19 distinct macrophage functions, all relevant for the development of atherosclerosis, associated inflammatory responses, and AMI post injury repair. This platform allows us to understand how certain environmental cues (**chapter 2 and 5**) or systemic patient environment (**chapter 3**) can affect macrophage functional profile and subsequently relate to CVD incidence.

In the next paragraphs, we will further discuss the implication of each function included in the MacroScreen in the context of atherosclerosis and AMI repair. Furthermore, we will discuss possible other functions the platform could cover.

Cholesterol metabolism

Cholesterol metabolism in macrophages is an important player during atherosclerosis, it involves excessive uptake of modified cholesterol and reduced reverse transport of cholesterol to HDL particles[3,4]. Studies in mice have shown that inhibition of lipid uptake by depletion of certain scavenger receptors results in a reduction of atherosclerotic plaque development. Babaev *et al.* showed that deletion of myeloid class A scavenger receptors in mice reduced the atherosclerotic lesion area by 60%[5]. A similar reduction in lesion area (76.5%) was observed after depletion of CD36, a class B scavenger receptor, and this was attributed to reduced

foam cell formation by 60%[6]. Additionally, increased reverse cholesterol transport, called cholesterol efflux, from macrophages has also shown beneficial effects on atherosclerosis. Stimulation of liver X receptor α (LXRα) in macrophages leads to upregulation of ATP binding cassette A1 (ABCA1), a cholesterol transporter, and was seen to reduce atherosclerotic lesions[7–9], confirming that regulation of cholesterol metabolism in macrophages is crucial in atherosclerosis. Unsaturated fatty acid, such as palmitic and linoleic acid (included in **chapter 2**) can downregulate ABCA1 and ABCG1 expression, via inhibition of agonist binding to LXRα[10]. Furthermore, the pro-inflammatory micro-environment of the atherosclerotic plaque is also able to regulate foam cell formation, as interleukin-12 (IL-12) and IL-18 were shown to reduce ABCA1 expression, and thus cholesterol efflux by macrophages[11].

Mitochondrial functions

During the process of foam cell formation, free cholesterol can disrupt mitochondrial functions in macrophages[12]. Mitochondria are cell organelles exerting a myriad of functions essential for cell homeostasis, including regulation of ATP production, reactive oxygen species (ROS) production or apoptosis signal[13] and macrophage mitochondrial dysfunction has been associated with inflammation in cardiovascular disease. Yu *et al.* observed a reduction in mitochondrial oxygen consumption and mitochondrial DNA (mtDNA) copies in human atherosclerotic necrotic cores and fibrous cap. This observation was also made in ApoE$^{-/-}$ mice fed a high-fat diet and increasing mitochondrial respiration by restoring mtDNA copy number through overexpression of Twinkle, a mitochondrial helicase, led to smaller and more stable plaques[14]. In the context of cardiac repair after MI, three days post infarct, cardiac macrophages have been observed to exhibit dysregulated mitochondrial function, as shown by e.g. upregulation of genes involved in oxidative phosphorylation, hinting to a macrophage metabolic switch[15].

ROS production

ROS production is a critical function of macrophages. To combat infection, phagocytes, including macrophages, release massive amounts of reactive oxygen species, a process known as respiratory burst[16]. Macrophage-derived ROS are produced by mitochondria e.g. fatty acid oxidase (NOX4), in endosomes (NOX1), in cytosol, or at the cell membrane (e.g. via NADPH oxidases (NOX4 and 5) and extracellularly (myeloperoxidases etc)). Excessive intra- and extracellular ROS has been repeatedly linked to atherosclerosis development[16,17]. Xu *et al.* showed that

deletion of NOX1 and NOX2 leads to reduced ROS macrophage production and defective macrophage differentiation from monocytes[18]. Furthermore, a recent study showed that reduction of atherosclerotic plaque by treatment with the antioxidant flavonoid quercetin in ApoE$^{-/-}$ mice was attributable to decreased ROS production by macrophages[19]. Additionally, LDLR$^{-/-}$ mice irradiated and transplanted with mitochondrial catalase transgenic mouse bone marrow, known to have reduced mitochondrial oxidative stress, showed less macrophage oxidative stress in atherosclerotic lesions and in turn, smaller plaque size[20]. ROS production is also critical to cardiac repair following MI, as ROS was associated with the amount of necrosis after MI and overexpression of superoxide dismutase in mice led to improved cardiac function[21]. In addition, bone marrow derived macrophages were found to be the main source of increasing expression of gp91phox, also known as NOX2, during the first stages of cardiac repair[22].

Cytokine production

Next to ROS production, macrophages also produce a broad range of cytokines during cardiovascular disease. Cytokines are effector proteins that will act as pro or anti-inflammatory signals and are abundantly found in atherosclerotic lesions or in the infarcted heart[23]. In atherosclerosis, cytokines are first released by macrophages after oxLDL uptake and this will, in turn, become a vicious circle of inflammation, as cytokines will further activate macrophages and other cell types to release more cytokines progressively. Pro-inflammatory cytokines, such as Interferon-γ (IFNγ) and tumor necrosis factor (TNF) have pro-atherogenic properties, as TNFα-deficient or IFNγ-deficient ApoE$^{-/-}$ mice showed reduced atherosclerotic plaque size, mostly attributed to less foam cell formation, expression of other pro-inflammatory markers[24] and decreased lipid content[25]. Contrarily, anti-inflammatory cytokines, such as IL-13 or IL-10, commonly exert anti-atherogenic effects by modulating macrophage phenotype[26] and lipid metabolism[27], respectively. Similarly, during cardiac repair following MI, macrophages first release a range of pro-inflammatory cytokines, such as IL-1β and TNF-α, and this is followed by a switch to an anti-inflammatory state, mediated by anti-inflammatory cytokines, such as IL-4 and IL-10[28]. This switch seems beneficial, as treatment of mice after MI with IL-10 led to increased polarization to M2 macrophage and improved cardiac functions, such as ejection fraction and left ventricular dilation[29].

Inflammasome activation

Another key pro-inflammatory cytokine produced by macrophages is IL-1β. IL-1β is the result of the macrophage inflammasome activation in response to cholesterol crystals and oxLDL[30,31]. The inflammasome is a protein complex containing one nucleotide-binding oligomerization domain-like receptors (NLR) protein, an apoptosis-associated speck-like (ASC) protein and a cysteine protease caspase-1 and has widely been reported to play an important role in the progression of atherosclerosis. Indeed, in a $LDLR^{-/-}$ mouse model, Duewell *et al.* transplanted bone marrow lacking NLRP3, ASC or IL-1β and observed a regression in atherosclerosis development by 69%[32]. Similarly, mice lacking ASC and subjected to ischemia/reperfusion injury displayed smaller infarct size, and improved cardiac repair[33]. Prior to the secretion of IL-1β in macrophages, the inflammasome complex activates caspases, which will then turn on the programmed cell death machinery or apoptosis.

Apoptosis and phagocytosis

Apoptosis is another key process in the development of atherosclerotic lesions and after MI. Macrophages have been shown to undergo apoptosis at all stages of atherosclerosis[34,35], however, macrophage apoptosis will affect lesion progression distinctively depending on plaque stage[34,36,37]. In early mouse and human lesions, macrophage apoptosis is thought to protect against plaque progression, as they are cleared by phagocytosis, preventing the formation of a necrotic core[38]. In contrast to advanced lesions, where apoptotic cell clearance by phagocytosis is defective, leading to the formation of larger necrotic cores consisting mostly of lipids and apoptotic cell debris[36,39,40]. Impairing phagocytosis by depleting phagocytic receptors such as Mertk or Lrp1 has shown to increase apoptosis and plaque size[41,42]. Second, macrophages help at resolving the inflammation in the post-MI response by phagocyting apoptotic myocytes[28]. Impaired macrophage phagocytosis after MI was linked to differed inflammation resolution and disrupted cardiac repair[43].

Giant cell formation

Another intriguing and less known macrophage function is the formation of giant cells. Multinucleated giant cells result from the fusion of macrophages and have only been observed in calcified regions of human atherosclerosis[44]. Furthermore, cathepsin K expressing giant cells, were previously observed in the central atheroma, close to the media, where they cleave elastin fibers and facilitate smooth

muscle cell migration. In **chapter 4**, we have investigated the effect of Mcl-1 myeloid deletion in atherosclerosis and observed a great increase in giant cell formation. However, the exact mechanism and contribution to atherosclerosis remains to be elucidated.

