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Phenotyping of Circulating Monocytes in Coronary Artery Diseases

Dissertation for applying
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Contents

Abbreviations

A

ACS	Acute coronary syndrome
AHA	American Heart Association
ALD	Alcoholic liver disease
ALT	Alanine transaminase
AP	Alkaline phosphatase
AST	Aspartate transaminase

B

BMI	Body mass index
BNP	B-type natriuretic peptide
BSA	Bovine serum albumin

C

CAD	Coronary artery disease
CANTOS	Canakinumab Anti-Inflammatory Thrombosis Outcomes Study
cDNA	Complementary deoxyribonucleic acid
CHD	Coronary heart disease
CI	Confidence interval
CIRT	Cardiovascular Inflammation Reduction Trail
CKD	Chronic kidney disease
Ct	Cycle threshold
CVD	Cardiovascular disease

D

DC	Dendritic cells
DM	Diabetes mellitus
DMSO	Dimethylsulfoxide

DNA	Deoxyribonucleic acid

E

EB	Ethidium Bromide
ECLIA	Electrochemiluminescence immunoassay
EDTA	Ethylene diamine tetraacetic acid
e.g.	for example
ELISA	Enzyme-linked immunosorbent assay
EMIL	**E**chinococcus **M**ultilocularis and other medical d**i**seases in **L**eutkirch
etc.	et cetera

F

FCS	Fetal calf serum
FDR	False discovery rate
FFA	Free fatty acids
FGF-21	Fibroblast growth factor 21
FL	Fatty liver disease
FLI	Fatty liver index
FPG	Fasting plasma glucose
FSC	Forward scatter

G

GGT	γ-glutamyl transferase
GM-CSF	Granulocyte colony stimulating factor

H

HbA1c	Hemoglobin A1c
HDL	High-density lipoprotein
HDL-C	High-density lipoprotein cholesterol
HIV	Human immunodeficiency virus

HLH	Helix-loop-helix
HOMA-IR	Homeostasis model assessment-estimated insulin resistance index
hs-CRP	High sensitive C-reactive protein

I

ICAM-1	Intercellular adhesion molecule 1
i.e.	id est
IFN	Interferon
IHD	Ischemic heart disease
IL	Interleukin
IPA	Ingenuity pathways analysis
IR	Insulin resistance

L

LDL	Low-density lipoprotein
LDL-C	Low-density lipoprotein cholesterol
LPS	Lipopolysaccharide
LURIC	LUdwigshafen RIsk and Cardiovascular Health

M

MCP-1/CCL2	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
MFI	Median fluorescence intensity
MI	Myocardial infarction
MMPs	Matrix metalloproteinases

N

n.s.	Not significant
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis

NK	Nature killer

O

OR	Odds ratio
ox-LDL	Oxidised LDL

P

PAI-1	Plasminogen activator inhibitor 1
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor

Q

qRT-PCR	Quantitative real-time reverse transcription polymerase chain reaction

R

RANTES,	Regulated upon activation, normal T-cell expressed and secreted
RCPH	Research Center for Prevention and Health
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RTC	Reverse-transcription controls

S

SD	Standard deviation
SE	Standard error
SHBG	Sex hormone binding globulin
SSC	Side scattor

T

T2DM	Type 2 diabetes mellitus
TAE	Tris-Acetate-EDTA
TG	Triglyceride
TGF	Transforming growth factor
TLRs	Toll like receptors
TNF	Tumor necrosis factor

V

VCAM-1	Vascular cell adhesion molecule 1
vs.	versus

W

WC	Waist circumstance
WHO	World Health Organization
WHR	Waist-to-hip ratio

β	Standardized regression coefficient

1. Introduction

1.1. A short overview of Coronary artery disease (CAD)

Coronary artery disease (CAD) also known as coronary heart disease (CHD) or ischemic heart disease (IHD) is characterized by atherosclerosis in the epicardial coronary arteries. According to the World Health Organization (WHO) 2013 statistics report based on CAD deaths world-wide between 1995 and 2009, CAD (Finegold et al. 2013) is the single largest cause of death, causing 12.7% of total global mortality. Notably as well, a 20-fold CAD mortality difference between countries was observed (Finegold et al. 2013). The American Heart Association (AHA) 2014 statistics report (Go et al. 2014) showed that the 2010 overall rate of death attributable to cardiovascular disease (CVD) was 235.5 cases per 100,000 and the death rates attributable to CVD was 31.9% in the United States. In particular CAD alone caused around 1 of every 6 deaths in 2010 (Go et al. 2014). Although CAD mortality rates have declined in many high-income countries, the burden of disease remains high. It is estimated that approximately every 34 seconds, one American has a coronary event, and around every minute and 23 seconds, an American will die of a coronary event (Go et al. 2014). Therefore, a better understanding of the complex pathogenesis of CAD is required for the identification of new therapy targets.

1.2. Pathophysiology of CAD

CAD is a chronic process associated with formation and progression of the so-called atherosclerotic plaques throughout life. The process of atherosclerosis is depicted in Figure 1. The most widely accepted theory of the pathophysiology is vascular low grade inflammation, and the vascular inflammation plays a critical role in formation of atherosclerosis genesis, activity and plaque stability. Viruses as well as numerous bacterial signatures such as nucleic acids and peptidoglycan have been observed in human atherosclerotic plaques (Laman et al. 2002). A broad variety of bacterial colonization have been detected in atherosclerotic lesions of

patients with CAD (Ott et al. 2006). Another evidence for the presence of inflammation in CAD is that a significant number of macrophages are seen in ruptured plaques. In clinical practice, elevated levels of high sensitive C-reactive protein (hs-CRP) was found in CAD patients without systemic inflammation, and hs-CRP has been considered as a prognostic biomarker for cardiovascular events (Sabatine et al. 2007). In addition, several ongoing studies including Canakinumab Anti-InflammatoryThrombosis Outcomes Study (CANTOS) and Cardiovascular Inflammation Reduction Trial (CIRT) directly investigate the potential impact of anti-inflammatory treatments in reducing vascular events (Ghattas et al. 2013). Recently, a report from the population-based prospective cohort at the Danish Research Center for Prevention and Health (RCPH) combined meta-analysis indicated that several different pro-inflammatory cytokines (Interleukin (IL)-6, IL-18, etc.) are each associated with CAD risk independent of conventional risk factors. These findings provide further evidence of the inflammation hypothesis in CAD (Kaptoge et al. 2014).

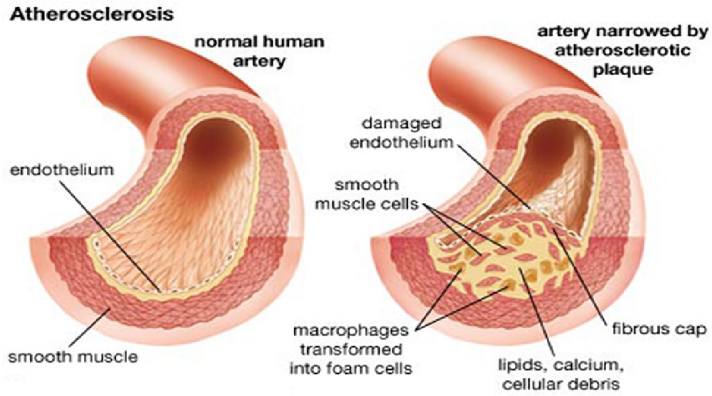

Figure 1 The process of atherosclerosis. The damaged endothelium caused by the oxidized LDL molecules triggers a cascade of immune responses, fatty streaks containing atherogenic lipoproteins and macrophage foam cells form between the endothelium and internal elastic lamina. With the development of fatty streaks, an intermediate lesion containing lipid core, layers of smooth muscle and connective tissue matrix eventually forms a fibrous cap. Plaque rupture exposes the impaired lumen, results in platelet adherence, aggregation, and progressive luminal narrowing. Taken from http://www.tappmedical.com/atherosclerosis.htm.

1.3. Major risk factors for CAD

CAD has a number of well-known risk factors. Generally, they can be classified as: fixed factors (such as age, family history, race (Meadows et al. 2011), etc.) and

modifiable factors (such as smoking (Rea et al. 2002), hypertension (Allen et al. 2012; Chobanian et al. 2003; Vasan et al. 2001), obesity (Rexrode et al. 1998), type 2 diabetes mellitus (T2DM) (Howard et al. 2002; Paynter et al. 2011), dyslipidemia (LaRosa et al. 2005), non-alcoholic fatty liver disease (NAFLD), etc.). They also can be divided into traditional risk factors and non-traditional risk factors. The major risk factors for CAD are summarized in Figure 2.

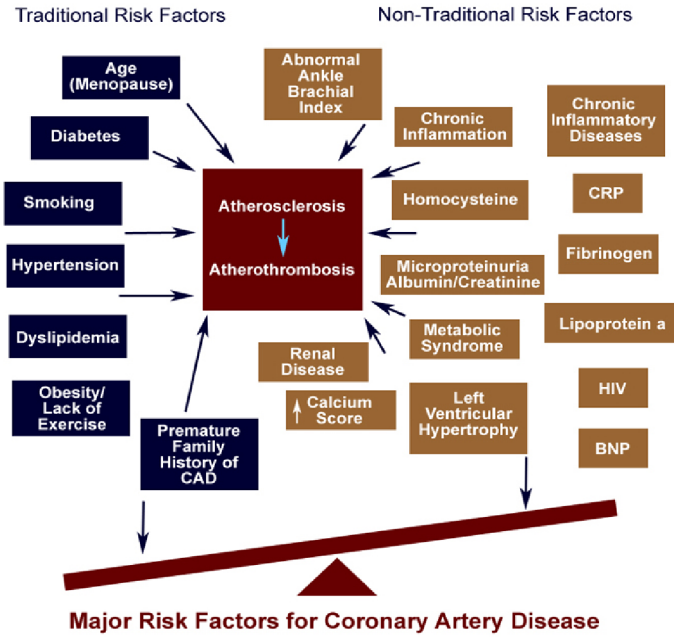

Figure 2 Major risk factors for coronary artery disease. The traditional risk factors provide greater contribution than the expanding list of non-traditional biomarkers for predicting future cardiovascular events. BNP, B-type natriuretic peptide; CRP, C-reactive protein; CAD, coronary artery disease; HIV, human immunodeficiency virus. Taken from http://emedicine.medscape.com/article/164163.

1.4. Non-alcoholic fatty liver disease (NAFLD) and CAD

1.4.1. NAFLD as an independent risk factor for CAD

NAFLD is characterized by a spectrum of liver disease that encompasses simple steatosis, steatohepatitis, fibrosis, cirrhosis and hepatocellular carcinoma. NAFLD, as rapidly becoming one of the most prevalent liver disease (around 20%-30%) (Anstee et al. 2011; Browning et al. 2004; Cobbold et al. 2010; Musso et al. 2011; Ratziu et al. 2010) worldwide, has been highlighted as an independent risk factor for CAD even in the absence of metabolic syndrome and confounding disease (hypertension, diabetes mellitus (DM), etc.)(Gastaldelli et al. 2009). Several studies

reported that NAFLD increases the instability of coronary plaques, the risk of having coronary calcification and developing T2DM (Assy et al. 2010; Gastaldelli et al. 2009; Sung et al. 2012). Recently two long-term population-based cohort studies have provided more evidence to clarify that NAFLD is an early independent predictor for carotid atherosclerotic disease and improvement of NAFLD reduce the risk of developing DM (The International Liver Congress, 2014). As a modifiable and novel non-traditional risk factor for CAD, NAFLD is now attracting more attention.

1.4.2. The pathophysiological links between NAFLD and CAD

The pathophysiological links between NAFLD and CAD are intricate. They are always interacted with T2DM together. Potential mechanisms of the interplay between the three disease conditions may originate from the development of expanded and inflamed visceral adipose tissue, which can provide a series of factors potentially involved in NAFLD as well as atherogenesis and insulin resistance (IR), i.e. decreased levels of adiponectin, increased levels of free fatty acids (FFA) as well as proinflammatory cytokines (Badman et al. 2007; Shoelson et al. 2007; Shulman. 2014). In IR state, the inflamed adipose tissue becomes resistant to the antilipolytic effect of insulin, and lipolysis is increased resulting in the increased influx of FFA to liver, promoting hepatic triglyceride (TG) synthesis and increased hepatic steatosis. In turn, hepatic steatosis induces intrahepatic inflammation through activation of NF-κB pathways that exacerbate IR, finally setting up a vicious cycle (Anstee et al. 2013; Fabbrini et al. 2010; Stefan et al. 2008; Tilg et al. 2008; Yki-Jarvinen. 2010). See more details in Figure 3.

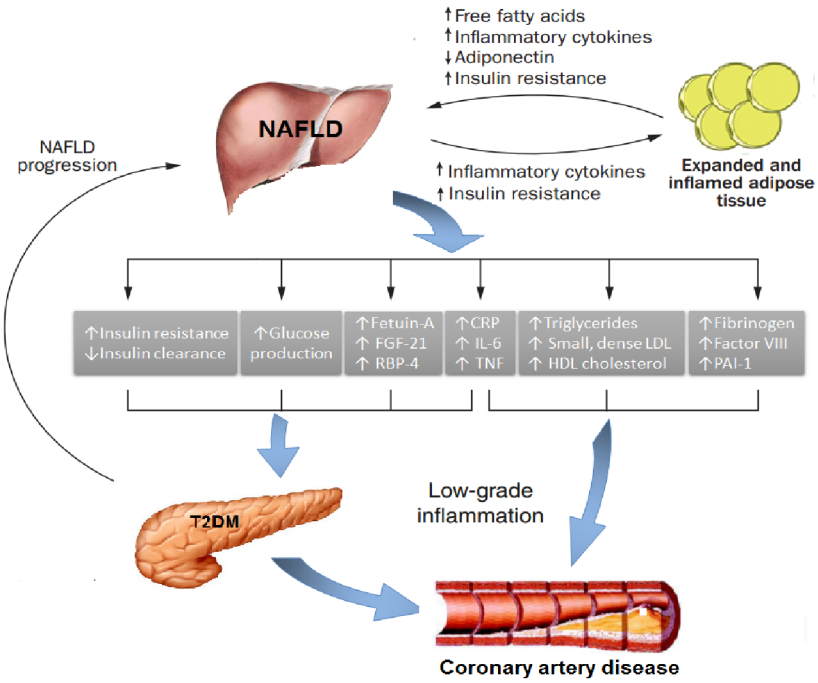

Figure 3 The putative mechanisms underlying the link between NAFLD, T2DM and CAD. NAFLD contributes to coronary artery disease via atherogenic dyslipidemia (high levels of triglycerides and small, dense LDL particles, low levels of HDL cholesterol), insulin resistance, dysglycemia and increased secretion of many proinflammatory markers (i.e, CRP, IL-6, TNF) and hemostatic-fibrinolytic factors (i.e. fibrinogen, factor VII, PAI-1). In addition, NAFLD contributes to developing T2DM by increased glucose production in the liver and exacerbating insulin resistance possibly through proinflammatory factors and some liver-associated proteins such as fetuin-A, FGF-21 and RBP-4. In turn, T2DM is a well-known risk factor for the progression of NAFLD. NAFLD, CAD and T2DM are all strongly associated with low-grade inflammation. CAD, coronary artery disease; CRP, C-reactive protein; FGF-21, fibroblast growth factor 21; HDL, high-density lipoprotein; IL-6, interleukin-6; LDL, low-density lipoprotein; NAFLD, non-alcoholic fatty liver disease; PAI-1, plasminogen activator inhibitor 1; RBP-4, retinol binding protein 4; T2DM, type 2 diabetes mellitus; TNF, tumour necrosis factor. Derived from Nat Rev Gastroenterol Hepatol, 2013, 10(6):330-344.

Overall, all of these metabolic abnormalities directly or indirectly contribute to the formation of plaque and atherosclerosis. However, the various links and interdependencies between the three disease entities are not fully understood. Since NAFLD may be considered an intermediate process or pre-CAD, fatty liver disease could become a potential target for preventing cardiovascular events.

1.5. Gender disparities in CAD

1.5.1. Emerging female-specific attention for CAD

Men are at greater risk of CAD than women in mid-age, and the risk for women increases after menopause. This disparity was regarded as the female advantage.

Actually, CAD once viewed as a man's disease has been underestimated in women in the past (Healy. 1991). More women than men have died due to cardiovascular events annually since 1984 (Wenger. 2012). In fact, CAD is the major cause of mortality for women worldwide. Compared to their male counterparts, women may only have 7~10 years later onset of clinical manifest CVD (Maas et al. 2011). Strinkingly, women with CAD have much worse outcomes than men: women with angina have a doubled morbidity and mortality; women with CAD (especially <50 years) have a doubled mortality after myocardial infarction (MI) (Vaccarino et al. 1999), although the sex gap was narrowing along with the improvements in treatment (Vaccarino et al. 2009); women with MI are more likely to have recurrent MI and to be subsequently disabled by heart failure (Wenger. 2003). There may be some differences in pathophsiology process of CAD between women and men. The gender disparities in CAD attract more specific attention and research.

1.5.2. Potential causes of the gender disparities in CAD

Although it is inperfect in past studies, especially the inadequate proportion (around 30%) of women enrolled in trials and lack of gender-stratified analysis in some studies (Wenger. 2012), they provide some clues about the potential causes of the gender disparities. It is hypothesized that estrogen may delay the onset of atherosclerosis in women for 7~10 years (Maas et al. 2011). In addition, the patterns and progression of atherosclerosis are different in women at different ages: more diffuse pattern of atherosclerosis and plaque erosion at middle age, while the classical pattern of plaque rupture is more common in older women and men (Burke et al. 2001; Burke et al. 1998; Frink. 2009; Shaw et al. 2009). The plaque erosion may lead to distal embolization of the microvascular instead of the obstruction in the epicardial coronary arteries. The absence of obstructive coronary disease is thought to be an emerging paradigm for women in MI with adverse outcomes (Bairey et al. 2006). Moreover, the symptom presentations are different among women and men, more women with atypical symptom than men are underdiagnosed and undertreated (Canto et al. 2007; Dey et al. 2009; Pope et al.