Other functions

In conclusion, we built a platform able to investigate major macrophage functions in CVD, however macrophages exert some other key functions that are not (yet) included in the platform. First, macrophage foam cell formation also depends on cholesterol efflux. However, our platform does not yet include a cholesterol efflux assay and it would be important to complement our lipid uptake assay with a measure of reverse cholesterol transport, e.g. by micro scaling an existing efflux assay[45]. Second, human macrophages also act as antigen presenting cells and express major histocompatibility complex class II (MHC II), as well as Human Leukocyte Antigen-DR isotype on their surface to elicit an adaptive immune response[46]. Furthermore, macrophage MHC II expression can be regulated by autophagy, a process of self-digestion to maintain energy homeostasis and macrophage autophagy has been shown to be athero-protective in the later stage of the disease[47]. Therefore, measuring macrophage antigen-presentation and autophagy capacity could be indicative of macrophage performance in CVD. Third, macrophage migration to tissue is a key process in CVD. To be able to infiltrate and migrate in tissue, macrophages employ focal proteolysis, secreting several proteases, such as matrix metalloproteinases or cathepsins[48]. Additionally, macrophage metalloproteinases also regulate the extra cellular matrix turnover after AMI[49]. Therefore, assessment of macrophage migration and proteolytic activity would be an important addition to the MacroScreen. Lastly, hypoxia or the lack of oxygen has been suggested to promote necrotic core formation in atherosclerosis[50] and is also observed in the heart after AMI. In response to hypoxia macrophages, will stabilize hypoxia-inducible factor 1, and subsequently polarize to pro-inflammatory macrophage[51]. Measuring macrophage hypoxic response in the MacroScreen would therefore also complement the function panel.

Capturing macrophage functional plasticity with the MacroScreen

In **chapter 2**, we have shown that macrophage transcriptional plasticity is reflected in their functional behaviour, resulting in five functional clusters of macrophage activation. At first, macrophages were thought to present in two phenotypes: M1 (pro-) and M2 (anti-inflammatory macrophages). M1 polarization is induced by pro-

inflammatory cytokines, such as LPS and IFN-γ and hence M1 macrophages exert pro-inflammatory functions. On the other hand, macrophages are polarized towards M2 by a broad range of anti-inflammatory cytokines (e.g. IL-4, IL-13, IL-10), to exert tissue remodelling and wound healing functions. Over the years, accumulating evidence suggested that the dichotomous macrophage polarization model was incomplete and, depending on the activating stimulus and functional profile, the M1-M2 bipolar activation was further refined to include M2a, M2b, M2c and M2d subgroups and other subtypes (M4, Mox, Mhem and M(Hb))[52,53]. All these macrophage subtypes were observed in atherosclerotic lesions, with M1-like macrophages being more abundantly found in rupture-prone plaque regions or unstable plaque segments; whereas M2-like macrophage were found in the adventitia or stable plaque regions[54,55]. Moreover, Mhem and M(Hb) macrophages were associated with intraplaque haemorrhages[56], whereas Mox macrophages was suggested to be induced by oxidised phospholipids and showed pro-atherogenic features[57]. Next to their high heterogeneity, plaque macrophages appear to be very plastic and are able to switch phenotypes depending on their environment[58]. Indeed, M1 and M2 macrophages can reverse their phenotype, when exposed to M2 and M1 stimuli, respectively[59,60]. Taken altogether, this shows that macrophages respond differently depending on stimulus and that their polarization profile is complicated. Furthermore, most of the polarization studies are performed *in vitro* using at most two stimuli and this setting obviously does not reflect the complexity of the *in vivo* situation. In the infarcted heart environment, there is a great amount of effector molecules able to activate macrophages. Using our platform, we were able to study individual stimuli to identify specific activation pathways related to certain functions, but we also included combinations of stimuli (**chapter 2**) to reflect chronic inflammation and human diseased serum to reflect the systemic environment (**chapter 3**). This allows better *in vitro* mimicking of complex activation profiles observed *in vivo*.

The use of different types of macrophages in the Macroscreen

In our newly developed functionomics platform (**chapter 2 to 5**), we made use of monocyte-derived macrophages (MDM). However, this is not the only type of human macrophages. In this section, we will discuss source-dependent differences in makeup of macrophages, and repercussions thereof for the MacroScreen application.

Monocyte-derived vs tissue-resident macrophages

It is nowadays widely accepted that there are two principal types of macrophages: tissue-resident macrophages, mainly of embryonic origin, and inflammatory macrophages derived from blood monocytes and recruited to the site of inflammation[61]. Tissue-resident macrophages originate from the yolk sac or fetal liver during embryonic development and are seeded into the respective tissues before birth, maintained independently of monocytes[61–63]. Not only do they differ regarding their origin, tissue-resident macrophages and recruited macrophages were also seen to exert different functions. Indeed, embryonic macrophages were shown to exert tissue remodelling and homeostatic functions, whereas MDMs mainly have inflammatory functions, such as pathogen killing and host defence[64], serving as reservoir for temporary repopulation of macrophage-depleted tissue.

Resident macrophages exert a variety of homeostatic functions under normal conditions, such as phagocytosis of apoptotic cells[62], lipid homeostasis in the liver[65] or electric conductance in the heart[66]. Upon infection, but also during cardiac repair after AMI and chronic sterile inflammation (e.g. atherosclerosis), macrophages were initially thought to originate from blood born monocytes[67], which after recruitment differentiate into (often short-lived) inflammatory macrophages. More recent studies suggest resident macrophage proliferation as main source of macrophage pool maintenance in atherosclerotic plaques[68,69], and in the heart[2,70]. Accordingly, resident hematopoietic progenitor cells were shown to reside in murine adventitia[71,72]. Furthermore, proliferating macrophages were observed in all stages of atherosclerosis, however, this process was mostly prominent in early lesions where almost 50% of replicating macrophages derived from resident macrophage proliferation[68]. In addition, resident macrophage proliferation can affect atherosclerosis, as Gage *et al.* showed that increased atherosclerotic plaque burden after disruption of LXRα phosphorylation was attributed to lesion-resident macrophage proliferation. Yet, these results must be interpreted with caution, as recent doubts were emitted regarding that the clonal expansion of plaque macrophage pool might be overestimated[73].

Our *in vitro* functionomics platform described in **chapter 2-5** is closely modelling inflammatory macrophages as it focuses on monocyte-derived macrophages isolated from healthy volunteers and differentiated for seven days using macrophage colony-stimulating factor (M-CSF).

iPSC derived macrophages to mimic tissue-resident macrophages

One could argue that our platform does not mimic the tissue-resident macrophage phenotype. Isolating human tissue-resident macrophages is laborious and the number of isolated cells is usually limited. Furthermore, care should be taken when working with isolated tissue-resident macrophages, as their transcriptional profile is affected by the isolation procedure, as shown by Gosselin *et al.* with human and mouse microglia, the brain-resident macrophages[74]. One attractive alternative to study the tissue-resident macrophage phenotype would be the use of human induced pluripotent stem cells (iPSC) derived macrophages (iMacs). iMacs are generated *in vitro* with a protocol that mimics the *in vivo* situation in the yolk sac[75]. They have been shown to resemble human and mouse yolk sac[76,77] and fetal liver[78] derived tissue-resident macrophages, especially in terms of transcription profiles. To program iMacs into specific tissue-resident macrophages, two approaches have been proposed[75]. First, iMacs can be cultured in the presence of effector molecules, such as specific cytokines. For instance, Abud *et al.* successfully produced brain-resident macrophages, a.k.a. microglia cells, by exposing iMacs to CSF1, IL-34 and TGFβ. These iMac-derived microglia cells resembled real microglia, as they expressed microglia-specific genes and proteins and showed increased phagocytotic activity[79]. However, this technique lacks the complex milieu of the *in vivo* situation and cannot completely mimic real microglia[75]. Second, iMacs can be differentiated to tissue-resident macrophages by culturing them with other cell types in that particular tissue, such as astrocytes and neurons for microglia[79] or with complete organoids. In the context of cardiovascular inflammation, iMacs could be cultured with cardiomyocytes to recapitulate cardiac-resident macrophage functions or with smooth muscle and endothelial cells in a lipid-rich environment to acquire a plaque phenotype. Alternatively, to mimic the atherosclerotic plaque environment, iMacs could also be cultured in plaque conditioned medium or lysates. However, the functional similarity between iMacs-derived tissue resident macrophages and tissue-resident macrophages remains to be elucidated, and this could be done using the MacroScreen platform.