2000). Although women and men share major of classic CVD risk factors, the prevalence and the adverse outcomes of them such as hypertension, smoking, DM and obesity are different. Overall, the further biological mechanisms underlying the gender disparities are still not completely clear.

1.6. Monocytes

Monocytes constitute 3~8% of peripheral blood leukocytes in humans. After maturation and release from bone marrow, monocytes circulate in the bloodsteam for about two to three days and do not proliferate, then migrate into tissues with differentiation into different types of tissue resident macrophages or dendritic cells (DC) (Geissmann et al. 2010). As main component of the innate immune system, monocytes are responsible for exogenous bacterial, viral and fungal infections as well as endogenous inflammation mainly by phagocytosis. Beside inflammation, monocytes play important roles in angiogenesis and tissue remodeling after injury.

1.6.1. Monocytes classification and function in humans

In humans, monocytes are divided into three subgroups according to the expression of various surface receptors. The major subset i.e. “classical” monocytes is defined as high expression of CD14 and lack of CD16 expression (CD14++CD16-), they account for 80~85% of the total circulating blood monocytes. These monocytes also express CCR2, L-selectin, and CD64 (Ancuta et al. 2003; Weber et al. 2000; Wong et al. 2011; Ziegler-Heitbrock. 2007). Another human monocyte subset is identified as “non-classical” monocytes (around 10% of total circulating monocytes) by the low expression of CD14 and high expression of CD16 (CD14+CD16++). They don’t express CCR2 or L-selectin but express high levels of CX3CR1, CCR5 (Ancuta et al. 2003; Weber et al. 2000). Recently, a third human monocyte subset is defined as “intermediate” monocytes (around 5% of total circulating monocytes) with expression of CD14++CD16+, they can be discriminated from the non-classical monocytes by the expression of CCR2 (Shantsila et al. 2011), and they are predominant type of monocytes expressing Tie2 (an angiopoietin receptor) (Murdoch et al. 2007).

These three subsets differ significantly in function. The classical monocytes are professional phagocytes that ingest native LDL, generate reactive oxygen species (ROS) and secrete cytokines in response to lipopolysaccharide (LPS) (Mosig et al. 2009). The migration of classical monocytes depends on monocyte chemoattractant protein-1 (MCP-1/CCL2) secreted by resident macrohages. Therefore, they are considered inflammatory mediators. Non-classical monocytes are weak on phagocytosing oxidised LDL (ox-LDL) and don't generate ROS, but they secrete inflammatory cytokines such as tumor necrosis factor (TNF)-α, IL-1β and CCR3 once toll-like receptors (TLRs) dependent pathway activated (Belge et al. 2002; Cros et al. 2010; Mosig et al. 2009). Their attraction and recruitment to endothelial surfaces depends on fractalkine (a transmembrane-anchored adhesion receptor expressed on activated endothelial cells) (Ancuta et al. 2003). It is considered that non-classical monocytes might serve as patrolling cells and are invovled in the innate local surveillance of tissues. They can migrate to inflamed tissues or sites as the classical monocytes but might emigrate out of the sites after certain maturation while the classical monocytes are retained within the sites. The intermediate monocytes are increasingly reported in a wide range of pathological conditions. They do not generate ROS but secrete high secretion of TNF-α and IL-1β in response to LPS (Cros et al. 2010). Overall, the exact function of monocyte subsets in human has not been fully understood. Of note, most of current knowledge on these cells has been acquired from animal studies.

1.6.2. Monocytes in CAD

Monocytes have been identified to be involved in the process of CAD. They play important roles in the initiation and formation of atherosclerotic plaque, plaque destabilization as well as cardiac remodeling after acute coronary events (Ghattas et al. 2013) (see Figure 4). Activated circulating monocytes are recruited to the damaged/activated endothlium by inflammatory chemokines, TLRs and adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1). Then the monocytes transmigrate across the

endothelium, differentiate into macrophages that ingest ox-LDL and transform into foam cells (Collot-Teixeira et al. 2007). The accumulation of macrophages, foam cells, apoptotic foam cell debris, combined with smooth muscle migration and proliferation as well as platelets activation and aggregation, lead to the plaque formation and progressive atherosclerosis, finally thrombus formation. The activated macrophages also secrete cytokines, matrix metalloproteinases (MMPs), ROS and tissue factors, induce persistent inflammatory and platelets activation/ attraction, and exacerbate the progression of thrombus (Badimon et al. 2011). MMPs are considered to promote the destabilization of the fibrous cap leading to the plaque rupture (Newby et al. 2009). Classical monocyte is considered to be the predominant subset identified in atherosclerotic plaques.

Once plaque rupture, the downstream reaction is triggered. Monocytes affect the coagulation cascade resulting in thrombus propagation during the acute event (Altieri et al. 1988). Elevated monocyte-platelet aggregates in acute coronary syndrome (ACS) patients were reported (Tapp et al. 2012), and ACS patients show features of procoagulant monocyte activation under exposure of tissue factor (Altieri et al. 1988). Activated monocytes also induce the expression of inflammatory cytokines (e.g. TNF-α, IL-1, IL-6), platelet derived endothelial cell growth factor as a chemoattractant and mitogen for fibroblasts, macrophage colony stimulating factor (M-CSF) which is important for macrophage survival, transforming growth factor (TGF) α and β which contributes to fibrosis, as well as insulin-like growth factor (Lambert et al. 2008). Furthermore, monocytes are considered to involve the tissue injury via the acute inflammatory response activated by ROS leading to cell injury and necrosis. In addition, monocytes mature into DC and macrophages within the subintimal space that promotes inflammation and extracellular matrix destruction (Geissmann et al. 2010).

After the myocardial damage by hypoxia, numerous monocytes are recruited to the infarcted areas that promote phagocytosis, fibroblast accumulation, angiogenesis and tissue formation, which lead to cardiac remodeling and recovery. Different subsets may play different roles. It was reported that classical monocytes were

negatively related to left ventricular recovery after ACS and associated with poor outcome in stroke patients (Tsujioka et al. 2009; Urra et al. 2009), intermediate and non-classical monocytes had no effect on ventricular remodeling which contrasted with animal data (Nahrendorf et al. 2007). A recent study reported that intermediate monocytes were over 2.5 fold up-regulated in patients with ST-segment elevation MI while no changes of non-classical monocytes were observed (Tapp et al. 2012). An increased proportion of intermediate and non-classical monocytes were associated with better outcome in the stroke patients was observed (Urra et al. 2009). Combined to its increased expression of CD14 and CCR2, reduced expression of CD16, as well as high expression of some markers (e.g. VEGF receptor 2, CD163, CXCR4 and Tie2), intermediate monocytes are putative to have pro-reparative, pro-angiogenic, anti-inflammatory properties in cardiac recovery (Ghattas et al. 2013). However, some clinical studies indicated that high levels of intermediate monocytes have been associated with poor clinical outcome in future MI in stable CAD (Rogacev et al. 2012), and recurrence coronary events in patients with chronic kidney disease (CKD) (Rogacev et al. 2011). Collectively, the knowledge of human monocytes on cardiac remodeling is still limited. These data imply intriguing double-edged dynamics monocytes in cardiac remodeling. The balance between removing dead cells and prompt initiation of regeneration in appropriate timing may determine patients' outcomes. Indeed, most of our current knowledge in monocyte function comes from murine models, the studies in human are still with more emphasis on monocyte numbers, the exact function of monocyte subsets in human beings remains unclear.

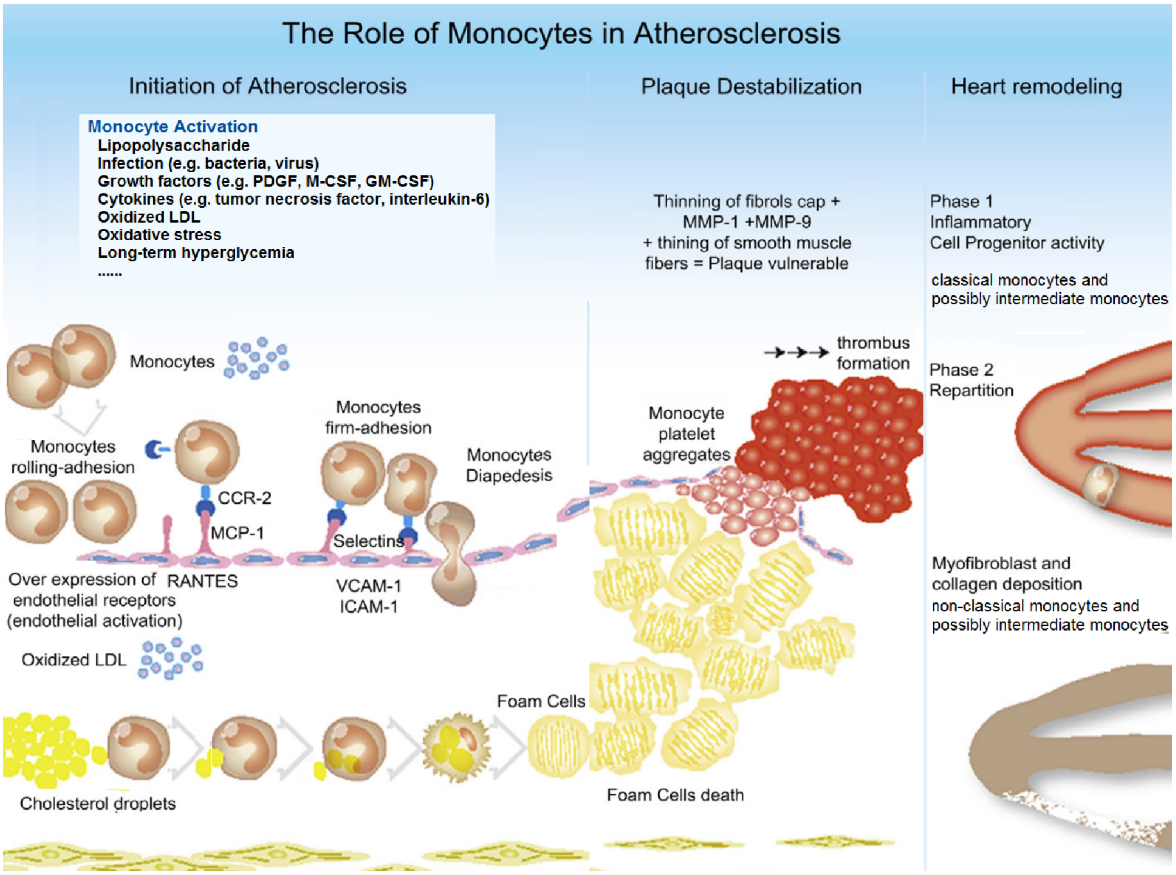

Figure 4 The role of Monocytes in atherosclerotic process. Circulating monocytes are prone to be activated by multiple factors such as lipopolysaccharide, infection, growth factors, pro-inflammatory cytokines, ox-LDL, oxidative stress, long-term hyperglycemia and so on. The dysfunction endothelial cells express exceeding inflammatory chemokines (MCP-1) and toll like receptors as well as adhesion molecules (VCAM-1, ICAM-1) that facilitate the monocytes accumulation and adhesion in the site of endothelial lesion. The monocytes transmigrate across the endothelium via diapedesis and MMPs assist activated by the NF-κB pathway. In subendothelial space, monocytes differentiate into macrophages that ingest oxidized-LDL via scavenger receptors and transform into foam cells. These cells undergo a process of apoptosis/necrosis that perpetuates the formation of further foam cells. The middle panel depicts events about plaque rupture including thinning of the fibrous cap and monocyte platelet aggregates. The right panel depicts cardiac repair. Classical and possibly intermediate monocytes reduce or abrogate to reduce inflammatory effect and remove myocytes form infarcted and peri-infarct areas in phase 1, and later in phase 2 non-classical and possibly intermediate monocytes alters the extracellular matrix remodeling by myofibroblast deposition and angiogenesis, leading to thinning of the infarcted cells. GM-CSF, granulocyte colony stimulating factor; ICAM-1, intercellular adhesion molecule; LDL, low-density lipoprotein; MCP-1, monocyte chemotactic protein-1; M-CSF, macrophage colony stimulating factor; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; RANTES, regulated upon activation, normal T-cell expressed and secreted; VCAM-1, vascular cell adhesion molecule. Modified from J Am Coll Cardiol, 2013, 62(17):1541-1551.

1.6.3. Monocytes in NAFLD

Kupffer cells are the liver resident macrophages (Naito et al. 1997). They have

been identified as one of key elements in pathogenesis of non-alcoholic steatohepatitis (NASH). Activation of its TLR4 signaling pathway drives Kupffer cells to release inflammatory cytokines and chemokines to initiate the inflammatory cascade (Brun et al. 2007). The inactivation of Kupffer cells can almost fully ameliorate inflammation, steatosis, and damage in NASH of mice (Miura et al. 2012; Rivera et al. 2007). Recently Satoh and co-workers found that Kupffer cells and the expression of CD14 on Kupffer cells were significantly up-regulated in postoperative NAFLD patients after pancreatoduodenectomy (Satoh et al. 2013). The number of CD14+ Kupffer cells increase with an increase in necroinflammatory grade and fibrosis stage of NAFLD (Tonan et al. 2012). Circulating monocytes, as “precursor”, may also participate and play key role in the process of NAFLD. A recent clinical study showed that the circulating monocyte fraction determined by standard blood cell counter was elevated in patients with NAFLD (Kim et al. 2011). However, a more thorough insight is lacking since the relationship between monocyte subsets and NAFLD has not been studied yet.

1.7. Study purposes

The purposes of the present work are as following:

(1) NAFLD reaching epidemic proportions has been identified as an independent risk factor for CAD; the first aim was to investigate the association between NAFLD and alteration in circulating monocyte subsets as a cellular hallmark of the CAD state.

(2) CAD patients display different outcomes over long-term follow-up, monocytes play key role in the cardiac remodeling. Therefore, the second aim was to identify key genes expressed on monocytes associated with clinical outcomes of CAD.

(3) Gender disparities in CAD prevalence and outcome do exist; the third aim was therefore to look for gender differenecs expressed on monocytes.

(4) Although CAD has been extensively studied, key pathways involved in the pathogenesis are not thoroughly understood, thus, the fourth aim was the

exploration of novel potential pathways related to the expression of CAD.

2. Materials and methods

2.1. Study cohort

2.1.1. Circulating monocyte phenome in NAFLD

Subjects enrolled in “circulating monocyte phenome in NAFLD” study were recruited from EMIL study cohort (**E**chinococcus **M**ultilocularis and other medical d**i**seases in **L**eutkirch), the population-based cohort first recruited in year 2002. The baseline investigation was performed from November to December, 2002 and comprised of 2,445 probands (Haenle et al. 2006). In 2013 follow-up was conducted with focus on extreme end variants, i.e. obese or overweight subjects with or without fatty liver disease (FL) and normal weight subjects with FL based on ultrasound examination. 484 subjects were enrolled in the EMIL follow-up study. Of 484 subjects, 73 probands were randomly selected to conduct extensive phenotyping of monocyte subsets. Figure 5 summarizes the subcohort examined in the study.

This study was conducted in conformity to basic principles of Helsinki Declaration. EMIL study was approved by ethics committee of the Landesärztekammer Baden-Württemberg and ethical committee of Ulm University, and written informed consent was obtained from all study participants.

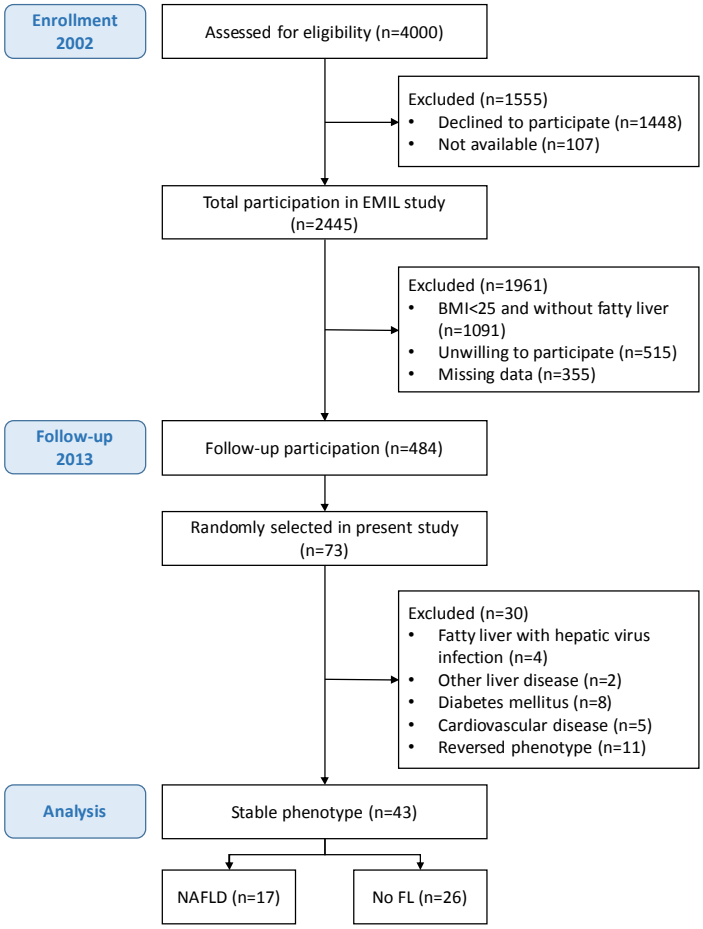

Figure 5 Flow of subjects across the NAFLD study. BMI, body mass index; EMIL, **E**chinococcus **M**ultilocularis and other medical d**i**seases in **L**eutkirch; FL, fatty liver disease; NAFLD, non-alcoholic fatty liver disease.