The use of cell lines in the MacroScreen

Isolating human $CD14^+$ monocytes from peripheral blood mononuclear cells (PBMCs) for our HCA platform is time consuming and cell yields were usually low, as monocytes only represent three to seven percent of the total PBMCs[80]. Furthermore, to avoid donor-to-donor variability reflected in cell phenotype, we always pooled five to seven donors per experiment. Another alternative to MDMs

to study *in vitro* human macrophage functional identity could be the use of immortalized cell lines, such as THP-1. THP-1 cells are monocyte-like cells obtained from acute monocytic leukemia[81] with stable genetic background, making them an interesting tool to study macrophage functions[82]. However, recent comparison between MDMs and THP-1 cells revealed that THP-1 cells were less sensitive to LPS stimulation[81], and that gene expression profiles do not fully correlate between THP-1 cells and MDMs[83,84]. This was confirmed at the functional level, as macrophage surface markers were only comparable for 41%[85]. Therefore, using THP-1 cells to assess cellular functions on our functionomics platform should be tested and compared to MDMs functional profile before switching to cell lines.

In conclusion, the use of MDMs in the MacroScreen does not reflect the full diversity of human macrophages. However, before using other cell types in the platform, such as cell lines to have a more controlled environment, or iMacs to mimic tissue-resident macrophages, these should be functionally tested and compared to MDMs functional signatures.

Humanized test platforms

The need for humanized test platforms

Biomedical research mainly relies on animal models such as mice, which represent a fast and easy readout for studying, amongst others, CVD inflammation including atherosclerosis, myocardial infarction repair or cardiometabolic disorders. Animal testing was traditionally thought to be an unavoidable step in fundamental research and drug discovery. However, it is increasingly clear that humans are not an inflated, big copy of mice and most results derived from animal testing cannot be directly extrapolated to humans[86]. Indeed, Pasterkamp *et al*. elegantly showed that the main mouse immune-associated genes and pathways causally associated with murine atherosclerosis had no association with human atherosclerotic plaque traits and subsequent incidence of coronary artery disease (CAD) or stroke[87]. Furthermore, many clinical trials testing drugs have failed due to grave side effects or drug efficacy issues, although the tested drugs were safe and effective in animal models[88–90]. Moreover for ethical reasons, increased efforts are being made to reduce the number of animals used in research (applying the 3R rule; replacement, refinement and reduction)[91]. We believe that our platform could be a good alternative to animal testing of innate immune responses, as it is based on human primary macrophages and it covers the major inflammatory processes involved in

the pathophysiology of atherosclerosis, including phagocytosis, oxLDL uptake and apoptosis (**chapter 2**). Furthermore, as we were able to show in **chapter 3**, our platform can detect changes in blood, which are able to alter macrophage functions relating to poor prognosis after an acute myocardial infarction. In addition, we were able to discover human immune genes directly linked to infarct size, showing the potential of the platform to unravel new mechanistic pathways linked to cardiovascular disease (**chapter 3**).

Goals of current humanized test platforms

In the last two decades, the field of humanized test platforms has gained growing interest and many platforms are currently being developed to serve several purposes in a broad range of research fields[92]. First, humanized test platforms were used to identify specific genes associated with biological processes. For instance, in the context of atherosclerosis, Domschkle *et al.* discovered four novel genes involved in LDL uptake in monocyte-derived macrophages[93]. Second, humanized test platforms can also be used to assess complex cell phenotypic changes after screening a compound library and to help selecting potential drug candidates, as illustrated by a study of Gao *et al.* who screened a library of 2,600 compounds and measured their impact on ABCA1 expression in HepG2 cells. They identified and validated 10 compounds that increased ABCA1 gene expression[94]. Third, humanized test platforms can be used as disease models to study mechanistic pathways and discover new potential drugs, e.g. Honarnejad *et al.* aberrantly expressed a mutant causing Alzheimer's disease in HEK293 cells to discover new drugs[95].

In this thesis, we developed a humanized high-throughput platform to model innate immune responses relevant to cardiovascular diseases with special emphasis on the functional behaviour of macrophages. The MacroScreen can detect, in a high-throughput and -content manner, 19 distinct functions, in contrast to mostly singular parameters measured by all the platforms described above.

Possible improvements of the MacroScreen

In the context of CVD, measuring macrophage function *in vitro* does not fully represent the complexity of the *in vivo* situation, which for atherosclerotic plaque contains, next to macrophages, smooth muscle and endothelial cells (ECs) and T-lymphocytes amongst others, and for myocardium, cardiomyocytes, fibroblasts and ECs. Therefore, it is important to stress that our platform neither covers cell-cell

interaction, nor cell-cytokine and cell-matrix interaction. Extracellular matrix components, such as proteoglycans, hyaluronic acid, and collagen deposition are instrumental in tissue remodelling processes in the atherosclerotic plaque or in the post-ischemic heart[96,97]. Thus, to better mimic the complexity of the *in vivo* pathophysiology of these diseases, some improvements could be made, which will be discussed below.

First, during atherogenesis, the endothelium becomes dysfunctional, leading to a weakened barrier integrity and leakage[98]. Endothelial cell dysfunction leads to the infiltration of blood monocyte to the vascular wall. During atherosclerosis, smooth muscle cells (SMCs), which under normal conditions exhibit a contractile phenotype, will undergo phenotypic switch to migrate from the media to the intima and proliferate, to produce collagen and form a fibrous cap[99]. Therefore, our innate immune platform could be complemented with EC and vSMC functional profiles, to create a humanized model of atherosclerosis cell functionality. Most of the processes included in the MacroScreen are also relevant to ECs and SMCs, such as apoptosis and cytokine production but some cell-specific functions should be added. Human SMCs from advanced atherosclerotic plaques have been shown to decrease proliferation and enter a state of senescence[100]. Furthermore, as explained above, during atherogenesis SMCs migrate to the intima to form a fibrous cap[101]. In addition, vSMCs can differentiate to osteoblast-like cells during the course of atherosclerosis and produce micro-calcification, which are known to promote plaque rupture[102]. Therefore, phenotype, proliferation, senescence, migration and calcification assays are the most obvious functionalities relevant to atherosclerosis and should be added to our functionomics platform to complement the current macrophage functional profile.

A second limitation of our platform is that all of the above described assays are based on two-dimensional (2D) monolayers. Three-dimensional (3D) cell cultures have emerged and offer a great opportunity to integrate cell-cell interaction, cell-extra cellular matrix (ECM) interaction, tissue oxygenation and nutrient gradients[103]. Three-D cultures were initially developed by growing single cell lines into spheroids. This was prominently used in the cancer field, where 3D spheroid cultures showed a high potential for drug discovery[103,104]. Later on, the spheroid approach was slowly abandoned in favour of organoids, which offer a better representation of cell heterogeneity as they were directly derived from human tissue or stem cells. Organoids are now widely used in biomedical research to grow

organs such as brains[105] and kidneys[106], but also tumors[107]. Interestingly, organoids have also been used in the field of cardiology to study the cardiac formation during organogenesis[108] and (congenital) cardiomyopathies[109]. More recently, even more refined multicellular organoid models have been designed such as endothelialized myocardium, consisting of endothelial cells bioprinted in a 3D hydrogel scaffold and seeded with cardiomyocytes to form a contracting myocardium[110]. Another example was established by Wimmer *et al.*, who successfully created a human blood vessel organoid composed of endothelial cells, pericytes, mesenchymal stem-like cells and haematopoietic cells as a model of diabetic vasculopathy[111]. They showed that exposure of blood vessel organoids to high concentration of glucose and cytokines led to the thickening of the basement membrane, a process also observed in diabetic patients, proving that this 3D model was structurally and functionally resembling the *in vivo* situation. The latter example, however, does not consider biomechanical forces, an important aspect of vascular disease. Recent advances in microfluidics have enabled the development of 3D blood vessels under flow[112]. For instance, van Duinen and colleagues developed a robust high-throughput 3D microvessel to assess barrier function under continuous flow[113]. Another recent example was brought by Poussin *et al.*, who established a human 3D model of endothelial microvessels under flow, able to measure leukocyte adhesion to the lumen[114].

Summarizing the above, the field of *in vitro* humanized test models for (diseased) vasculature and myocardium is increasingly growing to allow rapid, cheap and more reliable way to study atherosclerosis and AMI, respectively. The strength of the MacroScreen, compared to other humanized platforms, lies in the development of a multi-parametric platform, able to measure 19 distinct macrophage functions in a high-throughput way, as opposed to the measurement of a single function in current platforms. Our platform is a fast and cheap way to measure cell functionality, as compared to organoids. However, one should realize that the MacroScreen is an *in vitro* static 2D model and lacks the physiological relevance of the dynamic 3D *in vivo* situation. To mimic the complexity of the *in vivo* situation and capture the full spectrum of cellular functions involved in CVD-related innate immunity, co-culture of macrophages with other cell types, such as SMCs and ECs or deployment of cell free (artificial or tissue based) matrices to form a “3D plaque on a chip” and further functional analysis could be performed.