2.1.2. Gene expression profiling of monocytes in CAD

2.1.2.1. Discovery set

Subjects enrolled in “gene expression profiling of monocytes in CAD” microarray study were recruited from the LURIC study cohort (**LU**dwigshafen **RI**sk and **C**ardiovascular Health), which is an ongoing prospective study of 3316 individuals who were referred for coronary angiography to Ludwigshafen Heart Center in South-West Germany and were recruited between July 1997 and January 2000 (Winkelmann et al. 2001). There was a follow-up for all-cause and cardiovascular mortality in 2010, 2580 probands with complete information recorded about the vital information, 755 deaths and 1825 survival. 316 of 2580 probands were collected

enough total ribonucleic acid (RNA) in our lab. Of total 316 participants, the mortality of 51 probands was due to cardiac causes including sudden death, fatal myocardial infarction, congestive heart failure, death after intervention to treat CAD and other death due to CAD, 50 probands died due to fatal stroke, infection, cancers and other causes for death, while the rest of 215 probands survived. The subjects including 30 deceased due to cardiac causes as well as 41 survivors matched with age, gender and general status as far as possible were enrolled in this gene expression profiling study. Additionally, 10 healthy Caucasian donors from Ulm were also recruited in this study. Figure 6 summarizes the subcohort examined in this study.

The LURIC study was conducted in accordance with the basic principles of Helsinki Declaration and was approved by the ethics committee at the“Ärztekammerm Rheinland-Pfalz”. Written informed consent was obtained from all study participants.

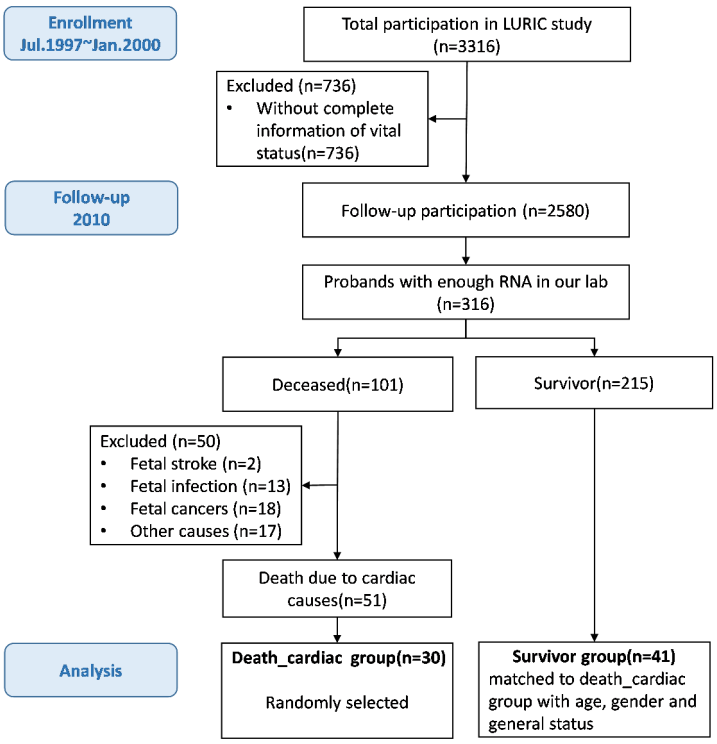

Figure 6 Flow of subjects across the microarray study. RNA, ribonucleic acid; LURIC, **LU**dwigshafen **RI**sk and **C**ardiovascular Health.

2.1.2.2. Replication set

To avoid a confounding effect just be addressing Swabian patients, 8 patients were recruited from Medicine I - Cardiology, Pneumology, Angiology and Internal

Medicine Intensive Care Center, University Hospital Aachen. The diagnosis of CAD was based on coronary angiography. Except the 8 patients from Aachen, namely 5 patients with T2DM and CAD, 1 patients with CAD but no DM (excluded because of no DM),1 patient without CAD or DM (excluded) and 1 patient without coronary angiography (excluded because of unclear diagnosis), the blood samples were taken by phlebotomy of a superficial cubital vein. In addition, total RNA samples from 5 healthy donors in our lab dataset were selected (see Table 1). All of the subjects were Caucasians.

The study was conducted in accordance with the basic principles of Helsinki Declaration and was approved by the ethics committee of University Hospital Aachen, and written informed consent was obtained from all study participants.

Table 1 Group settings in replication set

CAD+ T2DM+	Healthy control
n	n
5	5

Note: "+" represents that subject was suffered from this disease, "-" represents that subject did not have this disease. CAD, coronary artery disease; T2DM, type 2 diabetes.

2.2. Materials

2.2.1. Microarray Gene Chip

Affymetrix GeneChip® Human Gene 1.0 ST Array	Affymetrix, Santa Clara, CA, USA

2.2.2. Primers for qRT-PCR

All primers for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) were obtained from RT^2 qRT-PCR Primer Assay, QIAGEN, see Table 2.

Table 2 Primer list.

Gene symbol	Catalog #	RefSeq #
PPBP	PPH00533A-200	NM_002704
CD69	PPH00831F-200	NM_001781
CCL2	PPH00192F-200	NM_002982
EMP1	PPH16632B-200	NM_001423
SMAD7	PPH01905C-200	NM_005904
THBD	PPH02576A-200	NM_000361
TNFRSF12A	PPH17750C-200	NM_016639
ID1	PPH00317B-200	NM_002165
CD226	PPH18064A-200	NM_006566
CCL5	PPH00703B-200	NM_002985
CTSL1	PPH00113F-200	NM_001912
SDC4	PPH15118E-200	NM_002999
NAB2	PPH57666E-200	NM_005967
PIM1	PPH00222F-200	NM_002648
FFAR3	PPH14987A-200	NM_005304
LDLR	PPH00503E-200	NM_000527
CCR1	PPH00611F-200	NM_001295
MAPK14	PPH00750B-200	NM_001315
UBR2	PPH12128B-200	NM_015255
OGT	PPH19166A-200	NM_181673
ABL1	PPH00087E-200	NM_005157
HPRT1	PPH01018C-200	NM_000194

Note: All of the primers were obtained from RT^2 qRT-PCR Primer Assay, QIAGEN. The RefSeq number refers to the representative sequence used to design the enclosed primers. *ABL1* and *HPRT1* were performed as housekeeping genes, others were target genes. qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

2.2.3. Chemicals and Reagents

Acetic acid (100%)	Merk, Darmstadt, Germany
Agarose powder NEEO Ultra Quality	Roth, Karlsruhe, Germany
Anti-human CD14 Pacific blue	Biolgend, San Diego, CA, USA
Anti-human CD16 APC	Biolgend, San Diego, CA, USA
Anti-human CD36 PE	Biolgend, San Diego, CA, USA
Anti-human CD45 PerCP-cy5.5	Biolgend, San Diego, CA, USA
Anti-human CD9 FITC	Biolgend, San Diego, CA, USA
Anti-human HLA-DR PE-cy7	Biolgend, San Diego, CA, USA

BD Pharm Lyse™ Lysing buffer	BD Biosciences, San Diego, CA, USA
Beads buffer	0.5% BSA, 2 mM EDTA in PBS
Blocking buffer	1% BSA in PBS
β-mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA
Bovine serum albumin (BSA), pH 7.0	SERVA, Heidelberg, Germany
Cardio Phase hs-CRP	Siemens, Eschborn, Germany
CD14 Microbeads human kit	Miltenyi Biotec, Bergisch Gladbach, Germany
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO, USA
Ethylene diamine tetraacetic acid (EDTA)	Sigma-Aldrich, St. Louis, MO, USA
Ethidium Bromide (EB)	Sigma-Aldrich, St. Louis, MO, USA
Fetal calf serum (FCS)	Invitrogen, Carlsbad, CA, USA
Ficoll-paque plus	GE Healthcare, Chalfont St Giles, UK
Freezing-Medium	10% DMSO in 90% FCS
FTIC mouse IgG1, k isotype control	Biolgend, San Diego, CA, USA
Glycerol (100%)	Sigma-Aldrich, St. Louis, MO, USA
Hanks' salt solution	Biochrom AG, Berlin, Germany
Loading buffer	75% Glycerol, 0.5% Xylen cyanol FF in double-distilled water
Monocyte isolation kit II	Miltenyi Biotec, Bergisch Gladbach, Germany
Phosphate buffered saline (PBS) (1x), liquid, pH: 7.4	Invitrogen, Carlsbad, CA, USA
PE mouse IgG2a, k isotype control	Biolgend, San Diego, CA, USA
Penicillin/streptomycin	Invitrogen, Carlsbad, CA, USA
QIAGEN RNeasy Micro Kit	QIAGEN, Hilden, Germany
QIAGEN RNeasy Mini Kit	QIAGEN, Hilden, Germany
QIAGEN RT^2 First Strand Kit	QIAGEN, Hilden, Germany
QIAGEN RT^2 SYBR green mastermix	QIAGEN, Hilden, Germany

Quantikine HS IL-6 Immunoassay	R&D Systems, Wiesbaden, Germany
RPMI 1640 medium (1x), liquid	Invitrogen, Carlsbad, CA, USA
1kb ladder	Fermentas, Vilnius, Litauen
Tris Base Ultrapure	USB Corporation, MA, USA
Tris-Acetate-EDTA (TAE) buffer (50X)	24.2% Tris base, 5.71% Acetic acid, 0.05M EDTA in double-distilled water
Trypan blue	Sigma-Aldrich, St. Louis, MO, USA
Tuerk's solution	Merck, Darmstadt, Germany
Xylen cyanol FF (C.I 43545)	USB Corporation, MA, USA
Washing-Medium	100U/mL penicillin/streptomycin in RPMI 1640 medium

2.2.4. Labware

Biosphere® Filter tips (10µL, 20µL, 200µL, 1000µL)	SARSTEDT, Nuembrecht, Germany
Costar stripette serological pipet (5mL, 10mL, 25mL)	Corning Inc., Corning, NY, USA
Eppendorf reference pipette (10µL)	Eppendorf, Hamburg, Germany
Glass pasteur pipette (D812)	Poulten & Graf GmbH, Wertheim, Germany
Hemocytometer	Carl Roth GmbH, Karlsruhe, Gemany
MACS separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Micro tube (0.5 mL)	SARSTEDT, Nuembrecht, Germany
MicroAmp™ Fast optical 96-well reaction plate with barcode	AppliedBiosystems, Foster, CA, USA
MicroAmp™ Optical adhesive film	AppliedBiosystems, Foster, CA, USA
MS/LS column	Miltenyi Biotec, Bergisch Gladbach, Germany
Parafilm "M"	Pechney, Plastic Packaging, Chicago, IL, USA
Pipette tips (10µL, 200µL, 1000µL)	Brand, Wertheim, Germany

Pipette-boy	INTEGRA Biosciences, Fernwald, Germany
Pipette-man (20µL, 200µL, 1000µL)	Gilson, Villiers-le-Bel, France
Polypropylene Round-Bottom Tube (5mL, 14mL)	BD, Franklin Lakes, NJ, USA
Polypropylene tubes, conical bottom (50 mL, 15 mL)	Greiner Bio-One, Frickenhausen, Germany
QIAshredder spin column	QIAGEN, Hilden, Germany
Safe-lock tube (1.5 mL)	Eppendorf, Hamburg, Germany
SafeSeal-Tips®premium (10µL)	Biozym, Oldendorf, Germany
SafeSeal-Tips®professional (20µL, 200µL, 1000µL)	Biozym, Oldendorf, Germany
Serological Pipet (2 mL)	BD, Franklin Lakes, NJ, USA
Stericup and steritop vacuum filter cups (0.22µm Filter)	Millipore Corporation, Billerica, MA, USA

2.2.5. Laboratory equipment

7500 Fast Real-Time PCR system	Applied Biosystems, Darmstadt, Germany
BD LSRII flow cytometer	BD, Franklin Lakes, NJ, USA
Block Heather (SBH 130D)	Bibby Scientific Limited, Staffordshire, UK
Centrifuge (5417R)	Eppendorf, Hamburg, Germany
Centrifuge (multifuge 3S-R)	Heraeus, Hanau, Germany
Centrifuge(multifuge X3R)	Heraeus, Hanau, Germany
Dry block bath (Thermostat 5320)	Eppendorf, Hamburg, Germany
Owl™ Easy-Cast™ B1A Gel Electrophoresis Systems	Thermo Scientific, Wilmington, USA
Electrophoresis power supply (Macro Drive 1)	LKB, Bromma, Schweden
GenoSmart gel documentation system	VWR, Leuven, Belgium

Heating block HBT 130-2	Haep Labor Consult, Bovenden, Germany
Laminar flow hood (HA 2448)	Heraeus, Hanau, Germany
Magnetic stirrer (Ikama Rec-G)	Ika Labortechnik, Staufen, Germany
Mcrowave oven	Bosch, Gerlingen, Germany
Microscope (Olympus IMT-2)	Olympus, Tokyo, Japan
Nanodrop 2000	Thermo Scientific, Wilmington, USA
Nitrogen tank (Chronos 400)	Messer, Sulzbach ,Germany
Refridgerator	Liebherr, Bulle, Schweiz
Vortex shaker (REAX 2000)	Heidolph, Schwabach, Germany
Waterbath (3043)	Koettermann, Haenigsen, Germany

2.2.6. Software and internet resourses

7500 Fast System SDS software version 1.4	Applied Biosystems, Darmstadt, Germany
BD FACSDiva™ software version 6.1.2	BD Biosciences, Franklin Lakes, NJ, USA
Genesis software version 1.7.6	IGB-TUG, Graz, Austria
GraphPad PRISM® version 5.04	GraphPad Software, La Jolla, CA, USA
IBM SPSS statistical software version 21.0	IBM Inc., Chicago, IL, USA
Ingenuity Pathway Analysis (IPA) 2000-2013	Ingenuity Systems, Inc. CA, USA
GoMiner	http://discover.nci.nih.gov/gominer/index.jsp
RT² Profiler™ PCR Array Data Analysis	http://www.sabiosciences.com/pcrarraydataanalysis.php
STRING 9.1	http://string-db.org/

2.3. Methods

2.3.1. Clinical parameters

2.3.1.1. Circulating monocyte phenome in NAFLD

The EMIL follow-up investigation in the year 2013 followed procedures applied during the first survey in the year 2002 (Haenle et al. 2006). Briefly, the personal information, personal medical history, family history, alcohol consumption, dietary habits and so on were recorded through one standardized questionnaire and the structured interview. Body weight, body height, hip circumference and waist circumstance (WC) were measured. Then the body mass index (BMI) and waist-to-hip ratio (WHR) were calculated according to the WHO recommendations (A WHO Expert Committee, 1995).

After at least eight hours fasting, peripheral blood samples were obtained by phlebotomy from superficial cubital vein. Clinical laboratory parameters including lipids, liver enzymes and other biochemical values (TG, cholesterol, high-density lipoprotein cholesterol (HDL-C), LDL-C, γ-glutamyl transferase (GGT), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (AP), sex hormone binding globulin (SHBG), insulin and glucose) were performed using Cobas 8000 analyzer (Photometric measurement, electrochemiluminescence immunoassay; Roche Diagnostics, Rotkreuz, Switzerland). The fractions of leukocytes and its subsets were measured by the automated hematology system (Sysmex XE-5000, Sysmex Europe Inc., Norderstedt, Germany). The above laboratory tests were performed in the laboratory of University Hospital Ulm. In addition, hs-CRP was measured by immunonephelomtery (Cardio Phase hs-CRP, Siemens, Eschborn), and IL-6 was measured by enzyme-linked immunosorbent assay (Quantikine HS IL-6 Immunoassay, R&D Systems, Wiesbaden). The serum levels of hs-CRP and IL-6 were conducted in the laboratory of Professor Wolfgnag Koenig by Gerlinde Trischler in Laboratory for Molecular Cardiovascular Epidemiology and Preventive Cardiology, University of Ulm. The Fatty Liver Index

(FLI) was calculated by: FLI = ($e^{0.953*\log e(TG)+0.139*BMI+0.718*\log e(GGT)+0.053*WC-15.745}$)/(1+ $e^{0.953*\log e(TG)+0.139*BMI+0.718*\log e(GGT)+0.053*WC-15.745}$)*100 (Bedogni et al. 2006). The Homeostasis Model Assessment -estimated Insulin Resistance Index (HOMA-IR) was calculated by: HOMA-IR = glucose (mg/dL)* insulin (mU/L)/405 (Matthews et al. 1985).

The FL was diagnosed based on the sonographic comparison of renal and hepatic parenchyma, the assessment of dorsal echo attenuation by liver, the visibility of diaphragm and hepatic vessels. The degree of FL was graded as “severe”(grade 3), “moderate”(grade 2), “mild”(grade 1) and “none”(grade 0) (Hamaguchi et al. 2007). NAFLD was diagnosed with absence of the excessive alcohol consumption (the threshold is < 30g/day for male and < 20g/day for female) (Anstee et al. 2013) and the other reasons including hepatic virus infections and other liver diseases.

2.3.1.2. Gene expression profiling of monocytes in CAD

All the participants underwent standardised personal and family history questionnaire and laboratory examination including markers of endothelial dysfunction, inflammation, lipid metabolism and so on at baseline observation. Fasting venous blood was taken and blood or peripheral blood mononuclear cells (PBMC) were stored for later analysis (Winkelmann et al. 2001). CAD was diagnosed as following criteria: clinical symptoms as myocardial ischemia or stenosis of one ore more vessels ≥ 20% under coronary angiograms. Criteria for diagnosis of diabetes (according to American Diabetes Association 2012 criteria) were: subjects with elevated glycated hemoglobin A1c (HbA1c) (≥6.5%) or increased fasting plasma glucose (FPG) (≥126 mg/dL) or increased 2-h plasma glucose (2 hours after the 75 g glucose load ≥ 200 mg/dL).(American Diabetes Association. 2012) Furthermore, the subjects with diabetes history under treatment were considered as diabetic.