Patient macro-environment and CVD risk

Systemic environment and its link to CVD risk

Chapter 3 of this thesis highlights the potential of mapping the patient's systemic environment, defined as patient circulating plasma or serum containing a variety of effector molecules, for disease (prognostic) diagnosis, as exposure of macrophages to 20% serum of AMI patients led to functional as well as transcriptional changes that accurately correlated with infarct size four months after the event. This is probably attributable to all kind of effector molecules, released/contained in patient serum upon such major ischemic event. Currently, patient diagnosis relies on a few molecules or risk factors; however, we further show in **chapter 5** that measuring a higher number of features can lead to a great improvement of disease prediction. In **chapter 5**, we screened circulating lipids to discover new biomarkers for the development of obesity-related cardiovascular disease. The plasma lipid profiles, in particular of cholesterol ester and triglyceride content, have widely been shown to be predictive of cardiovascular event [115]. Lipidic molecules are not the only systemic predictors of CVD. Atherosclerosis is, as previously explained, seen as an inflammatory disease and measurement of systemic inflammatory cytokines has showed its potential in terms of event prediction. High sensitivity C-reactive protein (hs-CRP) and other pro-inflammatory cytokines such as IL-6, serum amyloid A and soluble intercellular adhesion molecule type I and chemokines, such as chemokine ligand 2 or 8, have been observed as independent predictors of CVD[116,117]. Furthermore, it was recently confirmed that targeting the inflammation by treating high-risk patients with an IL-1β antibody reduced the risk of CVD by 15%[118]. Since then, many studies are trying to investigate the role of different cytokines, such as IL-5, IL-6 receptor, IFN-γ and TNFα in disease prediction[119]. However, these biomarkers assume that patient populations are all similar and that a universal disease mechanism exists that applies to all patients, which can be captured by one single biomarker. In obesity, for example, increase in BMI leads to an increase in the most common lipid predictors (including LDL cholesterol, total cholesterol) and inflammatory markers[120,121]. Therefore, better prediction and subclassification of high-risk population and subsequent therapy tailoring is needed. Our lipid signature, confirmed in two independent cohorts of obesity, could, in the future, serve as new biomarkers for obesity-related cardiovascular disease.

The need for precision medicine

Obesity is a very heterogenous condition, available diagnosis tests and treatments for obesity-related comorbidities are not efficient in all patients. Thus, there is a need for more precise medicine. Precision medicine is defined as the concept of characterizing each patient separately to select more effective treatment. The recent advances in "-Omics" techniques has greatly helped to deeply characterize patient groups at several levels, such as genes, proteins, metabolites[122]. However, in a recent study, only 2.7% of gene loci were associated with BMI[123]. Furthermore, another genome wide association (GWAS) study showed that, in a population of 200,000 individuals, only 10.6% of coronary artery disease (CAD) could be explained by genetics[124]. This shows the need to integrate environment-derived cues into the characterization of high-risk individuals. Lipidomics, as performed in **chapter 5**, has already been used before to facilitate to reclassification of high-risk patients[125]. For instance, Fan *et al.* performed a comparison of metabolome profile between distinct subtypes of CAD and identified 89 differential metabolites classifying with an accuracy >90% in 2,324 patients[125]. A common limitation to such metabolomics approaches is the lack of causal assessment. Demonstration of causality of a marker in disease will not only facilitate development of specific therapeutic approaches, but it also warrants that any changes in marker level will reflect changes in disease risk. Investigating biomarker causality in disease development and progression can help understand which mechanistic pathways are altered and further design robust treatment. In an ideal situation, novel biomarkers could serve as risk predictors but could also be modifiable by specific therapies and subsequently reduce disease-risk. These risk factors could have the potential to better stratify risk population, where standard risk factors, such as CRP or LDL, lack precision. In this thesis (**chapter 5**), we have thoroughly assessed the implication of our lipid-signature in CVD development by investigating their presence in atherosclerotic plaques and their effect on macrophage functions using our "functionomics" platform. Further analyses on our predictive lipid-signature could help understand how these lipids dysregulate phagocytosis, how this is reflected in disease progression and how certain drugs could possibly reverse this phenotype to study risk reduction properties. In the future, biomarker research should be more centered on causality assessment as this allows better and more precise disease prevention.

Linking systemic inflammation to CVD risk using the MacroScreen: a monocyte centered view

Precision medicine uses many "-omics" platforms to deeply characterize patients, and our functionomics platform developed in **chapter 2** could help to further characterize patient phenotype by directly screening functionality of circulating monocytes after a cardiovascular event. In this thesis, we have used monocytes-derived macrophages from healthy volunteers, but one could think of adapting the platform to enable monocyte screening. AMI leads to an increased production and release of classical monocytes from the spleen, worsening atherosclerosis and, subsequently, the incidence of secondary CVD events during followup[118,126]. Moreover, the systemic blood environment was demonstrated to influence monocytes; for example, in patients with familial hypercholesterolemia, a genetic disorder leading to high level of circulating LDL, monocytosis was observed[127]. In addition, dyslipidemia also activated monocytes and regulated their adhesion to the endothelium[128]. Therefore, investigating the systemic environment in terms of monocyte functionality and linking it to secondary event would be of great interest. Monocytes represent 3-8% of total circulating leukocytes and are embedded in plasma, the patient's systemic environment[129]. Human monocytes are divided in three subsets based on their expression of CD14 and CD16. Classical monocytes ($CD14^{++}$ $CD16^{-}$), intermediate monocytes ($CD14^{+}$ $CD16^{+}$) and non-classical monocytes ($CD14^{+}$ $CD16^{++}$) have all been linked to CVD. In fact, classical[130] and intermediate[131] monocytes have been defined as independent risk predictors of CVD in separate cohorts, whereas $CD16^{+}$ monocytes have been correlated to mean intima-media thickness[130]. Moreover, recent research has shown that each monocyte subpopulation has distinct functions, e.g. classical and intermediate monocytes were shown to have high phagocytic capacities, whereas non-classical monocytes only had a limited phagocytic activity[132]. Characterizing the monocyte's functional phenotype using our HCA platform could help to determine how monocyte origin affects their functional profile, and whether monocytes from AMI patient are more prone to differentiate into functionally distinct macrophages than healthy patients. This could also help us to reveal and interfere with relevant mechanistic pathways involved in the differentiation of monocytes to functionally diverse macrophages.

Concluding remarks

In this thesis, we have developed a ready-to-use, high-content analysis platform able to detect macrophage functional heterogeneity in the context of CVD inflammation. This platform is constantly being improved and expanded by including other relevant macrophage functions, and by incorporating functionalities of other cell types relevant to atherosclerosis. In addition, we have proven that this platform can be deployed in different settings, such as the development of a reference map of macrophage functional activation, or in detection of AMI-linked macrophage functional and transcriptional signatures, opening new avenues for therapeutic research.

Macrophages transcriptional plasticity is now widely acknowledged, which has refashioned the previous oversimplified M1/M2 activation view. Using high content cell imaging, we demonstrated that macrophage transcriptional diversity has direct repercussions at the functional level. Furthermore, we revealed specific gene expression patterns linked to unique functions. This allowed us to build a unique function-based macrophage atlas. This atlas could, in the future, be used to directly predict macrophage functional activity in responses to drugs based on gene expression profile, and thus rapidly study drug efficacy.

Aside from their important role in atherosclerosis development and progression, macrophages are also key players in AMI repair. Exposure of monocyte-derived macrophages to AMI systemic environment appears to affect macrophage functions and this is also reflected in the transcriptional patterns. Furthermore, we successfully detected specific transcriptional programs associated with certain macrophage functions and poor prognosis.

Lastly, using our platform, we studied the functional implication of newly discovered biomarkers for the development of obesity-related CVD. Studying the causal relationship of biomarkers with CVD inflammation could help at discovering new therapeutic targets.

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Chapter 7

Summary | Samenvatting | Résumé

Summary

Cardiovascular diseases, mainly driven by atherosclerosis, are the leading cause of death in the world. Atherosclerosis is a chronic inflammatory and lipid-driven vascular disease, where inflammatory cells, lipids and fibrous tissue progressively accumulate in the inner wall of the arteries, forming an atherosclerotic plaque that can eventually rupture. This might restrict blood flow and cause myocardial infarction and/or stroke. Macrophages are instrumental in a variety of cardiovascular diseases, as they not only are the most abundant immune cell type in atherosclerosis, but also play a central role in ischemia-reperfusion injury and healing in the heart. Moreover, they are known to contribute to the low-grade inflammation typical of cardiometabolic diseases. The transcriptional heterogeneity and plasticity of macrophages have widely been demonstrated. However, to date, evidence of functional plasticity and its link to transcriptional activity is still missing. Having a detailed description of macrophage functional heterogeneity could help to understand their exact role under pathological condition. Therefore, in this dissertation, we examined the functional diversity of macrophages in the context of cardiovascular inflammation, such as atherosclerosis, acute myocardial infarction and cardiometabolic disease, and their potential role as disease sensors.

In **chapter 2**, we built a high-throughput platform able to detect key functions of macrophages in the context of cardiovascular inflammation. We developed and downscaled functional assays, all based on fluorescent probes, to detect a total of 19 functions, i.e. phagocytosis and apoptosis. We were able to show that macrophage transcriptional plasticity is directly translated to functional heterogeneity. Additionally, we generated a function-based atlas of human monocyte derived macrophage responses to a variety of established stimuli using our *in vitro* platform. The functional activation map can serve as a reference for future research on macrophage heterogeneity, but also to position disease-associated changes in functional pattern.

Next to their importance in atherosclerosis, macrophages are also key players of acute myocardial repair. **Chapter 3** investigated the use of our novel high-throughput platform to study the impact of systemic status of patients that suffered an acute myocardial infarction on macrophages. We discovered that, after exposure to acute myocardial infarction patient serum, macrophages exert specific functions, reflecting their activation state and cellular stress level. Moreover, RNA

sequencing analysis revealed specific genes highly correlating with acute myocardial infarction phenotype and clinical progression of the patient. Our results thus show that serum of patients with and acute myocardial infarction reprograms macrophage functionality, and this could help to understand how disease-compromised functions can have direct consequences on disease prognosis. This opens the way for new therapies to correct adverse functional changes.

In **chapter 4**, we described the use of the platform to examine functionality of macrophages after depleting an anti-apoptotic protein, Mcl-1, in a mouse model of atherosclerosis. Using our high-throughput platform, we showed that, macrophages lacking Mcl-1 showed a clear phenotype of increased lipid uptake capacity and apoptosis, which aligns with the phenotypic effects we observed in mice with Mcl-1 myeloid deficiency. Furthermore, we developed a novel assay for the platform to study the formation of giant cells, a hallmark of macrophages lacking Mcl-1 in our study.

In **chapter 5**, we studied the causal relationship of novel obesity-related CVD biomarkers and macrophage functions using our high-throughput platform. Obesity is one of the main risk factors of CVD, as it is linked to abnormal blood lipid profiles, and conventional classifiers for CVD are not accurate enough in risk prediction. Therefore, we performed lipidomics on two independent obese cohorts and discovered a new lipid signature, able to accurately predict disease outcome. We measured the functional profile of macrophages after exposure to these novel biomarkers and identified phenotypic changes, such as phagocytosis, hinting to a causal involvement in the disease process and rendering them potential therapeutic target for obesity-related CVD.

Finally, **chapter 6** addressed the main findings of this thesis and discusses their implications and future perspectives. This dissertation suggested that the novel platform able detect measure macrophage functional plasticity could, in the future, help to study the implication of macrophages in atherosclerosis and drug development.

Altogether, this thesis provided novel insights into the functional diversity of macrophages in cardiovascular disease inflammation, such as atherosclerosis, acute myocardial infarction and cardiometabolic diseases using a ready-to-use high-content analysis platform.

Samenvatting

Cardiovasculaire aandoeningen, voornamelijk het gevolg van aderverkalking, zijn de belangrijkste doodsoorzaak wereldwijd. Aderverkalking is een chronische ontsteking van de vaatwand, waarbij ontstekingscellen, vetten en fibreus materiaal zich opstapelen in de binnenste laag van een bloedvat. Dit leidt tot de vorming van een atherosclerotische plaque die uiteindelijk kan scheuren, wat de bloeddoorstroming kan beperken en een hartinfarct en/of beroerte kan veroorzaken. Macrofagen zijn van belang bij verschillende cardiovasculaire aandoeningen; niet alleen zijn ze het meest voorkomende celtype bij aderverkalking, ze spelen ook een centrale rol bij ischemia-reperfusie schade en herstelprocessen in het hart. Daarenboven is bekend dat ze bijdragen aan de laaggradige ontsteking kenmerkend voor cardiometabole aandoeningen. De transcriptionele verscheidenheid en de plasticiteit van macrofagen zijn uitvoerig aangetoond. Tot op vandaag is er echter geen bewijs voor een functionele plasticiteit en de daarbijhorende brug naar transcriptionele activiteit. Een gedetailleerde beschrijving van de functionele verscheidenheid van een macrofaag zou zijn precieze rol in ziekteprocessen kunnen verduidelijken. Om die reden hebben we in deze verhandeling de functionele diversiteit van macrofagen in de context van cardiovasculaire ontstekingsziekten, zoals aderverkalking, hartinfarct en cardiometabole aandoeningen, onderzocht, alsmede hun mogelijk gebruik als ziekte-sensoren.

In **hoofdstuk 2** bouwden we een high-throughput platform dat sleutelfuncties van macrofagen in een cardiovasculaire ontstekingscontext kan detecteren. We ontwikkelden en miniaturiseerden functionele testen, gebaseerd op fluorescente detectiemoleculen, zodat in totaal 19 functies, bijvoorbeeld fagocytose en apoptose, gemeten konden worden. We konden aantonen dat de transcriptionele plasticiteit in een macrofaag rechtstreeks relateert aan zijn functionele verscheidenheid. Daarnaast genereerden we met behulp van ons *in vitro* platform een op functie gebaseerde atlas van de reactiepatronen van humane monocyt-afgeleide macrofagen op een diverse set gekende stimuli. De functionele activatiekaart kan dienst doen als referentie voor toekomstig onderzoek naar macrofaag heterogeniteit, en ook als basis om ziekte-gerelateerde veranderingen in het functionele patroon op te positioneren.

Naast hun belangrijke rol in aderverkalking zijn macrofagen ook kritische spelers in het herstellen van het beschadigde hart na infarct. **Hoofdstuk 3** onderzocht of ons

nieuwe high-throughput platform geschikt was om de impact van de systemische omgeving van hartinfarctpatienten op macrofagen te bestuderen. We ontdekten dat macrofagen, na blootstelling aan serum van hartinfarctpatienten, specifieke functies vervulden die overeenkwamen met hun activatiestatus en cellulaire stressniveau. Daarenboven identificeerde RNA sequencing analyse bepaalde genen die heel sterk correleerden met het hartinfarct fenotype en met de klinische prognose van de patient. Onze resultaten laten zien dat het bloed van hartinfarctpatienten macrofagen programmeert tot het vervullen van andere functies. Ze kunnen bijdragen aan een beter begrip van hoe bepaalde functies die door ziekte worden gecompromitteerd onmiddellijke gevolgen kunnen hebben voor het verdere ziekteverloop. Dit zet de deur open naar nieuwe behandelingen die de ongewenste functionele veranderingen tegengaan.

In **hoofdstuk 4** beschrijven we gebruik van het platform om macrofaag functionaliteit te onderzoeken nadat een anti-apoptotisch eiwit, Mcl-1, werd verwijderd in een muizenmodel van aderverkalking. Met behulp van het high-throughput platform bewezen we dat macrofagen die Mcl-1 misten een duidelijk verhoogde vetopname capaciteit en verhoogde celdood vertoonden, gelijkaardig aan het fenotype geobserveerd in muizen met Mcl-1 deficientie in myeloide cellen. Daarnaast ontwikkelden we een nieuwe functionele test die aan het platform werd toegevoegd: de vorming van veelkernige reuzencellen, een in het oog springende eigenschap van de Mcl-1 deficiente macrofagen in deze studie.

In **hoofdstuk 5** bestudeerden we het oorzakelijk verband tussen nieuwe biomarkers voor overgewicht-gerelateerde cardiovasculaire aandoeningen en macrofaag functies met behulp van ons high-throughput platform. Overgewicht is een van de belangrijkste risicofactoren voor cardiovasculaire ziekten omdat het samenhangt met abnormale lipidenprofielen in het bloed. Standaard beslismodellen zijn hier niet voldoende accuraat in het voorspellen van cardiovasculair risico. Om die reden hebben we lipidomics uitgevoerd in twee onafhankelijke cohorten van mensen met overgewicht. We ontdekten een nieuwe lipidensignatuur die met grote precisie voorspelde hoe de ziekte verder verliep. We maten het functionele profiel van macrofagen na blootstelling aan deze nieuwe biomarkers en identificeerden fenotypische veranderingen, bijvoorbeeld in fagocytose, wat er mogelijks op wijst dat deze biomarkers oorzakelijk betrokken zijn in het ziekteproces en dus kandidaten kunnen zijn in het behandelen van overgewicht-gerelateerde cardiovasculaire aandoeningen.