2.3.2. PBMC isolation

PBMC isolation was performed by ficoll density gradient centrifugation (Figure 7). The whole blood from subjects or buffy coats was diluted 1:2 in Hanks' salt solution (Biochrom AG, Berlin, Germany) and 35 mL thereof overlaid carefully on 15 mL Ficoll-Paque Plus solution (GE Healthcare, Uppsala, Sweden). The centrifugation at 2000 rpm for 30 minutes without break resulted in formed different layers containing distinct cell types due to differential density. The interface layer containing the PBMC fraction was harvested, and washed twice with washing-medium (100 U/mL penicillin/streptomycin in RPMI 1640 medium) by centrifugation at 1800 rpm for 10 minutes and 1200 rpm for 10 minutes respectively, cell counting was performed by hemocytometer with Tuerk's solution (Merck, Darmstadt, Germany), then followed by aspiration of the supernatant and resuspension in PBS for downstream applications or freezing-medium (10% DMSO in 90% FCS) for cryopreservation.

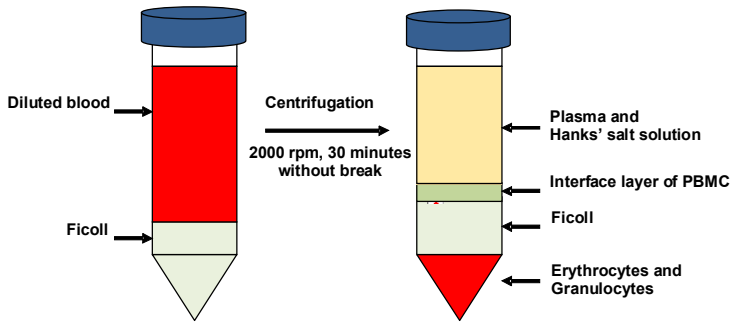

Figure 7 Isolation of PBMC by ficoll density gradient centrifugation. PBMC from either subjects or buffy-coat was isolated by using Ficoll-Paque Plus, the interface layer of PBMC was collected for further experiments. PBMC, peripheral blood mononuclear cell.

2.3.3. MACS cell separation

MACS technology has been considered to be the gold standard for cell separation. It is based on MACS microbeads, which are 50 nanometers superparamagnetic particles that are conjugated to high specific antibodies against a particular antigen on the cell surface. There were two methods to get monocytes by MACS cell separation, negative selection and positive selection in the research. In discovery set, the positive selection method was used. To avoid activating monocytes, the

negative selection method was used in replication set.

The positive selection of monocytes was performed using MACS separation kit for human CD14 monocyte (130-050-201; Miltenyi Biotec, Bergisch Gladbach, Germany). The surface marker CD14 is strongly expressed on most monocytes in the peripheral blood. Frozen PBMC were washed by PBS and beads buffer (0.5% BSA, 2 mM EDTA in PBS), then resuspended in 80 µL of beads buffer per 10^7 total cells. Subsequently, 20 µL of CD14 Microbeads per 10^7 total cells was added and incubated for 15 minutes at 4°C. Afterwards, cells were washed with beads buffer and resuspended in 500 µL of beads buffer for MACS separation. For MACS separation, a MS column was placed in the magnetic field of a MACS separator. The column was equilibrated by rinsing with 500 µL of beads buffer and the cell suspension was applied onto the column. The effluent containing unlabeled cells (negative fraction) was collected. The column was washed twice with 500 µL of beads buffer. Total effluent was collected. After removing the column from the magnetic separator, 1 mL of beads buffer was added onto the column, the CD14+ monocytes (positive fraction) were eluted by firmly pushing the plunger into the column. (Figure 8) The effluent containing CD14+ monocytes was centrifuged and the cell pellet was directly used for total RNA extraction.

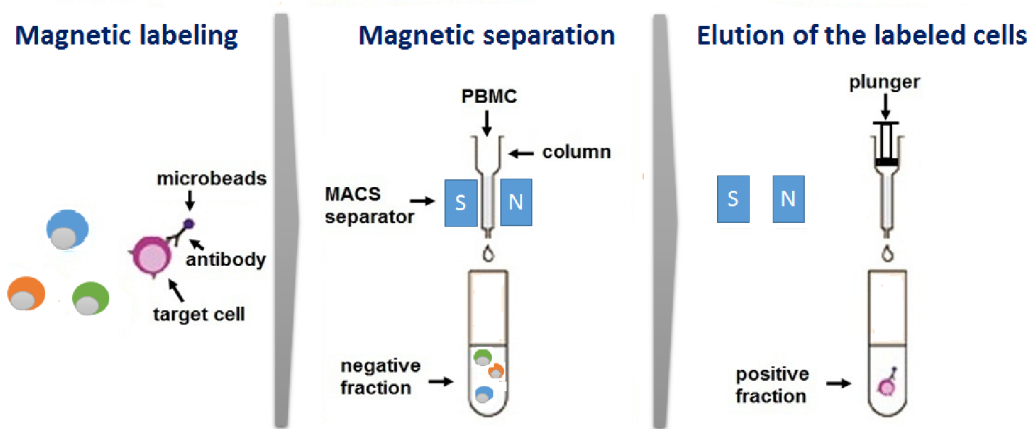

Figure 8 Basic principle of MACS cell separation. PBMC were labeled with indicated MACS microbeads at 4°C. Cells were applied onto the column placed in the magnetic field of a MACS separator and the flow-through was collected as negative fraction. Afterwards, the MACS column was removed from the magnetic field and the positively selected cells which were magnetically retained in column were eluted with beads buffer by using a plunger. PBMC, peripheral blood mononuclear cells; MACS, magnetic-activated cell sorting. Derived from

http://www.miltenyibiotec.com/.

The negative selection of monocytes was performed using MACS Monocyte isolation kit II (130-091-153; Miltenyi Biotec, Bergisch Gladbach, Germany), monocytes were isolated by depletion of non-monocytes, i.e. T cells, nature killer (NK) cells, B cells, DC and basophils, which are indirectly magnetically labeled using a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123 and Glycophorin A. In brief, frozen or fresh PBMC were washed by PBS and beads buffer, and resuspended in 30 µL of beads buffer per 10^7 total cells. Subsequently, 10 µL of FcR blocking reagent per 10^7 total cells and 10 µL of Biotin-antibody cocktail per 10^7 total cells were added and incubated for 10 minutes at 4°C. Afterwards, 30 µL of beads buffer per 10^7 total cells and 20 µL of Anti-biotin microbeads per 10^7 total cells were added and incubated for an additional 15 minutes at 4°C. Then, cells were washed with beads buffer and resuspended in 500 µL of beads buffer for MACS separation. For MACS separation, a MS/LS column was placed in the magnetic field of a MACS separator. The column was equilibrated by rinsing with 500 µL/3mL of beads buffer and the cell suspension was applied onto the column. The effluent containing unlabeled cells was collected. The column was washed twice with 500 µL/3mL of beads buffer. Total effluent containing monocytes was collected and directly used for total RNA extraction or other applications.

2.3.4. Total RNA extraction

Total RNA extraction was performed by QIAGEN RNeasy Mini Kit (74104; QIAGEN, Hilden, Germany) or RNeasy Micro Kit (74004; QIAGEN, Hilden, Germany) depending on cell amounts. For efficient lysis and homogenization, the pelleted monocytes which were loosed and thoroughly resuspended with 350 µL of buffer RLT were pipetted directly into a QIAshredder spin column (79656; QIAGEN, Hilden, Germany) placed in a 2 mL collection tube and centrifugated for 1 minute at 14,000 rpm twice. All the following centrifugation steps were performed at 14,000 rpm for 1 minute if not indicated otherwise. Subsequently, 1 volume (350 µL) of 70% ethanol

was added to the homogenized lysate with well pipetting for providing appropriate binding conditions. Afterwards, that lysate mixture including any precipitate was completely transferred to an RNeasy spin column placed in a 2 mL collection tube and centrifuged for making the RNA binding to the silica membrane. Then, on-column DNase digestion was performed by adding the premixture of 10 µL DNase I stock solution and 70 µL buffer RDD onto column membrane and incubating for 15 minutes at room temperature (20~30°C) after washing the spin solumn membrane by 350 µL of buffer RW1. After that, the spin membrane was washed by 350 µL of buffer RW1 and 500 µL of buffer RPE sequently. (Figure 9)

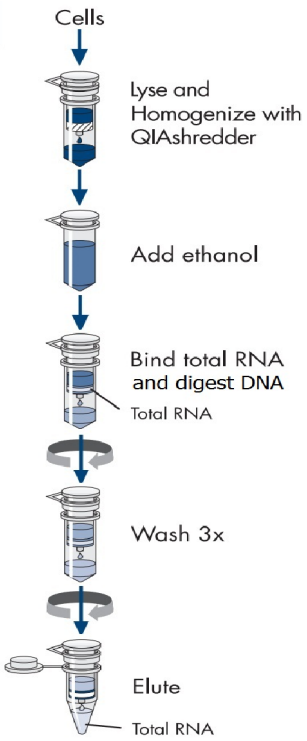

Figure 9 RNeasy Mini/Micro Procedure. The cell pellet was lysed with buffer RLT and homogenized with QIAshredder. Subsequently, 1 volume of 70% ethanol was added to the homogenized lysate with well pipetting for providing appropriate binding conditions. Then the mixed lysate was applied to an RNeasy Mini (RNeasy Mini Kit)/MinElute (RNeasy Micro Kit) spin column, where only the total RNA longer than 200 bases binds to the silica membrane. High-quality RNA was then eluted in appropriate volume of RNase-free water after DNase treatment and efficient washing step. DNA, deoxyribonucleic acid; RNA, ribonucleic acid. Modified from http://www.qiagen.com/.

There were some differences in the following steps between QIAGEN RNeasy Mini Kit or RNeasy Micro Kit. For RNeasy Mini spin column, the spin column membrane

was washed by additional 500 µL of buffer RPE, and total RNA was eluted into a new 1.5 mL collection tube in 50 µL of RNase-free water. For RNeasy MinElute spin column, the spin column membrane was washed by additional 500 µL of 80% ethanol; then, centrifuged for 5 minutes at 14,000 rpm with the lid open to dry the spin column membrane; finally, total RNA was eluted into a new 1.5 mL collection tube in14 µL of RNase-free water. The final total RNA reaction was placed on ice, and the concentration and purity of RNA was determined by Nanodrop 2000 before it was stored at -80°C. The quality control criteria for total RNA are as follows: A260: A230 ratio should be greater than 1.7; A260: A280 ratio should be greater than 1.8; concentration determined by A260 should be more than 50 µg/mL.

2.3.5. Agarose gel electrophoresis

The integrity of RNA was checked by means of electrophoresis on a 1% agarose gel. Briefly, 0.5 g agarose powder was dissolved in 50 mL 1x TAE buffer by heating and swirling. Then 1.7 µL EB was added into the dissolved agarose gel and mixed well. Afterwards, the cool agarose solution was poured onto the casting tray with the sample comb placed in. About 40 minutes later, the comb was removed when the gel had set, and the gel was oriented in the electrophoresis tank, which was filled with TAE buffer to submerge the gel. Subsequently, 4 µL of RNA or 10 µL of standard was mixed with 2 µL loading buffer and appropriate water. The final 10 µL of RNA premixture per each sample as well as 12 µL standards were loaded to the well. After that, the gel was run for about 25 minutes on 125 V voltages. Finally, the gel was placed under the ultraviolet light and photographed. The RNA size was determined by comparing their migration to that of standard. It was only performed in several samples of the replication set. For total RNA samples, thick 28S and 18S ribosomal RNA gel bands at an approximate mass ratio of 2:1 are indications of high integrity (Figure 10).

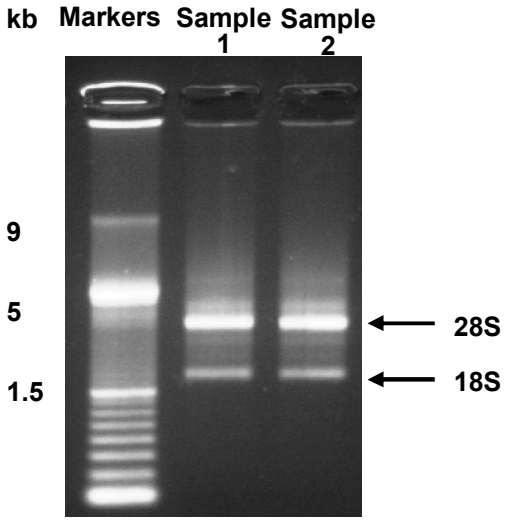

Figure 10 Integrity of total RNA. The 18S and 28S ribosomal RNA bands are clearly visible in the intact RNA sample. RNA, ribonucleic acid.

2.3.6. Microarray

Microarray technology is developed to study the expression of a large number of genes simultaneously. In present study, the Affymetrix GeneChip® Human Gene 1.0 ST Array (901087; Affymetrix, Santa Clara, CA, USA) was used to determine the gene expression profiling of human monocytes for whole transcript coverage. This cost-effective array interrogates 28,869 well-annotated genes with 764,885 distinct probes, which was designed based on the March 2006 human Genome sequence assembly (UCSC Hg18, NCBI build 36) with comprehensive coverage of RefSeq, Ensembl and putative complete CDS GenBank transcripts. The probes are distributed across the full length of the gene, providing a more complete and accurate picture of overall gene expression than most 3'-based expression array. The genes in the following situations including alternative splicing at the 3' end of the gene, utilization of a novel 3' terminal exon, alternative polyadenylation sites, nonpolyadenylated messages, truncated transcript, degraded samples and genomic deletion can be accurate represented on this array.

Microarray experiment and analyses were conducted by the group of Dr. Karlheinz Holzmann from Interdisciplinary Center for Clinical Research, Genomics Core

Facility, Ulm University, Germany. The relative fold change was used for measuring change in the expression level of a gene, fold change <-1.5 or >1.5 ($p<0.05$) was considered down-regulated or up-regulated expression for microarray analysis.

2.3.7. qRT-PCR

qRT-PCR is a highly sensitive and reliable method for gene expression analysis. The complementary deoxyribonucleic acid (cDNA) was synthesized from extracted total RNA by using QIAGEN RT^2 First Strand Kit (330401; QIAGEN, Hilden, Germany). RT^2 SYBR green mastermix (330529; QIAGEN, Hilden, Germany) was used for real-time polymerase chain reaction (PCR). All primers were obtained from QIAGEN RT^2 qPCR Primer (See Table 2). Gene expression profiles of genes of interest were determined by qRT-PCR with the 7500 Fast Real-Time PCR instrument from Applied Biosystems.

For cDNA synthesis, firstly each reaction (reaction volume: 10 µL): 2 µL buffer GE, 400 ng total RNA and appropriate volume of RNase-free water were mixed and incubated for 5 minutes at 42°C for eliminating genomic DNA .

Genomic DNA elimination mix:	
RNase-free water	variable
Buffer GE	2 µL
Total RNA (400ng)	variable
Total volume per reaction	10 µL

Subsequently, 10 µL reverse-transcription mix which was composed of 3 µL RNase-free water, 4 µL 5x buffer BC3, 1 µL control P2 and 2 µL RE3 reverse transcriptase mix was added to each previous reaction and incubated for exactly 15 minutes at 42°C. Then the reaction was immediately stopped by incubating at 95°C for 5 minutes. After that, the cDNA was diluted by 80 µL RNase-free water. All of the cDNA samples were stored at -20°C for later reaction.

Reverse-transcription mix:	
RNase-free water	3 µL
BC3 (*5X Reverse Transcription Buffer 3*)	4 µL
Control P2 (*Primer and External Control Mix*)	1 µL
RE3 (*Reverse Transcription Enzyme Mix 3*)	2 µL
Total volume per reaction	10 µL

Reaction mixtures for all gene expression assays were added in 96-well plate, of which one reaction contains 9.5 µL RNase-free water, 12.5 µL RT^2 SYBR green mastermix, 1 µL primer and 2µL of previous cDNA synthesis reaction. Afterwards, the 96-well plate was covered by optical adhesive film and centrifuged for 2 minutes at 3000 rpm to remove bubbles. The plate was stored at -20°C wrapped in aluminum foil for at least 4 hours but no more than 24 hours before it was conducted in the real-time cycler.

PCR components mix:	
RNase-free water	9.5 µL
RT^2 SYBR Green Mastermix	12.5µL
RT^2qPCR Primer Assay (10µM stock)	1 µL
cDNA synthesis reaction	2 µL
Total volume per reaction	25 µL

All samples from discovery set for validation were assayed in single well. All samples of replication set were assayed in duplicate. Each plate contained two housekeeping genes (*ABL1*, *HPRT1*), one negative control and one reverse-transcription control (RTC). (Figure 11)

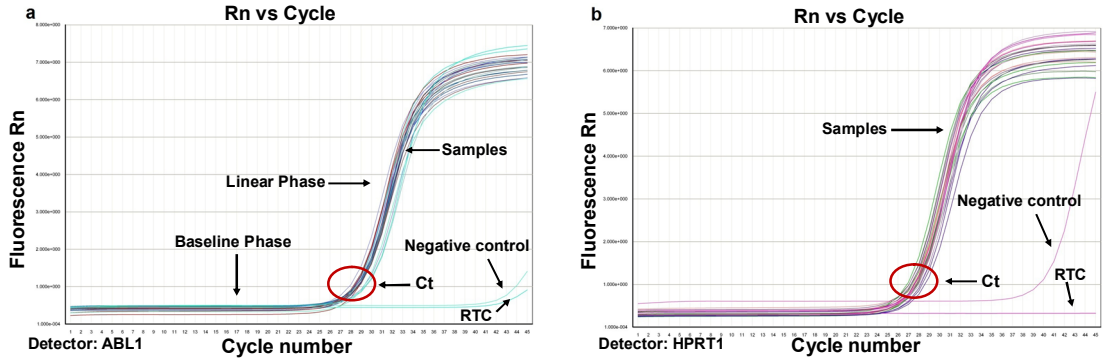

Figure 11 Presentation of qRT-PCR. *ABL1* and *HPRT1* were detected as housekeeping genes. qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; RTC, reverse-transcription control; Ct, cycle threshold.