Hoofdstuk 6 tenslotte keek terug op de belangrijkste bevindingen van deze thesis en bespreekt hun implicaties en toekomstperspectieven. Deze verhandeling suggereerde dat het nieuwe platform dat de functionele plasticiteit van macrofagen in kaart kan brengen in de toekomst zou kunnen worden ingezet om de betrokkenheid van macrofagen in aderverkalking en medicijnontwikkeling te bestuderen.

Samenvattend verschaft deze thesis nieuwe inzichten in de functionele diversiteit van macrofagen in cardiovasculaire aandoeningen, zoals aderverkalking, hartinfarct en cardiometabole ziekten, door gebruik te maken van een operationeel high-content analyse platform.

Résumé

Les maladies cardiovasculaires, souvent causées par des plaques d'athérome, sont la principale cause de décès dans le monde. L'athérosclérose est une maladie vasculaire inflammatoire chronique dans laquelle des cellules inflammatoires, des lipides et des tissus fibreux s'accumulent progressivement dans la paroi interne des artères, formant une plaque d'athérome qui risque de finalement se rompre. Le flux sanguin est alors restreint et peut causer un infarctus du myocarde et/ou un accident vasculaire cérébral. Les macrophages ont un rôle déterminant dans plusieurs maladies vasculaires, car ils constituent non seulement le type de cellules immunitaires le plus abondant dans les plaques d'athérome, mais ont aussi un rôle central dans les lésions d'ischémie-reperfusion et la régénération des tissus cardiaques. Ils sont également connus pour contribuer à la légère inflammation caractéristique des maladies cardiométaboliques. L'hétérogénéité transcriptionnelle et la plasticité des macrophages ont été largement démontrées. Cependant, à ce jour, aucune preuve de plasticité fonctionnelle liée à leur activité de transcription n'a été prouvée. L'accès à une description détaillée de l'hétérogénéité fonctionnelle des macrophages pourrait aider à mieux comprendre leur rôle précis dans le développement de maladies cardiométaboliques. Par conséquent, dans cette thèse, nous avons étudié la diversité fonctionnelle des macrophages dans le contexte des maladies cardiovasculaires, telle que l'athérosclérose, l'infarctus aigu du myocarde et les maladies cardiométaboliques.

Dans le **chapitre 2**, nous avons développé une plateforme à haut débit capable de détecter certaines fonctions clés des macrophages dans le contexte de l'inflammation cardiovasculaire. Nous avons mis au point des tests fonctionnels, tous basés sur des sondes fluorescentes, afin de détecter un total de 19 fonctions, telles que la phagocytose ou l'apoptose. Nous avons démontré que la plasticité transcriptionnelle des macrophages est directement traduite en hétérogénéité fonctionnelle. De plus, en utilisant notre plateforme *in vitro,* nous avons généré un atlas de fonctionnalités de macrophages, dérivés de monocytes humains, qui répertorie leur réponse à une variété de stimuli. Ce spectre d'activation fonctionnelle pourra servir de référence pour des recherches futures sur l'hétérogénéité des macrophages, mais également pour classer les changements de fonction associés à certaines maladies dans notre atlas.

En plus de leur importance dans l'athérosclérose, les macrophages sont également des acteurs clés de la cicatrisation cardiaque à la suite d'un infarctus aigu du

myocarde. Dans le **chapitre 3** nous avons utilisé notre nouvelle plateforme à haut débit pour étudier l'impact de l'état systémique des patients ayant subi infarctus du myocarde aigu sur les macrophages. Nous avons découvert qu'après être avoir été exposés au sérum de patients souffrant d'un infarctus aigu du myocarde, les macrophages exerçaient des fonctions spécifiques reflétant leur état d'activation et leur niveau de stress cellulaire. De plus, l'analyse du séquençage de l'ARN a révélé des gènes spécifiques étroitement corrélés au phénotype d'infarctus aigu du myocarde et à l'évolution clinique du patient. Nos résultats montrent donc que le sérum des patients atteints d'un infarctus aigu du myocarde reprogramme la fonctionnalité des macrophages. Cela pourrait aider à comprendre comment les dysfonctions cellulaires liées à certaines maladies peuvent avoir des conséquences directes sur le pronostic de la maladie et aider à développer de nouvelles solutions thérapeutiques pour rétablir les fonctions cellulaires.

Dans le **chapitre 4**, nous avons décrit l'utilisation de cette plateforme pour analyser la fonctionnalité des macrophages suite à la déplétion d'une protéine anti-apoptotique, Mcl-1, dans un modèle murin d'athérosclérose. En utilisant notre plateforme à haut débit, nous avons démontré que les macrophages dépourvus de Mcl-1 présentaient un phénotype précis d'augmentation de la capacité d'absorption des lipides et d'apoptose, ce qui correspond aux effets phénotypiques observés chez les souris présentant un déficit myéloïde en Mcl-1. De plus, nous avons développé un nouveau test pour la plateforme permettant d'étudier la formation de cellules géantes, caractéristique des macrophages dépourvus de Mcl-1 dans notre étude.

Dans le **chapitre 5**, nous avons étudiés la relation de cause à effet entre nouveaux marqueurs biologiques pour le développement de maladies cardiovasculaires et macrophages chez les obèses, en utilisant notre plateforme à haut débit. L'obésité est l'un des principaux facteurs de risque des maladies cardiovasculaires, car elle est liée à des profiles lipidiques sanguins anormaux, et les marqueurs classiques des maladies cardiovasculaires ne permettent pas une prédiction du risque suffisamment précise. Par conséquent, nous avons effectué une analyse lipidomique sur deux cohortes indépendantes obèses et avons découvert une nouvelle signature lipidique, capable de prédire avec précision l'évolution de la maladie. Nous avons mesuré le profil fonctionnel des macrophages après exposition à ces nouveaux marqueurs biologiques et identifié des modifications

phénotypiques, telles que la phagocytose, suggérant une implication causale dans le processus de la maladie.

Enfin, le **chapitre 6** a traité des principales conclusions de cette thèse et discuté de leurs implications et perspectives. Cette thèse suggère que la nouvelle plateforme capable de détecter et mesurer la plasticité fonctionnelle des macrophages pourrait, à l'avenir, aider l'étude de l'implication des macrophages dans les maladies cardiovasculaires et le développement de médicaments.

En conclusion, cette thèse a fourni de nouvelles informations sur la diversité fonctionnelle des macrophages dans le contexte de l'inflammation vasculaire liées au pathologies cardiovasculaires, telles que l'athérosclérose, l'infarctus aigu du myocarde et les maladies cardiométaboliques en utilisant une plateforme d'analyse à haut débit prête à l'emploi.

Chapter 8

Valorization

Valorization, defined as a "process that aims at enhancing societal impact"[1], is an important aspect of (academic) research. It is crucial for scientists to make the knowledge acquired during a PhD available and accessible to society both by transferring the results to potential stakeholders (e.g. pharmaceutical companies or other research centers) and if opportune, by translating them into possible news products that society could benefit from. In this chapter, we will present how the work described in this thesis has and will be transferred to society and how it, in the future, could help to reduce the socio-economic burden of cardiovascular disease.

Cardiovascular disease

Cardiovascular disease (CVD), mainly driven by atherosclerosis, is still the leading cause of death in the world[2]. In 2016, almost 18 million people died from CVDs globally, mostly attributable to coronary artery disease and stroke[3]. CVD has become an obvious economic burden, as the European Heart Network has estimated the total CVD-related cost to be €210 billion per year[2]. The main costs generated from CVD are related to healthcare of CVD patients (healthcare, medication, hospitalization), but also indirect costs, such as patient work productivity loss and intangible costs, e.g. costs of pain and suffering, are substantial. This highlights the need to rapidly discover new therapies or strategies to cure and study CVD. CVD research centers on two major topics: (1) investigations on mechanistic pathways involved in the pathophysiology of CVD; (2) translational studies aimed at CVD diagnosis, prevention and intervention, often based on findings from the basic science studies. In this thesis, we aimed to cover both areas of research, with **chapter 3 and 5** focusing on prediction and prevention of CVD, and **chapter 2 to 4** mainly focusing on understanding functional pathways involved in CVD inflammation.