The qRT-PCR program was set as Table 3. All qRT-PCR data were analysed with 7500 Fast System SDS software version 1.4 from Applied Biosystems. The Ct values were input to RT² Profiler™ PCR Array Data Analysis (http://www.sabiosciences. com/pcrarraydataanalysis.php) to calculate the relative fold change based on the $\Delta\Delta C_T$ method. The relative fold change was used for measuring change in the expression level of a gene, fold change <-2 or >2 ($p<0.05$) was considered down-regulated or up-regulated expression for qRT-PCR analysis.

Table 3 Standard qRT-PCR program

Cycles	Duration	Temperature	Comments
1	10 minutes	95°C	Activation of HotStart DNA Taq-Polymerase
45	15 seconds	95°C	
	1 minute	60°C	Fluorescence data collection
1	15 seconds	95°C	
	20 seconds	60°C	Dissociation (melting)
	15 seconds	95°C	curve analysis
	15 seconds	60°C	

Note: Cycles: 7500 Fast Applied Biosystems models. DNA, deoxyribonucleic acid; qRT-PCR: quantitative real-time reverse transcription polymerase chain reaction.

2.3.8. Multiparameter FACS

The protocol of monocyte phenotyping was adapted from Heimbeck et al (Heimbeck et al. 2010). In briefly, 100 µL of the peripheral blood was added to one 5 mL polystyrene round bottom tube. The erythrocyte lysis was performed with

using the BD Pharm Lyse™ Lysing buffer (555899; BD Biosciences, San Diego, CA, USA) before the staining. After lysis, samples were washed by 3 mL of PBS containing 1% BSA, then incubated for 20 minutes at 4°C with the premixed antibody cocktail: CD45 PerCP-Cy5.5 (304028, Clone HI30), HLA-DR PE-Cy7 (307616, Clone L243), CD14 Pacific Blue (301828, Clone M5E2), CD16 APC (302012, Clone 3G8), CD36 PE (336206, Clone 5-271), and CD9 FITC (312104, Clone HI9a). All antibodies and the isotype controls were purchased from the Biolegend, Germany. Prepared cells after staining were washed by 3 mL of PBS and resuspended in 300 µL PBS. Then the cells were analyzed by multiparameter flow cytometer (BD LSRII flow cytometer, USA) immediately in NAFLD study. Monocytes were gated by the sequential gating on CD45-positive leukocytes in scatter plots, HLA-DR versus CD14 staining and CD16 versus CD14 staining (Heimbeck et al. 2010) [Figure 12]. The surface marker levels of monocytes were measured as the median fluorescence intensity (MFI). Monocyte phenotyping was performed only in selected probands from EMIL follow-up study in 2013.

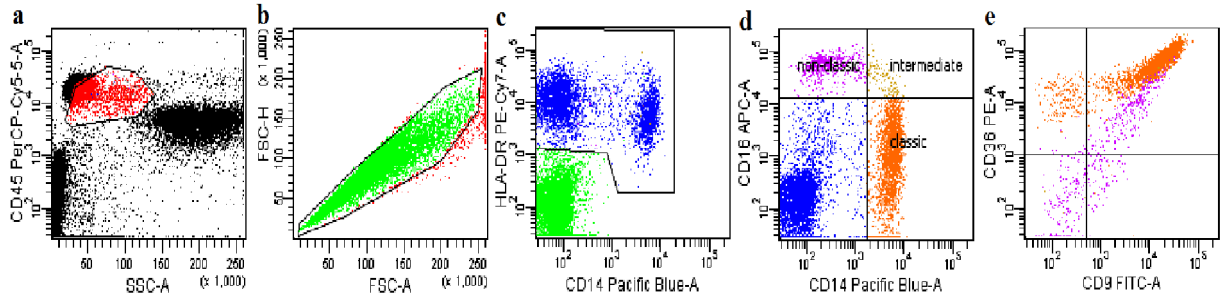

Figure 12 Monocyte populations by FACS. (a) CD45 versus SSC, (b) FSC-H versus FSC-A, (c) HLA-DR versus CD14, (d) CD16 versus CD14, (e) CD36 versus CD9 and quadrants expressed isotype control staining. CD14++CD16- represents classical monocytes, CD14++CD16+ represents intermediate monocytes, CD14+CD16++ represents non-classical monocytes. Monocyte surface marker levels were measured and quantified as the median fluorescence intensity. FSC, forward scatter; SSC, side scatter.

2.4. Statistical analysis

The categorical variables were presented as percentages. The continuous variables were presented as medians with interquartile range (for skewed distribution) or as means with standard deviation (SD) values (for normal distribution). Shapiro-Wilk

test was used to examine for the normal distribution. Comparisons of continuous variables between two groups were performed with independent-sample Student's t-test or Mann-Whitney U test according to their distributions. Comparisons of categorical variables between two groups were performed with χ^2 test. The comparisons of variables between the baseline and follow-up in NAFLD study were performed by Wilcoxon signed-rank test or Paired T test. In NAFLD study, variables which were statistically significant ($p<0.1$) in the univariate Linear or Logistic Regression analysis and several variables of interest with no signifcant were added to the multiple Linear or Logistic regression analysis by enter or forward stepwise method.

All statistical tests were two-tailed and the statistical significance was $p<0.05$. Statistical analysis of all data was performed using the IBM SPSS statistical software (version 21.0, IBM Inc., Chicago, IL).

3. Results

3.1. Circulating monocyte phenotypes in NAFLD

3.1.1. Descriptive analysis between NAFLD and no fatty liver disease

Of 73 probands selected with no alcohol abuse, 19 probands were excluded as follows: FL related to hepatic virus infection (4 probands), other liver diseases (2 probands), and diabetes mellitus (8 probands), cardiovascular events (5 probands) such as angina, MI and stroke, bypass surgery and peripheral arterial disease. Of 54 probands remained, the phenotype of 11 probands reversed over 11-year flow-up while the rest 43 probands kept the stable phenotype (Figure 5). Of 43 probands with constant phenotype, 17 probands were diagnosed as NAFLD while 26 with no FL. The follow-up characteristics and the most variables information of probands with constant phenotype are summarized in Table 4. There was no significant difference in BMI or age between NAFLD and no FL group, while the gender distribution of them was different. (The follow-up data of probands with reversed phenotype are given as Supplement Table 1). Our major analysis focused on the subjects with constant phenotype over the whole observation period unless noted otherwise.

In probands with stable phenotype, monocytes and its subsets fraction were different significantly between NAFLD and no FL group. Compared to the no FL group, the probands with NAFLD showed increased total monocyte fraction (8.7±2.8% vs. 7.0±1.5%, *p=0.028*) and count (0.6 [0.4-0.6] 10^9/L vs. 0.4 [0.3-0.5] 10^9/L; *p=0.035*), decreased classical monocyte fraction (73.4±10.1% vs.79.5±6.4%, *p=0.034*), elevated non-classical monocyte fraction (16.3±7.4% vs. 12.5±4.9%, *p=0.049*) and elevated total CD16+ monocyte fraction (26.6±10.1% vs. 20.5±6.4%, *p=0.036*) [Figure 13 a-d]. Additionally, there were no significant differences in the expression of CD36 (39231 [32627-45447] vs. 35772 [29163-51740]; *p=0.602*) and CD9 (3444 [1109-11058] vs. 4617 [1376-10758]; *p=0.766*) on monocytes between the two groups [Figure 13 e, f].

Table 4 Follow-up characteristics of enrolled subjects with stable phenotype in present study

Characteristics	NAFLD (n=17)	No fatty liver (n=26)	*P value*
Age (years)	58.2 ±8.0	54.3 ±12.4	*0.209*
Male gender, n(%) *	12 (70.6%)	7 (26.9%)	*0.011*
Degree of hepatic steatosis .Grade 1(n)	6	-	
Grade 2(n)	11	-	
Grade 3(n)	0	-	
Body mass index (kg/m^2)	30.7 (27.1 -32.3)	28.5 (26.6 -32.5)	*0.637*
Normal (<25) n(%)	0 (0)	2 (7.7%)	
Overweight (25~30) n(%)	8 (47.1%)	12 (46.2%)	*0.495*
Obese (>30) n(%)	9 (52.9%)	12 (46.2%)	
Waist circumstance (cm) **	104.0 (98.3 -108.5)	94.0 (85.4 -98.3)	*0.001*
Waist-to-hip ratio ***	0.96 ±0.08	0.84 ±0.08	*<0.001*
Liver function panel			
Aspartate transaminase (U/L) *	27 (24 -36)	24 (21 -27)	*0.029*
Alanine transaminase (U/L) **	29 (21 -51)	21 (18 -30)	*0.009*
γ-glutamyl transferase (U/L) **	38 (29 -82)	20 (16 -37)	*0.003*
Alkaline phosphatase (U/L)	65 ±14	64 ±17	*0.961*
Fatty liver index **	7.09 (1.91 -28.36)	1.15 (0.51 -3.68)	*0.005*
Metabolism panel			
Cholesterol (mmol/L)	5.7 (4.9 -7.1)	5.7 (5.1 -6.0)	*0.646*
LDL-C (mmol/L)	3.3 (2.6 -4.6)	3.4 (2.9 -4.0)	*0.960*
HDL-C (mmol/L)	1.4 ±0.4	1.6 ±0.4	*0.162*
Triglycerides (mmol/L) *	1.9 (1.1 -3.0)	1.1 (0.7 -1.8)	*0.031*
Insulin (mU/L) *	11.9 (7.9 -17.8)	7.3 (5.2 -12.0)	*0.028*
Glucose (mg/dL)	99 ±16	94 ±10	*0.189*
HOMA-IR *	2.80 (1.83 -4.60)	1.69 (1.13 -2.93)	*0.029*
Sex hormone binding globulin(nmol/L)	37.31 (26.38 -79.10)	60.09 (37.83 -75.81)	0.124
Inflammatory panel			
High sensitive c-reactive protein(mg/L)	3.11 (1.11 -5.85)	1.93 (0.72 -5.27)	*0.378*
Interleukin-6 (pg/mL)	1.73 (0.90 -3.09)	1.13 (0.83 -1.72)	*0.184*
Leukocyte count (10^9/L)	6.3 (5.2 -7.9)	6.0 (5.2 -7.2)	*0.737*
Leukocyte subtype			
Lymphocyte (%)	33.2 (28.9 -41.2)	30.4 (25.3 -33.2)	*0.096*
Neutrophil (%) *	52.5 ±11.5	58.9 ±7.2	*0.030*
Monocyte (%) *	8.7 ±2.8	7.0 ±1.5	*0.028*
Eosinophil (%)	2.4 (1.4 -3.8)	1.7 (1.2 -2.8)	*0.258*
Basophil (%)	0.8 ±0.4	0.9 ±0.4	*0.548*
Lymphocyte count (10^9/L)	2.3 ±0.9	2.0 ±0.5	*0.172*
Neutrophil count (10^9/L)	3.2 (2.4 -4.4)	3.4 (3.0 -4.8)	*0.345*
Monocyte count (10^9/L) *	0.6 (0.4 -0.6)	0.4 (0.3 -0.5)	*0.035*
Eosinophil count (10^9/L)	0.2 (0.1 -0.2)	0.1 (0.1 -0.2)	*0.242*
Basophil count (10^9/L)	0.1 ±0.0	0.1 ±0.0	*0.764*
Neutrophil/ Lymphocyte ratio	1.60 (1.08 -2.03)	2.03 (1.72 -2.50)	*0.050*

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------followed by previous page (Table 4 continued)

Note: Values were expressed as means with standard deviation (normal distribution) or as medians with interquartile ranges (skewed distribution) for continuous variables and number of subjects (percentage) for categorical variables. Shapiro-Wilk test was used to examine for normal distribution. Comparisons of continuous variables between two groups were performed with independent-sample Student's t test or Mann-Whitney U test. Comparisons of categorical variables between two groups were performed with χ2 test.* represented *p<0.05*, ** represented *p<0.01*, *** represented *p<0.001*. NAFLD, non-alcoholic fatty liver disease; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment-estimated insulin resistance.

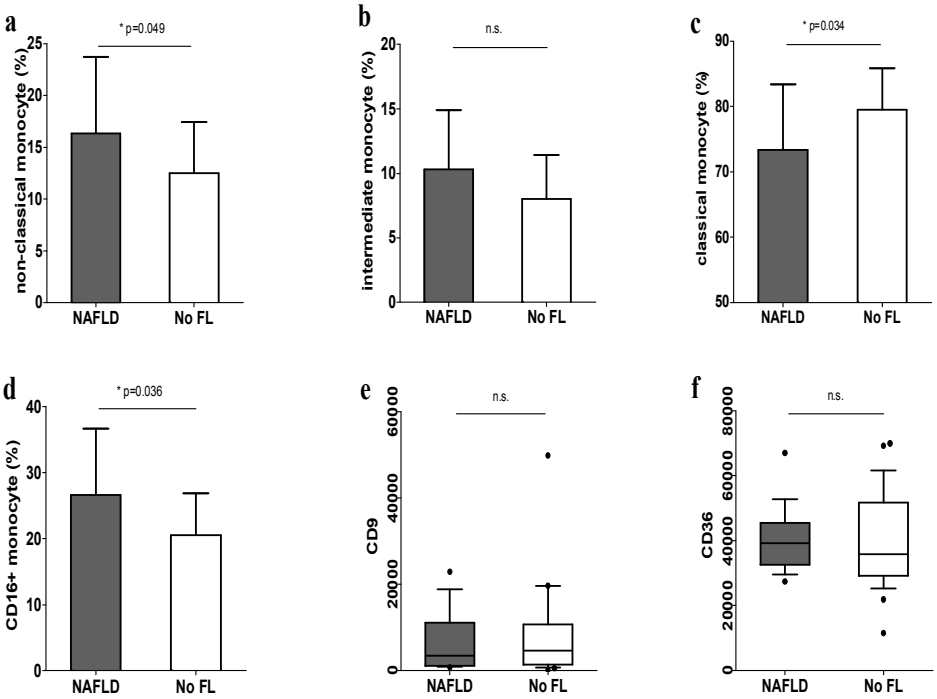

Figure 13 Monocyte populations in NAFLD study (a) non-classical monocyte fraction, (b) intermediate monocyte fraction, (c) classical monocyte fraction, (d) total CD16+ monocyte fraction, (e) CD9 MFI and (f) CD36 MFI on monocytes. Data following normal distribution were represented as bar graph with mean and standard deviation, while data following skewed distribution were represented as box plot with median and 25th to 75th percentiles (boxes) and 10th to 90th percentiles (whiskers). Independent-sample Student's t test or Mann-Whitney U test was used to determine significant differences (**p<0.05*) between two groups. * represented *p<0.05*. NAFLD, non-alcoholic fatty liver disease; FL, fatty liver disease; MFI, median fluorescence intensity; n.s., not significant.

The probands with NAFLD and no FL showed differences in some classical markers well-known to be related to obesity. The subjects with NAFLD had an increased WC compared to controls (104.0 [98.3-108.5] cm vs. 94.0 [85.4-98.3] cm; *p=0.001*),

increased WHR (0.96±0.08 vs. 0.84±0.08, *p<0.001*). Metabolic dysfunction was confined to the NAFLD probands with increased triglycerides (1.9 [1.1-3.0] mmol/L vs. 1.1 [0.7-1.8] mmol/L; *p=0.031*), significantly elevated insulin levels (11.9 [7.9-17.8] mU/L vs. 7.3 [5.2-12.0] mU/L; *p=0.028*) and an elevated HOMA-IR index (2.80 [1.83-4.60] vs. 1.69 [1.13-2.93]; *p=0.029*). As we expected, ALT (29 [21-51] U/L vs. 21 [18-30] U/L; *p=0.009*), AST (27 [24-36] U/L vs. 24 [21-27] U/L; *p=0.029*), GGT (38 [29-82] U/L vs. 20 [16-37] U/L; *p=0.003*) and FLI (7.09 [1.91-28.36] vs. 1.15 [0.51-3.68]; *p=0.005*) were significantly elevated in probands with NAFLD compared to the controls. No significant differences in serum levels of hs-CRP and IL-6 were found [Figure 14].

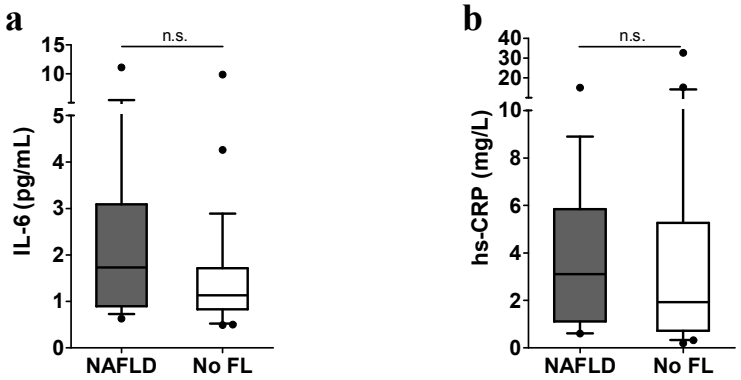

Figure 14 Serum levels of IL-6 and hs-CRP. IL-6 and hs-CRP followed skewed distribution, they were represented as box plot with median and 25th to 75th percentiles (boxes) and 10th to 90th percentiles (whiskers). Mann-Whitney U test was used to determine significant differences (p <0.05) between two groups. NAFLD, non-alcoholic fatty liver disease; FL, fatty liver disease; IL-6, interleukin-6; hs-CRP, high sensitive C-reactive protein; n.s., not significant.