As described above, valorization can be accomplished via two main tracks: "societal transfer of results" and "economical transfer". We have achieved the former by presenting the main results described in this thesis at (inter)national conferences. In addition, making scientific knowledge accessible to everyone is another key aspect of societal transfer. During my PhD, I was selected to give a Ted-talk at the PhD-student course organized by the Dutch Heart Foundation in 2017, improving my communication skills to present scientific research to a non-scientific audience. We further intend at disseminating the acquired knowledge to cardiovascular

patients by giving lectures via the Harteraad (https://www.harteraad.nl/). Moreover, we aim at publishing the main results of this thesis in peer-reviewed, preferably open access, scientific journals; in fact **chapter 4 has** already been published in an open-access journal, and **chapter 5** is under review at European Heart Journal. Datasets will be deposited in repositories that are publicly available, such as GEO. The published results could then be used as starting point or reference for the scientific community in future research. As to the second track, the translation of results into potential diagnostic or therapeutic strategies to detect and treat CVD, we aim at conducting an active Intellectual Property IP protection strategy to allow successful knowledge exploitation of the projects described in this thesis. Together with carefully selected external partners, such as biotech companies, we will develop a cost-effective utilization strategy of, for example, our MacroScreen platform or our predictive lipid-signature. The below paragraphs will expand the different valorization opportunities of the main results of this thesis.

Bringing the predictive lipid signature to the market

In **chapter 5**, we have profiled circulating lipids in two independent obese patient cohorts and built a prediction model of future CVD. Obesity is a well-known risk factor for CVD; it accounts for approximately 30% of the cases[4]. This is explained by the "bi-angular relationship" between CVD and obesity; obesity is both directly and indirectly linked to CVD due to the abnormal blood lipid profile and to its relation to other important risk factors of CVD, such as hypertension and diabetes, respectively. However, current risk factors, such as cholesterol levels, blood pressure or glucose levels, lack predictive power for the development of CVD in the obese population to be useful for individual risk assessment. The lipid-signature described in **chapter 5,** has unprecedented predictive power for future CVD and could be a valuable tool for early detection of high-risk obese patients, motivating them to lifestyle changes, instead of costly life-long medication, such as statins, or pricey surgical operations, thereby reducing the economic burden of CVD.

Current risk prediction is performed by calculating risk scores, based on many clinical parameters. Ideally, our multi-lipid CVD risk signature could be translated into a fast and accurate *in vitro* diagnostic (IVD) test for plasma samples of obese patients, which then would be used in the clinics to further help in individual CVD prediction. However, translating these findings to an accurate IVD test requires further study to prove the diagnostic value of our prediction model. First, future

studies will have to reproduce and validate these findings in even larger cohorts, as patient numbers still were rather limited for both our discovery (n=78) and validation cohort (n=200). Second, current lipid profiling is performed using mass spectrometry. This technique requires specialized infrastructure and is expensive and time-consuming, making it not feasible for point-of-care testing. Therefore, further studies aim at developing an IVD assay, which could for example rely on aptamer technology, to detect our lipid signature. Aptamers arrays are spotted with single strand DNA or RNA (ssDNA/ssRNA), that have a unique tertiary structure able to specifically bind to a single lipid[5] and have already showed their potential for biomarker discovery[6]. Together with specialized companies, such as Novaptech or Eurogentec, which have experience with production of aptamer-based arrays for diagnostic purposes, we will develop and validate a diagnostic product at different location sites and on a high number of biological samples. This easy-to-use diagnostic tool could directly be used by general practitioners and in the hospital, and lead to early identification of high-risk CVD patients, early individualized intervention and CVD prevention. Next to this, this diagnostic tool will help monitor medical treatment efficacy and will be used as an outcome measurement for clinical trials and intervention studies.

Potential therapeutic solutions

Current medication for CVD involves lipid lowering therapy, including statins[7], and antihypertensive treatment. Despite their effectiveness with a 20% reduction in CVD risk, lipid lowering therapies are unable to prevent future cardiovascular events in a significant number of patients[8]. More recently, intervention in IL-1β signaling with a monoclonal antibody on top of statin treatment, in subjects with low grade inflammation led to a supplementary reduction of 14% in CVD incidence[9]. However, this was accompanied by increased infection numbers and, importantly, no difference in overall mortality was observed after IL-1β antibody treatment. This emphasizes the need to discover new therapeutic targets to decrease CVD risk and develop new diagnosis tools to enable detection of high-risk profile at a reversible stage of the disease.

In **chapter 5**, we discovered an 18-lipid signature predicting the incidence of CV events in the obese population. Using our MacroScreen platform, we could show that some of our lipid predictors were affecting key functions of macrophages, the main immune cell type implicated in atherosclerosis, hinting at a possible causal

relationship. Moreover, these lipids were enriched in unstable, as compared to stable human atherosclerotic plaques. Future research aims at studying the implication of these lipids in disease development and progression, by interacting with their synthesis *in vivo* for example. This could potentially lead to the discovery of new key players in atherosclerosis related CVD and, subsequently, open new avenues for therapeutic solutions. As statins fail at reducing the risk in a majority of patients, despite their profound effect on cholesterol and, indirectly, on other lipids levels[10], novel drugs should be generated. To target specific lipids, one could think of generating molecules interacting with their synthesis pathway by inhibiting a key enzyme, for example.

Another potential target for future intervention was unveiled in **Chapter 4,** where we investigated the impact of a deficiency of Mcl-1, an anti-apoptotic protein, on atherosclerosis. We were able to convincingly show that Mcl-1 is essential for survival of macrophages, as well as neutrophils. Additionally, Mcl-1 was implicated in macrophage lipid uptake and subsequent macrophage fusion capacity, resulting in the formation of multinucleated giant cells (MGCs). Evidence for the presence of MGCs in human atherosclerotic plaques is scarce; and the description of a clear role in the pathogenesis of disease is lacking. One study described MGCs to express high level of cathepsins, enabling the rupture of the elastic lamina and facilitating the migration of smooth muscle cells during atherosclerosis[11]. Clearly, more research is needed to understand the implication of MGCs in atherosclerosis and their exact role in disease development and progression. Second, small molecules targeting Mcl-1 already exist, as Mcl-1 is an interesting target to treat cancer, due its high expression in tumor cells and role in cell survival[12,13]. However, as we showed, targeting Mcl-1 in an animal model of atherosclerosis led to severe neutropenia, and had no effect on atherosclerotic lesion size. Therefore, targeting Mcl-1 will most probably not lead to successful therapeutic strategies; yet, this could be used as a model of neutropenia or giant-cell enriched atherosclerosis to further investigate MGCs implication in atherosclerosis.

Novel human centered validation strategies in CVD research

Funding for cardiovascular disease research is sizable[14]. Between 2010 and 2012, funding for CVD research (both academic and in private company setting) amounted around €876 million in Europe[14]. CVD research is nowadays mainly performed on animal models, which are expensive, and time consuming and do

not, in every aspect, represent human disease. Therefore, building a humanized test platform to capture cardiovascular inflammation, such as atherosclerosis and AMI, in *vitro* (**chapter 2 to 5**) could help reducing the costs of CVD fundamental research and drug development. First, **chapter 2** showed that macrophages are functionally reprogrammed after exposure to various stimuli, such as several cytokines or fatty acids. This led to the identification of a macrophage activation functional map, which could serve as reference for further studies. Second, **chapter 3** investigated the functional responses of macrophages after exposure to AMI patient serum. We observed that macrophages functionally respond to AMI systemic environment and these functionalities were also linked to poor prognosis, four months after the infarct, showing the potential utility of this platform as a fast and cheap drug screening platform. One could think of assessing the effect of compound libraries on key functions of macrophages related to AMI systemic environment, and subsequently select the most interesting target for further analysis. Importantly, our platform will never serve a diagnostic purpose, as it is too time-consuming and labor intensive for clinical use.

Altogether, this demonstrates that our platform has great potential to quickly study the implication of macrophages in CVD inflammation and to screen for modulators of critical macrophage functions for drug development purposes. The platform is ready-to-use, however future research aims at improving its physiological complexity, as it is currently based on two-dimensional monolayers of cells and this lacks the cell-cell interaction and 3-dimension aspects of the human body.