3.1.2. Association between monocyte and NAFLD

To investigate the relationship between NAFLD and monocytes fraction, the multiple logistic regression was conducted. Since WHR contributed strongly (odds ratio (OR)=4.47E8; 95% confidence interval (CI) =3451.757-5.80E13, *p=0.001*) to NAFLD phenotype in the univariate logistic regression and might blunt the contribution of the other variables, which may be due to the overfitting of small sample size, therefore it was excluded from multiple regression analysis. Other variables such as gender, WC, ALT, AST, GGT, TG, neutrophile fraction, lymphocyte fraction, non-classical monocyte fraction, classical monocyte fraction, intermediate

monocyte fraction, total monocyte fraction, cholesterol, LDL-C, HDL-C, HOMA-IR were all added to multiple logistic regression model with the forward stepwise method. The analysis revealed that total monocyte fraction, non-classical monocyte fraction and WC significantly contributed on NAFLD (*p=0.015, 0.047, 0.013* respectively). Since "gender" was a confounder, "gender" was incorporated into the reanalysis with three significant variables by the enter method. It revealed that total monocyte fraction (OR=1.698; 95% CI=1.010-2.854; *p=0.046*), non-classical monocyte fraction (OR=1.172; 95% CI= 1.002-1.372; *p=0.047*) and WC (OR=1.137; 95% CI=1.024-1.263; *p=0.017*) were independent risk factors of NAFLD (Table 5).

Table 5 Multiple logistic regression analysis associated with NAFLD

No.	Variable	β	SE	OR	95% CI for OR	*p*
1	Waist circumstance	0.127	0.051	1.136	1.028 - 1.255	*0.013*
	Non-classical monocyte fraction	0.158	0.080	1.171	1.002 - 1.369	*0.047*
	Total monocyte fraction	0.520	0.214	1.682	1.107 - 2.556	*0.015*
2	Gender	-0.064	1.065	0.938	0.116 - 7.572	*0.952*
	Waist circumstance	0.128	0.054	1.137	1.024 - 1.263	*0.017*
	Non-classical monocyte fraction	0.159	0.080	1.172	1.002 - 1.372	*0.047*
	Total monocyte fraction	0.529	0.265	1.698	1.010 - 2.854	*0.046*

Note: No. means the order of analysis. No.1 analysis included the variables (gender, waist circumstance, AST, ALT, GGT, TG, lymphocyte fraction, neutrophil fraction, total monocyte fraction, non classical monocyte fraction, intermediate monocyte fraction, classical monocyte fraction, fatty liver index) which were significant (*p<0.1*) in the univariate logistic regression analysis and the variables of interest (LDL-C, HDL-C, cholesterol, HOMA-IR) with no significant in the model by forward stepwise method, WC, non-classical monocyte fraction, total monocyte fraction were the variables in the equation finally. No.2 analysis included "gender" and the three variables above in model by enter method. ALT, alanine transaminase; AST, aspartate transaminase; CI, confidence interval; GGT, γ-glutamyl transferase; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment-estimated insulin resistance index; LDL-C, low-density lipoprotein cholesterol; NAFLD, non-alcoholic fatty liver disease; OR, odds ratio; SE, standard error; TG, triglyceride; WC, waist circumstance; β, standardized regression coefficient.

3.1.3. Association between monocyte and other variables

To investigate the relationship of classical monocyte fraction, non-classical monocyte fraction and total monocyte fraction with other variables, the multiple linear regression analysis was performed. As we mentioned previously, the significant variables in the univariate analysis and the variables of interest were

added to analysis by the enter method. The result of multiple linear regression was shown in Table 6. As well-known, monocytes were strongly associated with gender. After controlling of all confounders, classical monocyte fraction and the non-classical monocyte fraction were both significantly associated with WHR (β=-1.206, *p=0.015;* β=1.025, *p=0.022*), while it was not powered enough to detect an association between total monocyte fraction with WHR (β=0.600, p=0.212). The non-classical monocyte fraction also showed weak association with HOMA-IR (β=-0.371, *p=0.033*) and total monocyte fraction showed association with ALT (β=-0.667, *p=0.037*).

Table 6 Multiple linear regression of non-classical monocyte fraction, classical monocyte fraction, total monocyte fraction with other variables

variable	non-classical monocyte fraction		classical monocyte fraction		total monocyte fraction	
	β	*P*	β	*P*	β	*P*
Gender	-0.613	*0.010*	0.705	*0.008*	0.706	*0.008*
Age	-0.061	*0.700*	0.278	*0.122*	-0.131	*0.461*
Body mass index	-0.194	*0.543*	0.202	*0.566*	0.092	*0.795*
Waist circumstance	-0.625	*0.238*	0.314	*0.587*	-0.378	*0.518*
Waist-to-hip ratio	1.025	*0.022*	-1.206	*0.015*	0.600	*0.212*
Aspartate transaminase	-0.159	*0.413*	0.180	*0.402*	-0.166	*0.443*
Alanine transaminase	0.165	*0.550*	0.219	*0.473*	-0.667	*0.037*
γ-glutamyl transferase	0.215	*0.390*	-0.485	*0.086*	0.296	*0.291*
Fatty liver index	0.420	*0.161*	-0.231	*0.479*	0.081	*0.805*
Cholesterol	0.230	*0.823*	-0.610	*0.593*	-0.825	*0.474*
LDL-C	-0.511	*0.611*	0.936	*0.400*	0.697	*0.533*
HDL-C	-0.274	*0.563*	0.369	*0.481*	0.569	*0.284*
Triglycerides	-0.086	*0.912*	0.332	*0.700*	0.583	*0.504*
HOMA-IR	-0.371	*0.033*	0.300	*0.111*	0.202	*0.281*
Interleukin-6	0.090	*0.613*	0.138	*0.485*	-0.346	*0.090*
High sensitive c-reactive protein	-0.220	*0.234*	0.133	*0.512*	-0.053	*0.795*

Note: Variables that showed significant association with non-classical monocyte fraction/classical monocyte fraction/total monocyte fraction and variables of interest were all included in this multiple linear regression analysis by enter method. HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment-estimated insulin resistance; LDL-C, low-density lipoprotein cholesterol; β, standardized regression coefficient.

3.1.4. Association between monocyte and the severity of NAFLD

The non-classical monocyte fraction elevated according to NAFLD severity (i.e. no FL, grade 1, grade 2), 12.5%, 12.6%, 18.4%, respectively; the classical monocyte fraction decreased according to the NAFLD severity, 79.5%, 78.3%, 71.2%, respectively; the total monocyte fraction increased according to the NAFLD severity, 7.0%, 8.0%, 9.2%, respectively. However, the significant differences only existed between probands with no FL and probands with moderate NAFLD in all comparisons, see Figure 15.

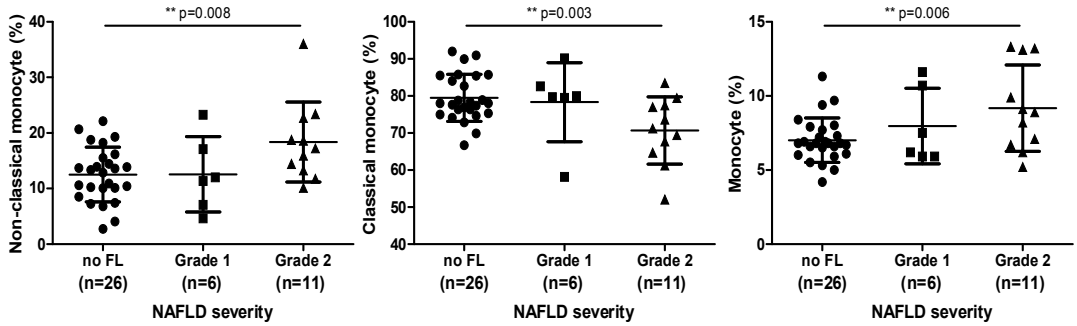

Figure 15 Association between monocyte and the severity of hepatic steatosis. All data was represented as scatter plot with mean and standard deviation. ** represented p<0.01. NAFLD, non-alcoholic fatty liver disease; FL, fatty liver disease.

3.1.5. Comparison between reversed phenotype and constant phenotype

There was no significant difference in inflammatory markers (neutrophil fraction, leukocyte count, total monocyte fraction, monocyte subset fraction, IL-6 and hs-CRP) between probands with the reversed no FL (the phenotype reversed from NAFLD to no FL) and stable no FL. The comparison between probands with reversed NAFLD (the phenotype reversed from no FL to NAFLD) and stable NAFLD was not conducted because of the insufficient sample sizes (see Supplement Table 2).

3.1.6. Comparison between baseline and follow-up

Since the measurement methods were different between baseline and follow-up, we only compared variables which used the same methods including BMI, WHR, WC, IL-6 and hs-CRP (Figure 16). The result showed that BMI, WHR, WC were

significantly elevated in the follow-up of probands with stable NAFLD than baseline. All data including probands with the reversed phenotype was displayed in Supplement Table 3.

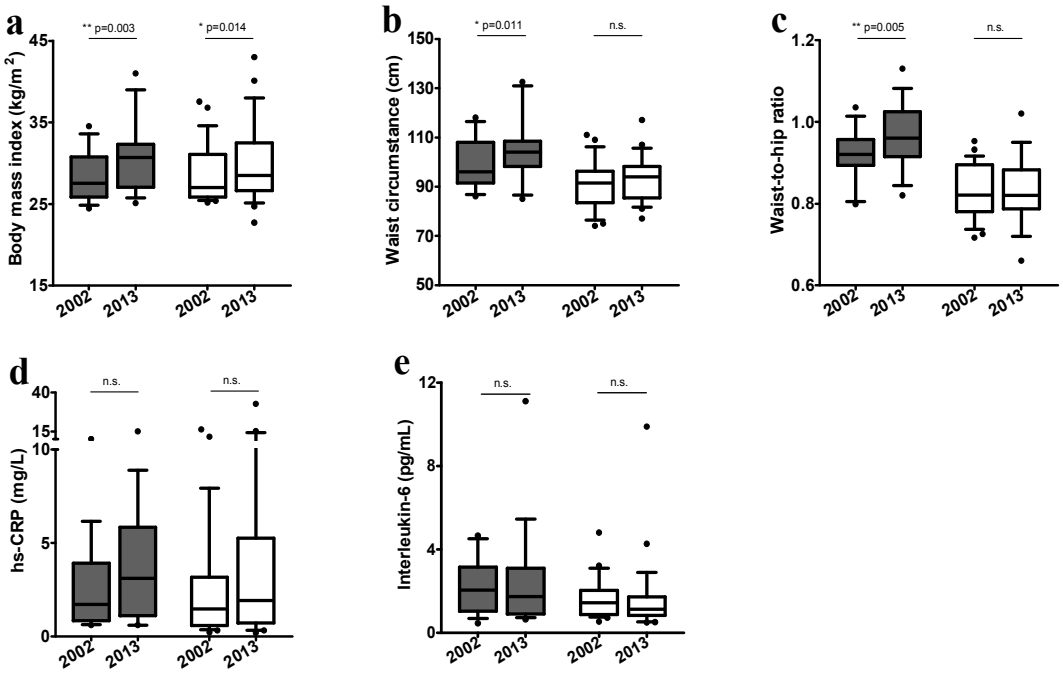

Figure 16 Comparisons between baseline and follow-up. Comparisons between baseline and follow-up. All the data were represented as box plot with median and 25th to 75th percentiles (boxes) and 10th to 90th percentiles (whiskers). The first two boxes represented the same subjects (n=17) with NAFLD; the latter two boxes represented the same subjects (n=26) without FL. * represented $p<0.05$, ** represented $p<0.01$. NAFLD, non-alcoholic fatty liver disease; FL, fatty liver disease; hs-CRP, high sensitive C-reactive protein.

3.2. Monocyte gene expression in CAD

3.2.1. Discovery set

3.2.1.1. Basic clinical characteristics

The baseline characteristics of 71 patients enrolled in present study are shown in Table 7. All of them were diagnosed as CAD by coronary angiograms at baseline observation. 30 CAD patients died due to cardiac causes, while 41 CAD patients survived over follow-up of 7.9 ± 3.8 (mean ± SD) years without having a major cardiac event. The 10 healthy controls aged 20 to 47 years were also Caucasians without CAD and diabetes. In two disease groups (death and survivor), there was no significant difference in BMI, HbA1C, total cholesterol, TG, gender, smoke, T2DM and hypertension except age. The deceased subjects were older compared to the survivors (68.8±5.6 vs. 65.2±5.2, *p=0.006*).

Table 7 Baseline characteristics of enrolled subjects with coronary artery disease

Characteristics	Death (cardiac causes) n=30	Survivor n=41	*P value*
Age	68.8±5.6	65.2±5.2	*0.006*
Body mass index (kg/m^2)	27.1± 3.5	28.0±2.5	*0.209*
Male n (%)	22 (73.3%)	22 (53.7%)	*0.137*
Type 2 Diabetes n (%)	24 (80.0%)	34 (82.9%)	*0.766*
HemoglobinA1C (%)	6.9±1.0	6.5±0.8	*0.139*
Smoke n (%)	24 (80.0%)	25 (61.0%)	*0.120*
Hypertension n (%)	19 (63.3%)	28 (68.3%)	*0.800*
Total cholesterol (mg/dL)	204.9±43.4	211.8±45.1	*0.523*
Triglycerides (mg/dL)	166.4±113.1	170.6±75.9	*0.852*

Note: Values were expressed as means with standard deviation for countinous variables and number of subjects (percentage) for categorical variables. Death (cardiac causes) represented patients died due to cardiac causes.

3.2.1.2. Microarray analysis

3.2.1.2.1. CAD with death (cardiac causes) versus CAD with survivor

The result of differentially expressed genes in CAD with death due to cardiac causes versus survivor was shown in Supplement Table 4. No matter the integral

analysis or stratification analysis, there were only a few differential genes. Most of them were small nucleolar RNA or other genes with high false discovery rate (FDR, >0.05) and have small relative fold change (<2). There was no differentially expressed gene in this comparison.

3.2.1.2.2. CAD Female versus CAD Male

The analysis of CAD Female versus Male showed the similar situation with the analysis in CAD with death due to cardiac causes versus survivor, see Supplement Table 5. After exlusion of genes with FDR (> 0.05) or small relative fold change (<2), the replicated genes and small nucleolar RNA, only the genes including *RPS4Y1, DDX3Y, EIF1AY, UTY, ZFY, KDM5D* and *TXLNG2P* (Table 8) which are linked to Y chromosome showed significantly differential expression in this comparison. These differentially expressed genes were caused by gender background not CAD.

Table 8 Differential gene expression in CAD Female versus Male

Gene symbol	Male versus Female (relative fold change)			
Y-chromosome	**Type 2 Diabetes**	**No diabetes**	**Death (cardiac causes)**	**Survivor**
RPS4Y1	16.48	16.72	15.73	17.17
DDX3Y	21.53	19.34	20.28	21.98
EIF1AY	16.3	18.48	16.74	16.71
UTY	10.35	9.86	10.1	10.54
ZFY	5.72	6.47	5.49	6.16
KDM5D	3.68	2.94	3.48	3.58
TXLNG2P	2.09	2.34	2.04	2.2

Note: Death (cardiac causes) represented patients died due to cardiac causes. The false discovery rate was less than 0.05 and p value was less than 0.05. CAD, coronary artery disease.

3.2.1.2.3. CAD with diabetes versus CAD without diabetes

The analysis in CAD with diabetes versus without diabetes is summarized in Supplement Table 6. After exclusion of the replicated genes, small nucleolar RNA and genes with high FDR (>0.05) or small relative fold change (<2), there was no significant differentially expressed gene too.

3.2.1.2.4. CAD versus Healthy control

The amount of differentially expressed genes in CAD versus healthy control is indiacted in Supplement Table 7. There were differences in the result between integral analysis and stratification analysis. Given the differences in all comparisons, to look for the most potential differential genes, the comparisons between the differential genes of all subgroup and total groups were performed. Finally, 186 identical differentially expressed genes with the same regulation pattern were observed, 77 up-regulated genes and 109 down-regulated genes, Figure 17 showed the heatmap of these 186 genes.

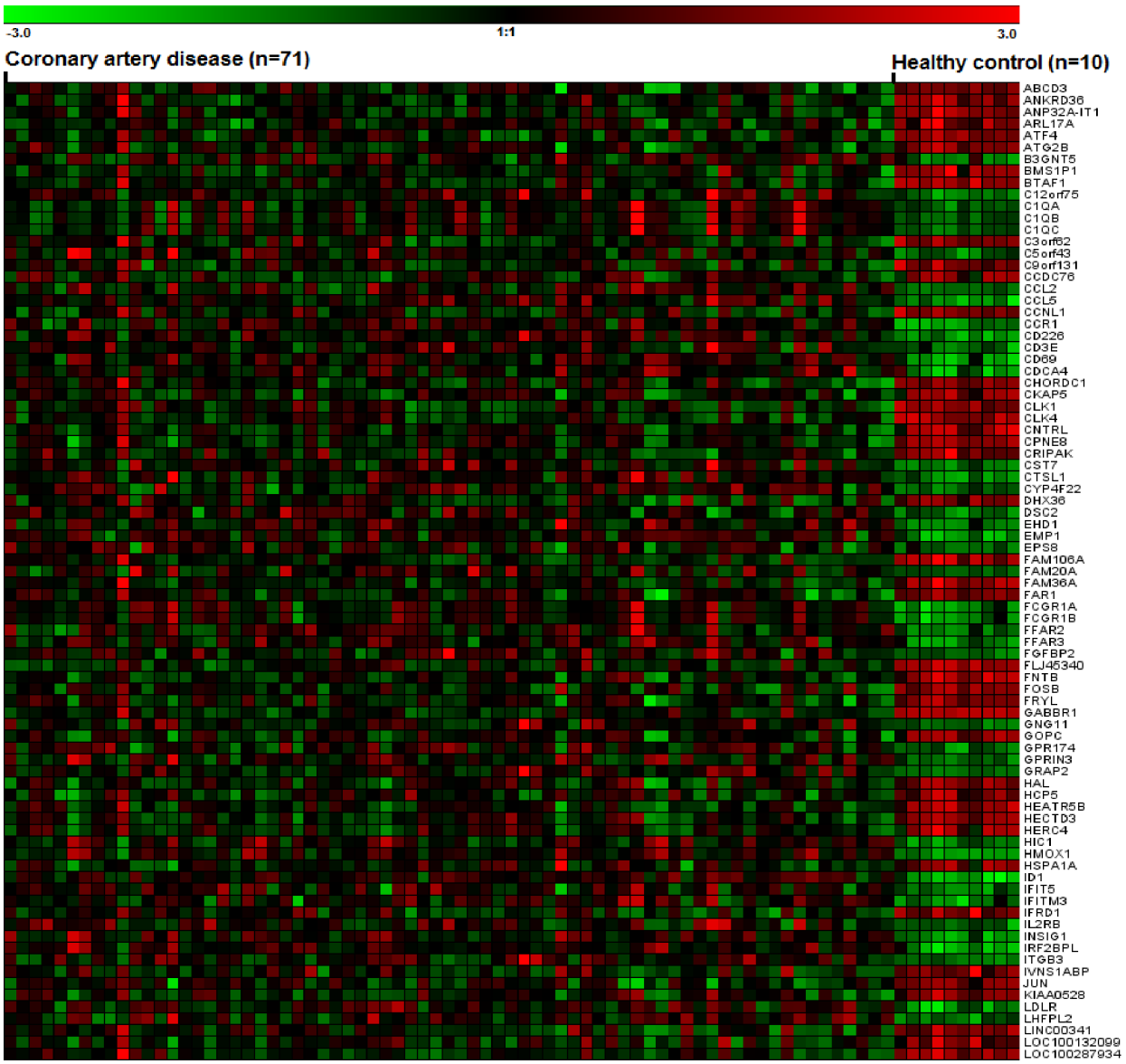

Figure 17 The heatmap of 186 identical differentially expressed genes in the comparison between CAD and healthy control. **------continued on next page**

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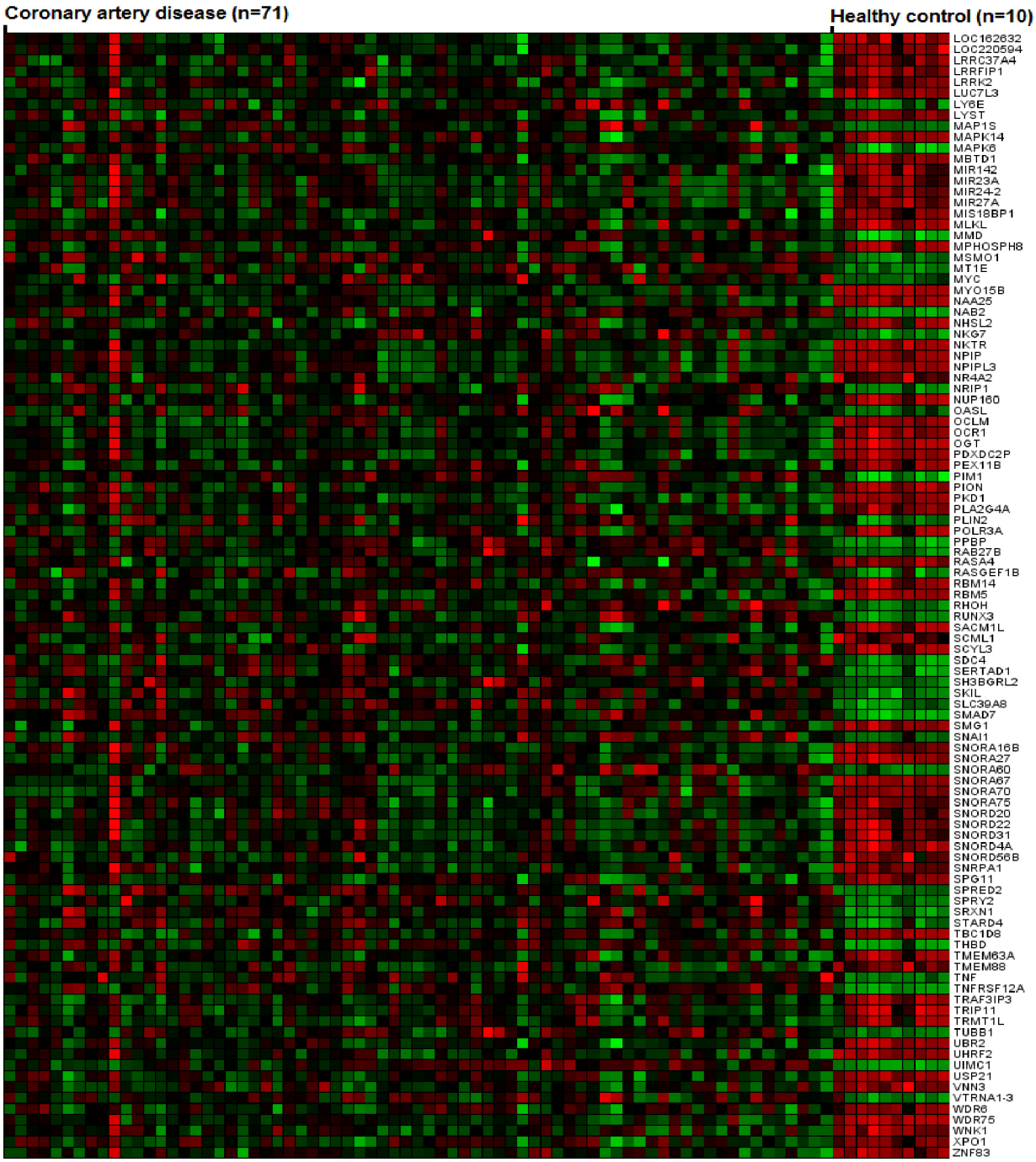

Figure 17 (continued) The heatmap of 186 identical differentially expressed genes in the comparison between CAD and healthy control. Red indicates that the gene transcription is highly expressed, while the green indicates that the gene transcription is lowly expressed. Row indicates differential gene, column indicates experiment (case). This figure contained 71 patients with CAD and 10 healthy controls. CAD, coronary artery disease.

The identical differentially expressed genes were inputed into the Ingenuity Pathway Analysis (IPA) database. Top diseases of these genes were related to inflammatory response, infectious disease, renal and urological disease,

connective tissue disorders and immunological disease. Top molecular and cellular functions of these genes were related to cellular function and maintenance, cell death and survival, cellular growth and proliferation, cellular development and cellular movement. Figure 18 showed the top ten significant canonical pathways involved.

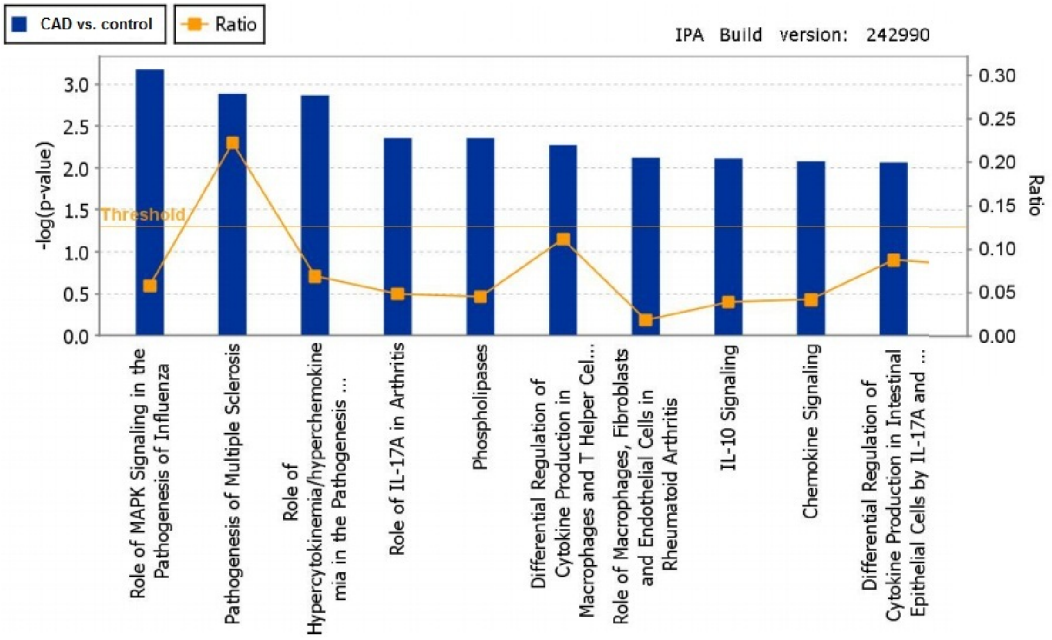

Figure 18 Top ten significant Canonical Pathways involved. This analysis was based on the data from the integral analysis of CAD versus healthy control. The ratio is calculated as the number of genes from our dataset that overlap with the Canonical Pathway in question divided by the total number of genes that are represented in that Canonical Pathway- it is meant to measure the amount of overlap. Significance is calculated in the same way as functional analysis- it is meant to measure the confidence of overlap. The threshold value indicates that p value is 0.05, above the threshold line means that p value is less than 0.05. The figure was adapted from IPA analysis. CAD, coronary artery disease; IPA, Ingenuity Pathways Analysis.

We investigated function interactions using the IPA and found 26 significant differential genes involved in CVD and cardiovascular system development and function. Of 26 differentially expressed genes, 17 genes were associated with inflammatory response while 8 of them were also related to lipid metabolism. Except that, *CD69* which was related to inflammatory response was significantly up-regulated in our dataset (relative fold change 5.770, p=1.00E-08, FDR 1.00E-08). All information of FDR and relative fold change presented here was based on the data from the integral analysis of CAD versus healthy control. Table 9 showed the

27 differential genes information in our data. Of 27 identified differentially expressed genes, 20 genes including *PPBP, CD69, CCL2, EMP1, SMAD7, THBD, TNFRSF12A, ID1, CD226, CCL5, CTSL1, SDC4, NAB2, PIM1, FFAR3, LDLR, CCR1, MAPK14, UBR2*, and *OGT* (relative fold chage ≥ 2 or ≤ -2) were selected as candidate genes for further study. Major of them were top regulated genes in CAD patients.

Table 9 The 27 of differentially expressed genes involved in cardiovascular disease, cardiovascular system development and function.

Gene symbol	Relative fold change	*p-value*	False discovery rate
PPBP	6.520	*<1.00E-07*	<1.00E-07
CD69	5.770	*<1.00E-07*	<1.00E-07
CCL2	5.410	*1.75E-05*	7.43E-05
EMP1	5.200	*<1.00E-07*	<1.00E-07
SMAD7	5.120	*<1.00E-07*	<1.00E-07
THBD	4.170	*<1.00E-07*	<1.00E-07
TNFRSF12A	3.450	*<1.00E-07*	<1.00E-07
ID1	3.390	*<1.00E-07*	<1.00E-07
CD226	2.990	*<1.00E-07*	<1.00E-07
CCL5	2.790	*<1.00E-07*	<1.00E-07
CTSL1	2.600	*<1.00E-07*	<1.00E-07
SDC4	2.550	*<1.00E-07*	<1.00E-07
NAB2	2.540	*<1.00E-07*	<1.00E-07
PIM1	2.480	*<1.00E-07*	<1.00E-07
FFAR3	2.440	*1.00E-07*	7.55E-07
LDLR	2.180	*<1.00E-07*	<1.00E-07
CCR1	2.120	*<1.00E-07*	<1.00E-07
RUNX3	1.840	*1.00E-07*	7.55E-07
HMOX1	1.820	*<1.00E-07*	<1.00E-07
GRAP2	1.810	*5.40E-06*	2.70E-05
TLR1	1.720	*7.00E-07*	4.54E-06
ATF4	-1.720	*<1.00E-07*	<1.00E-07
PLA2G4A	-1.790	*5.00E-07*	3.31E-06
MAPK14	-2.040	*<1.00E-07*	<1.00E-07
UBR2	-2.040	*<1.00E-07*	<1.00E-07
mir-24-2	-2.56	*1.00E-07*	7.55E-07
OGT	-3.13	*<1.00E-07*	<1.00E-07

Note: All information of false discovery rate and fold change presented here was based on the data from the integral analysis of CAD versus healthy control. CAD, coronary artery disease.

3.2.1.3. qRT-PCR validation

20 candidate genes were validated by qRT-PCR. *ABL1* and *HPRT1* were detected as housekeeping genes. Of 71 CAD patient samples, 38 patient samples as well as the whole 10 healty controls were selected for validation by the qRT-PCR. The direction and magnitude of the relative fold changes of the microarray and qRT-PCR were comparable as depicted in Figure 19 (P<0.05). *PPBP, CD69, CCL2, EMP1, SMAD7, THBD, TNFRSF12A, ID1, CD226, CCL5, CTSL1, SDC4, NAB2, PIM1, FFAR3, LDLR* and *CCR1* were up regulated in CAD patients. In contrast, *MAPK14, UBR2* and *OGT* were down regulated in CAD patients.

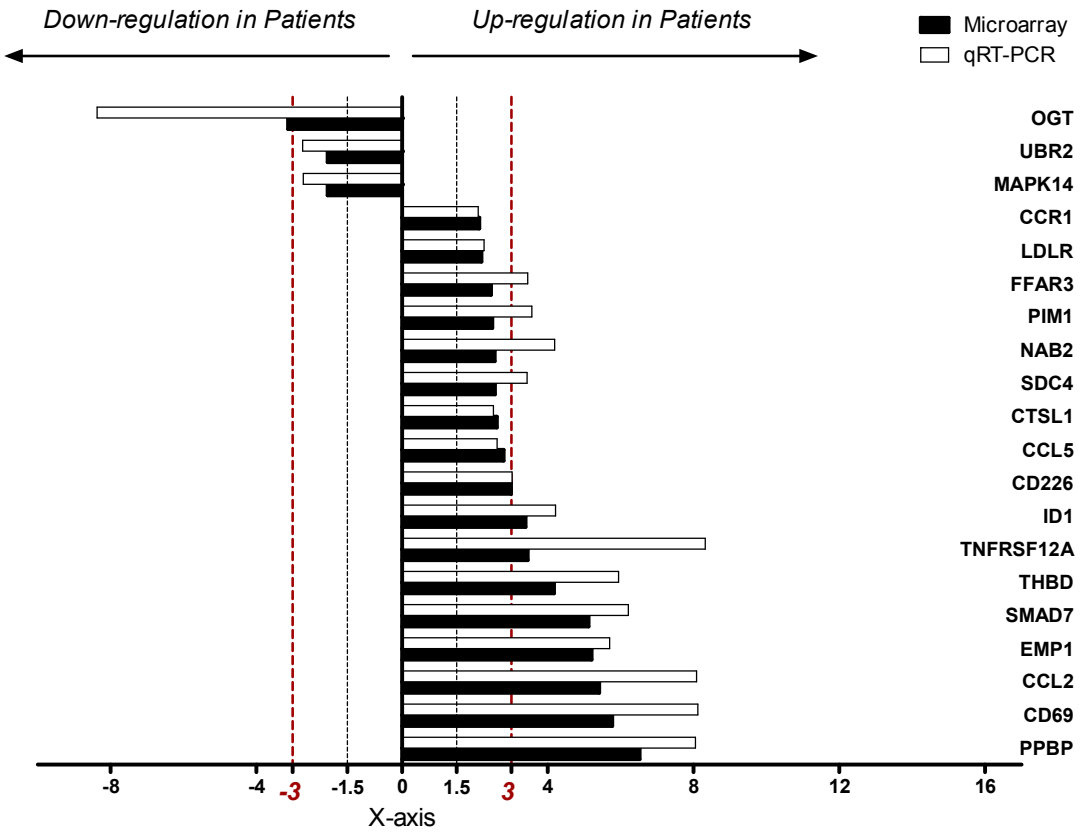

Figure 19 candidate genes expression in CAD versus healthy controls validated by qRT-PCR in discovery set. The X-axis depicts the relative fold changes in gene expression in CAD compared to controls. For each gene the first blank bar depicts the relative fold change in qRT-PCR, the second black bar depicts the relative fold change in microarray. A relative fold change between -1.5 and 1.5 indicates no effect. A relative fold change more than -3 and 3 indicates overt regulation. qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

3.2.2. Replication set

The age (presented as median with interquatile range) of group CAD+T2DM+, healthy control was 62.8 (59.4-70.6) and 36.1 (27.8-37.2), respectively (Table 10). No further clinical parameters were detected.

Table 10 Characteristics of enrolled subjects in replication set

Characteristics	CAD+T2DM+ n=5	Healthy control n=5
Age	62.8 (59.4-70.6)	36.1 (27.8-37.2)
Body mass index (kg/m^2)	33.3 (29.3-38.9)	-
Male (n)	3	3
Smoke (n)	2	-
Hypertension (n)	4	0

Note: "+" represents that subject was suffered from this disease. CAD, coronary artery disease; T2DM, type 2 diabetes.

The differential genes validation in replication set by qRT-PCR was shown in Table 11. In replication validation, only *CTSL1* showed the comparable regulation as discovery set (fold change 2.64, p=0.027). The genes including *PPBP, CCL2, SMAD7, THBD, TNFRSF12A, CD226, CCL5, PIM1, LDLR* were upregulated (fold change>2). The gene *MAPK 14* showed opposite regulation in replication set (fold change 2.09, p=0.044).

Table 11 Differentially expressed genes in dicovery set and replication set

Gene Symbol	Discovery set CAD versus Healthy			Replication set CAD+T2DM+ versus Healthy	
	Microarray fold change	qRT-PCR fold change	*P* (qRT-PCR)	qRT-PCR fold chage	*p*
PPBP	6.52	8.05	*0.003*	29.83	*0.484*
CD69	5.77	8.12	*0.019*	1.99	*0.672*
CCL2	5.41	8.08	*0.012*	2.03	*0.251*
EMP1	5.20	5.70	*0.011*	-1.49	*0.770*
SMAD7	5.12	6.21	*0.047*	3.13	*0.278*
THBD	4.17	5.94	*0.004*	4.04	*0.117*
TNFRSF12A	3.45	8.32	*0.003*	3.09	*0.119*
ID1	3.39	4.21	*<0.001*	-2.46	*0.776*
CD226	2.99	3.02	*<0.001*	9.81	*0.461*
CCL5	2.79	2.61	*0.010*	4.91	*0.897*
CTSL1	2.60	2.51	*<0.001*	2.64	*0.027*
SDC4	2.55	3.43	*0.005*	1.83	*0.184*
NAB2	2.54	4.19	*<0.001*	1.63	*0.318*
PIM1	2.48	3.56	*0.004*	3.46	*0.127*
FFAR3	2.44	3.45	*0.027*	-1.32	*0.653*
LDLR	2.18	2.25	*0.039*	2.57	*0.311*
CCR1	2.12	2.09	*<0.001*	1.19	*0.545*
MAPK14	-2.04	-2.71	*<0.001*	2.09	*0.044*
UBR2	-2.04	-2.73	*<0.001*	1.32	*0.181*
OGT	-3.13	-8.37	*<0.001*	1.63	*0.004*

Note: "+" represents that subject was suffered from this disease. Relative fold change <-2 or >2 was considered down-regulated or up-regulated expression for qRT-PCR analysis. P value less than 0.05 indicates statistical significance. CAD, coronary artery disease; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; T2DM, type 2 diabetes.