Conclusion

In conclusion, the work presented in this thesis aimed to tackle the socio-economic burden of CVD at several levels. We have developed a new tool, able to characterize macrophage functions in the context of CVD inflammation. This tool could, in the future, help reduce the use of animal models and improve the translational problem linked to animal studies. Furthermore, we have identified a predictive lipid signature for the development of obesity related CVD, which shows promise for stratifying obese individuals with increase CVD risk and may also have therapeutic potential. Lastly, we have investigated the impact of Mcl-1 in atherosclerosis, and its implication in giant cell formation opening new avenues to manipulate MGC formation in atherosclerosis, and potentially other diseases where MGCs play a role

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Appendices

List of abbreviations

2D	2-dimensional
3D	3-dimensional
AMI	Acute myocardial infarction
AT	Adipose tissue
ASC	Apoptosis-associated speck like
AUC	area under the curve
ABCA1	ATP binding cassette A1
B-cell	B lymphocyte
BP	blood pressure
BMI	Body mass index
BMDM	Bone marrow derived macrophage
BMT	Bone marrow transplantation
CRP	C reactive protein
CCA	Canonical correlation analysis
CVD	Cardiovascular disease
CEA	Carotid endarterectomy
CER	Ceramide
CCL	Chemokine (C-C motif) ligand
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
CE	Cholesteryl ester
CD	Cluster of differentiation
CODAM	Cohort on Diabetes and Atherosclerosis Maastricht
CAD	Coronary artery disease
CHD	Coronary heart disease
CCM	Correlation coefficient matrix
DC	dendritic cell
DM	diabetes mellitus
DAG	Diacylglycerol
EC	Endothelial cells
FH	Familial hypercholesterolemia
FCS	Fetal calf serum
FRS	Framingham study
GO	Gene ontology
GWAS	Genome wide association study
GC	Glucocorticoids
GM-CSF	Granulocyte macrophage colony stimulating factor
HE	Hematoxylin and eosin

HCA	high content analysis
HDL	High-density lipoprotein
iMacs	Iduced pluripotent stem cells derived macrophages
IC	Immune complexe
iPSC	Induced pluripotent stem cells
IFNβ	Interferion-β
IFNg	Interferon-g
IL	Interleukin
KO	Knock-out
LA	Lauric acid
LiA	Linoleic acid
LPS	Lipopolysaccharide
LR	Logistic regression
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPC	Lysophosphaditycholine
LPA	Lysophosphatidic acid
M-CSF	Macrophage colony stimulating factor
MMP	Metalloprotease
mtDNA	Mitochondrial DNA
MAG	Monoacylglycerol
MCP-1	Monocyte chemoattractant protein 1
MDM	Monocyte-derived macrophage
MGC	Multinucleated giant cell
Mcl-1	Myeloid cell leukemia 1
MI	Myocardial infarction
NK	Natural killer cell
NET	Neutrophil extracellular trap
NMOC	Non-mitochondrial oxygen consumption
NLR	Nucleotide-binding oligomerization domain-like receptors
ORO	Oil Red O
OA	Oleic acid
oxLDL	oxidized low-density lipoprotein
PA	Palmitic acid
P3C	Pam3CSK4
PLS-DA	Partial least square discriminant analysis
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PMN	Polymorphonuclear leukocyte
PCA	Principal componenet analysis
PCSK9	Proprotein converstase subtilisin/kexin type 9
PGE2	Prostaglandin E2
RF	Random forest
ROS	Reactive oxygen species
qPCR	Real time polymerase chain reaction
ROC	Receiver operator characteristic
RPMI	Roswell park memorial institute medium
SMC	Smooth muscle cell
SM	Sphingomyelin
SD	Standard deviation
SEM	Standard error of the mean
sLPS	Standard LPS
SA	Stearic acid
SHIP	Study of Health in Pomerania
SVM	Support vector machine
T-cell	T lymphocyte
TUNEL	Terminal deoxytransferase dUTP nick-end labeling
3D	Three dimensions
TPP	TNF+PGE2+P3C
TC	Total cholesterol
TAG	Triacylglycerol
TG	Triglycerides
TAM	Tumor associated medium
TNF-α	Tumor necrosis factor α
2D	Two dimensions
T2DM	Type 2 diabetes mellitus
upLPS	Ultra-pure LPS
VCAM	Vascular cell adhesion molecule
vSMC	Vascular smooth muscle cell
VLDL	very-low density lipoprotein
WGCNA	Weighted gene co-expression network analysis
WTD	Western type diet
WBC	White blood cells

WT	Wild type

Appendices

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Margaux

Appendices

Curriculum Vitae

Personal information

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Education

09/2015 - 11/2019	**PhD training in biomedical sciences** Dept. Of Pathology, Maastricht University Medical Center The Netherlands
09/2012-06/2014	**Biomedical Sciences (MSc)** Maastricht University The Netherlands
09/2009-06/2012	**Biotechnology (Bsc) - Magna Cum Laude** La Haute Ecole de la Province de Liège Belgium

Research/Work experience

11/2019 – now	**R&D Project Manager** Diagenode Diagnostics Liège, Belgium
09/2015 - 11/2019	**PhD training in biomedical sciences** Dept. Of Pathology, Maastricht University Medical Center The Netherlands Promoter: Prof. Dr. Erik. AL Biessen co-promoter: Dr. Judith C Sluimer and Dr. Lieve Temmerman The Macroscreen Platform: Capturing Cardiovascular Disease Inflammation In Vitro
10/2013-06/2014	**MSc Senior practical training** Dept. Of Cardiology, Maastricht University, The Netherlands Title: A Deeper Look Into the Heart during Sepsis in the Mouse
02/2013-07/2013	**MSc Junior practical training** Dept. Of Neurology, Maastricht University, The Netherlands

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09/2011-01/2012	**Bachelor practical training** Dept. Of Oncology, Janssen Pharmaceutica, Beerse, Belgium Title: Identification and Characterization of Cell Lines Expressing a Specific Tyrosine Kinase and Development of Cellular Assays for Screening Small Compounds

Additional Activity

04/2019	**International ESM-EVBO 2019 conference** Organizer of the Young Investigator Program
2016-2019	**PhD Academy Maastricht** Social Committee member
2015-2019	**PhD representative committee "I'MCARIM"** Chair of the committee

Publications

Low human and murine Mcl-1 expression leads to a pro-apoptotic plaque phenotype enriched in giant-cells.
MAC Fontaine, MM Westra, I Bot, H Jin, AJPM Franssen, M Bot, SCA de Jager, I Dzhagalov, Y-W He, BJM van Vlijmen, MJJ Gijbels, CP Reutelingsperger, TJC van Berkel, JC Sluimer, L Temmerman, EAL Biessen
Scientific Reports, 2019

A Novel Plaque Enriched Long Non-coding RNA in Macrophage Phagocytosis Regulation (PELATON)
J Hung, JP Scanlon, AD Mahmoud, J Rodor, M Ballantyne, **MAC Fontaine**, L Temmerman, J Kaczynski, K Connor, R Bhushan, EAL. Biessen, DE Newby, JC Sluimer, AH Baker
ATVB, 2019

Cardiovascular event prediction by machine learning: identification of a blood-based lipid signature to stratify obese subjects
MAC Fontaine, T Aliyev, R Sikkens, MMJ van Greevenbroek, CJH van der Kallen, CG Schalkwijk, CDA Stehouwer, J Dehairs, R Derua, E Waelkens, M Nauck, E Hammer, U Völker, A Henneman, M Dörr, H Völzke, S Zylla, JC Sluimer, Y Mengerink, L Temmerman, EAL Biessen.
Submitted

Towards a function-based model of macrophage plasticity: the MacroScreen platform
MAC Fontaine, H Jin H, T Ulas, J Schultze, J Huckriede, M Rousch, E Wijnands,JC Sluimer,L Temmerman, EAL Biessen
In Preparation

The systemic acute myocardial infarction environment reprograms human macrophages
MAC Fontaine, H Jin, M Gagliardi, M Rousch, E Wijnands, G Andersen, Bente Halvorsen, EAL Biessen, L Temmerman
In Preparation

Awards and selected talks

May 2019	**European Atherosclerosis Society (EAS) Meeting 2019. Maastricht, the Netherlands** Selected e-poster presentation: "Barcoding the lipidome in the obese: who's at increased risk for the development of cardiovascular disease?"
April 2019	**ESM-EVBO2019 conference. Maastricht, the Netherlands** Selected oral presentation: "Barcoding the lipidome in the obese: who's at increased risk for the development of cardiovascular disease?"
Nov. 2018	**Best poster prize** CARIM symposium day, Maastricht, The Netherlands
Nov. 2018	**Best poster prize** Pathology research day, Valkenburg, The Netherlands
Oct. 2018	**Poster prize** PhD Training course, Dutch Heart Foundation, Papendal, The Netherlands
April 2018	**24th Annual Scandinavian Atherosclerosis Conference, Humlabaek, Denmark** Selected oral presentation: "Barcoding the adipokines in the obese: Who's at risk for the development of cardiovascular disease?"

Nov. 2017	**1st oral presentation prize** Pathology research day, Valkenburg, The Netherlands
Nov. 2017	**Netherlands Vascular Biology meeting, Biezenmortel, the Netherlands** Selected oral presentation: "Barcoding the adipokines in the obese: Who's at risk for the development of cardiovascular disease?"
Oct. 2017	**Poster prize** PhD Training course, Dutch Heart Foundation, Papendal, The Netherlands
Oct. 2017	**Audience prize oral presentation** PhD Training course, Dutch Heart Foundation, Papendal, The Netherlands