4. Discussion

4.1. Circulating monocyte phenome in NAFLD

The result of our study showed an elevation of non-classical monocyte fraction and total monocyte fraction in NAFLD patients. The non-classical monocyte fraction and total monocyte fraction were the independent risk factors for NAFLD. The classical monocyte fraction and non-classical monocyte fraction were strongly associated with body composition (WHR), total monocyte fraction showed this trend too.

The imbalance of monocyte/macrophages as critical components of innate immune system has been reported in animal models (Mikita et al. 2011; Moore et al. 2013; Sakaguchi et al. 2011; Smith. 2013; Wan et al. 2014). Data from mouse models of alcoholic liver disease (ALD) and NAFLD showed that mice that are resistant to the effects of alcohol or a high-fat diet had a high M2:M1 Kupffer cell ratio, whereas mice that developed liver lesions has a high M1:M2 Kupffer cell ratio, and further research showed that M2 Kupffer cells isolated from these resistant mice promoted apoptosis of M1 Kupffer cells *in vitro* via paracrine activation of arginase (Smith. 2013). In those models mediator released by Kupffer cells was found to play key roles in progression of these disease. Due to the lack of liver biopsies we can't perform a histopathological examination as well as mechanistic studies. However, by using the ultimate model system, the human being, we could clearly reveal that an inflammatory monocyte phenotype is a key signal (biomarker) in subjects with fatty liver disease.

Monocytes are divided into three subsets in humans. Classical monocytes were more studied in past time, while the other two subsets were less known, especially the intermediate monocytes. The classical monocytes are considered as professional phagocytes. The CD16+ monocytes including intermediate monocytes and non-classical monocytes are considered as the "proinflammatory" cells and were found elevated in lots of inflammatory status, e.g. active Crohn's disease (Grip et al. 2007), erysipelas (Horelt et al. 2002), rheumatoid arthritis (Kawanaka et al.

2002) and cutaneous leishmaniasis (Soares et al. 2006). The CD16+ monocytes appear to be a more mature version (Ziegler-Heitbrock et al. 1993). In humans, non-classical monocytes are also considered to be patrolling cells that are involved in the local surveillance of tissues (Ghattas et al. 2013). The function of CD16+ monocytes seems ambivalent and likely depends on the disease stage, and the biological significance between non-classical monocytes and intermediate monocytes is still unclear. In several reports on CAD subjects, total CD16+ monocyte fraction were found to be elevated compared to the controls (Kashiwagi et al. 2010; Wildgruber et al. 2009). The presence of CD16+ monocytes was related to coronary plaque vulnerability in the patients with stable angina pectoris (Kashiwagi et al. 2010), whereas the alteration of monocyte subsets was independently related to cardiovascular events in patients with CKD (Heine et al. 2008; Rogacev et al. 2011). In human inflammatory status including CVD, circulating monocyte subsets have been proposed as a valuable marker (Yang et al. 2014). Our data showed the independent elevated total monocyte fraction and non-classical monocyte fraction in NAFLD patients, however the classical inflammatory parameters such as IL-6 and hs-CRP were found not be increased in comparison to no fatty liver group. In view of this result, we suggest that the total monocyte fraction and non-classical monocyte fraction are more sensitive biomarkers as compared to IL-6 and hs-CRP in the disease process of deregulated fat deposition.

Along this line our study indicated the strong correlation with the classical anthropometric marker of obesity and classical monocyte fraction and non-classical monocyte fraction. WHR was used to estimate abdominal obesity. Elevated WHR is associated with a greater risk of circulatory mortality in both older men and women (Price et al. 2006). Recently Rogacev and co-workers reported high CD16+ monocyte counts were significantly associated with both higher BMI and increased carotid intima media thickness, a significant univariate association between the CD16+ monocytes and both subclinical atherosclerosis and obesity in low-risk subjects was revealed (Rogacev et al. 2010). Our results support thepossible link between monocyte and obesity. Combined with previous result of total monocyte

fraction and non-classical monocyte fraction as the independent risks for NAFLD, the total monocyte fraction and non-classical monocyte fraction might be valuable potential biomarkers for predicting prognosis of NAFLD.

The association between total monocyte fraction and non-classical monocyte fraction with the severity of NAFLD was also studied in our study. There were significant differences only between subjects with no FL and with moderate NAFLD in all comparisons. Because no subjects with severe NAFLD were included in our study, the association between monocytes and the severity of NAFLD can't be concluded. However, the results may indirectly point toward the potential role of the non-classical monocyte fraction and the total monocyte fraction as markers of NAFLD severity. In our study alterations of circulating monocytes were associated to at least moderate NAFLD but not mild NAFLD.

As previously mentioned, multiple lines of evidence support the high degree of interplay between NAFLD and CAD. Patients with NAFLD always have multiple risk factors for CAD, some well-established risk factors (Ratziu et al. 2010) including insulin resistance, dyslipidemia, dysglycemia, hypertension and obesity, some nontraditional risk factors such as increased uric acid (Li et al. 2009; Sirota et al. 2013), decreased adiponectin (Targher et al. 2006), increased proinflammatory markers (e.g. CRP, IL-6) and so on. Furthermore, NAFLD has been demonstrated as the independent risk for CAD (2014). Abundant evidences indicate that patients with NAFLD have increased carotid artery intima-media thickness (Sookoian et al. 2008), impaired flow-mediated vasodilatation. NAFLD increases the instability of coronary plaques, the risk of having coronary calcification and developing T2DM (Assy et al. 2010; Gastaldelli et al. 2009; Sung et al. 2012). Therefore, NAFLD may be perceived as a pre-status of CAD. In view with our results, the non-classical monocyte fraction and total monocyte fraction are sensitive and valuable potential biomarkers for low-grade inflammatory status and predicting the prognosis of NAFLD. Given the important role of monocytes in CAD, it may be extrapolated that the non-classical monocyte fraction and total monocyte fraction might be potential biomarkers for assessing the status of CAD.

In our study we also looked for the expression of CD9 and CD36 on monocyte. CD9 is a cell glycoprotein, which belongs to the family of tetraspanin and widely distributed on an extensive range of cells and tissues, including B cells, activated T cells, platelets and most carcinomas (Maecker et al. 1997). CD9 has been implicated in various biological functions containing cell adhesion, differentiation, metastasis, signal transduction and endothelial regeneration, as well as platelet activation and aggregation (Ko et al. 2006; Maecker et al. 1997; Mangin et al. 2009; Nakazawa et al. 2008). The expression of *CD9* gene in circulating monocytes was up-regulated in diabetic patients compared to healthy controls (Beyan et al. 2010). CD36, an 88 kDa glycoprotein, is expressed on multiple cells and tissues including monocytes/macrophages, adipocytes, microvascular endothelial cells, platelets, hepatocytes, smooth muscle cells and so on. CD36 as one of scavenger receptors play key roles in lipid metabolism, platelet activation and atherogenesis (Collot-Teixeira et al. 2007). In our study, both CD36 and CD9 were found to be highly expressed using multiparamterer FACS analysis on the monocytes but no difference between NAFLD and controls could be seen.

As we expected, a panel of NAFLD associated markers including the variables (increased WHR, WC, insulin, TG, HOMA-IR) indicative of dysmetabolism and the variables (ALT, AST, GGT, FLI) indicative of alterations in the liver function were found to separate the two studied cohorts. Given well-known evidence reported (Armutcu et al. 2013), we considered them as the classical markers which confirmed NAFLD.

In conclusion, our study revealed alterations in monocyte subsets in peripheral blood as a marker of a dysmetabolic state with aberrant fat deposition in the liver. In view of biology of the monocyte lineage, it may well be that the circulating inflammatory monocytes are directly involved in the initiation and the progression of NAFLD. The marker sets described in our study might be of considerable interest to be used as the prognostic marker panel as well as the druggable target of both pro-inflammatory and dysmetabolic states.

4.2. Monocyte gene expression in CAD

The aim of this part was to identify potential key players in the disparities of long-term outcomes and gender in CAD patients, as well as novel players in the pathogenesis of in general. To address this issue we studied the gene expression profiles of circulating monocytes of CAD patients with different status (death due to cardiac events vs. survivor, male vs. female, T2DM vs. no DM) and compared the profiles with healthy controls. Our result did not show significant differentially expressed genes related to long-term outcomes, gender and T2DM in patients with CAD. However, we found that 20 candidate genes (relative fold change was more than 2 or less than -2) including *PPBP, CD69, CCL2, EMP1, SMAD7, THBD, TNFRSF12A, ID1, CD226, CCL5, CTSL1, SDC4, NAB2, PIM1, FFAR3, LDLR, CCR1, MAPK14, UBR2, OGT* involved in CVD and cardiovascular system development and function, of which most of genes were also associated with inflammatory response and lipid metabolism, were significantly differentially expressed in CAD patients compared to healthy controls. However, only *CTSL1* was confirmed in an independent small replication set of CAD patients recruited from Aachen.

4.2.1. Up-regulation of CTSL1 in CAD patients versus healthy control

CTSL1 i.e. cathepsin L is located on human 9q21.33. The protein encoded by this gene is a lysosomal cysteine proteinase associated with potent collagenolytic and elastinolytic activities, which has been involved in several pathologic processes, including myofibril necrosis in myocardial ischemia and in myopathies, and in the renal tubular response to proteinuria. Most tissues and cells express basal levels of cathepsin L. However, it is observed that inflammatory cytokines e.g. IL-6, interferon (IFN)-γ induce increased expression of cathepsin L in cultured human lung epithelial cells (Gerber et al. 2001), muscle (Gallardo et al. 2001) and macrophages (Lah et al. 1995; Liu et al. 2006). Several studies have shown that cathepsins B and L were present in the cytoplasm and nuclei of apoptotic macrophages within human atheroma (Li et al. 2001); increased expression of

cathepsins L localized to lesional smooth muscle cells, endothelial cells and macrophages in human abdominal aortic aneurysms and atheromata, and patients with coronary artery stenosis showed higher serum cathepsin L levels than those without lesions (Liu et al. 2006); cathepsin L deficiency reduced diet-induced atherosclerosis in LDL receptor-knockout mice with decreased macrophage and lymphocytes infiltration, as well as decreased collagen and elastin degradation (Kitamoto et al. 2007); cathepsin L was significantly increased in atherosclerotic plaques with formation of the necrotic core and rupture of the cap in quantitative immunohistochemical analysis of human carotid atherosclerotic lesions, human monocyte-derived macrophages from CAD patients showed significantly increased levels of cathepsin L, cellular lipids and apoptosis (Li et al. 2009). Except the expression of resident macrophages in lesion or plaques reported, our result showed that the gene *CTSL1* expression of circulating monocyte was up-regulated in CAD patients. Although the potential role of cathepsin L in CAD remains unclear thoroughly, our result confirms the fact that cathepsin L is implicated in the pathogenesis of CAD again. Figure 20 depicts the network of proteins interactived with CTSL1.

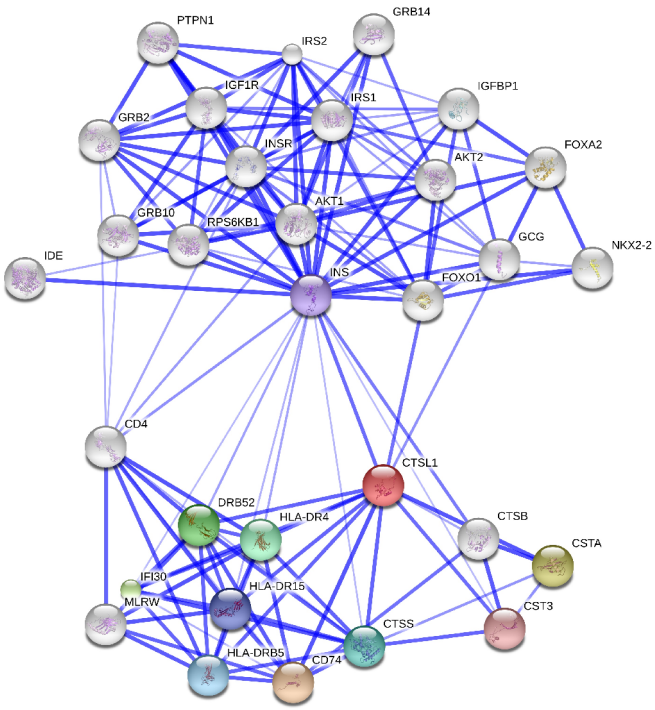

Figure 20 The network of proteins interactived with CTSL1. This is the confidence view, stronger associations are represented by thicker lines. Generated by STRING 9.1.

4.2.2. Potential functions of candidate genes not confirmed

The panel of 19 candidate genes initially identified could not be confirmed in the replication set. There are several potential factors may cause the different results in replication set. To avoid a confounding effect just be addressing Swabian patients, probands in replication set were recruited from Aachen. Although all fasting whole blood samples were collected in EDTA-tube and processed as soon as possible, the isolation of PBMC from whole blood of Aachen samples was not possible to be processed within 6 hours of sampling like LURIC samples because of long-distance transport. Normally it took at least 24 hours, or even longer. That may affect viability and activation of monocytes. Furthermore, the handling of samples was not totally identical. To avoid activating monocytes and get more yields, monocytes were isolated from fresh PBMC by negative selection in Aachen samples. However, monocytes were isolated from frozen PBMC by positive selection in LURIC samples. This may lead to a little different in the purity and activation of monocytes. Another important factor could be that three patients from Aachen were also suffered from depression and taken antidepressants, which may affect the gene expressions in monocytes. Therefore, further validation in more replication samples is needed.

Using a literature search, the majority of the initially identified genes may be implicated in the progression of CAD, especially *PPBP, CD69, CCL2, CCL5, CCR1*，*LDLR*, *MAPK14, THBD* and *SDC4.* These genes have been found to be related to inflammatory and lipid metabolisms. Secondly, the five candidate genes including *EMP1, SMAD7, TNFRSF12A, ID1* and *OGT* were all more than 3 fold regulated in CAD patient compared to healthy controls. Besides the 20 candidate genes, *HMOX1*, which was slightly regulated in every subgroup comparison of our discovery data, is interesting too. Figure 21 depicts the network of proteins encoded by these genes. They are valuable for further research.

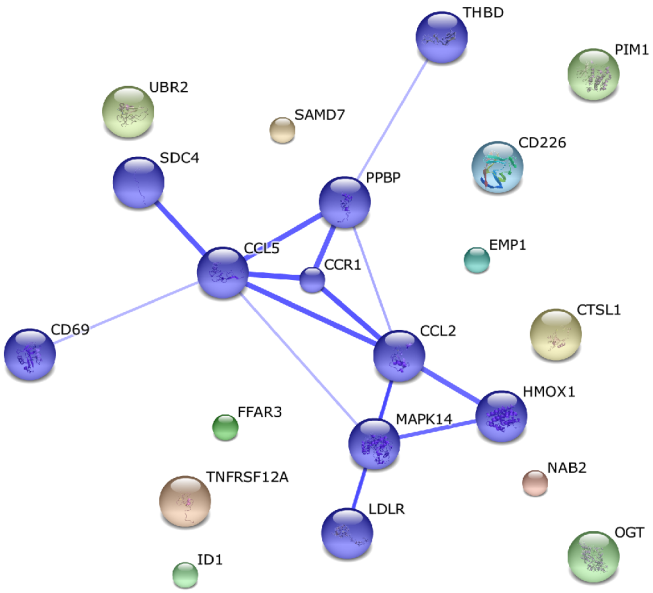

Figure 21 The network of proteins encoded by corresponding genes. This is the confidence view, stronger associations are represented by thicker lines. Generated by STRING 9.1.

4.2.2.1. Major candidate genes of interest

PPBP also known as *CXCL7* or *CTAP-III* i.e. pro-platelet basic protein is located in human 4q12-q13. The protein encoded by this gene is a platelet-derived growth factor that belongs to the CXC chemokine family. Chemokine PPBP is the most abundant representative among the chemokines stored and secreted by platelets. There are several known molecular variants of PPBP including platelet basic protein, connective tissue activating peptide III, β-thromboglobulin and neutrophil-activating peptide-2 (NAP-2). Only NAP-2 display chemotactic activity while other variants may be considered precursors of NAP-2 even though they also have other specific biological function (Gleissner et al. 2008). PPBP is a potent chemoattractant and activator of neutrophils. In addition, it has been shown to induce various cellular processes including DNA synthesis, mitosis, glycolysis, intracellular cAMP accumulation, prostaglandin E2 secretion, and synthesis of hyaluronic acid and sulfated glycosaminoglycan, as well as the formation and secretion of plasminogen activator by synovial cells. There is little direct evidence for PPBP related to proatherogenic process. Recently, PPBP was observed in mouse carotid arteries after wire injury, and it showed to induce adhesion of endothelial progenitor cells after arterial injury through receptor CXCR2 (Hristov et

al. 2007). However, one study showed that *PPBP* was down-regulated in pre-lesion and aged mouse endothelium (Erbilgin et al. 2013). In our discovery set, *PPBP* was 6.52 (8.05 fold validated by qRT-PCR) fold up-regulated in CAD patients versus healthy control. So far, there is less evidence based on human tissues and cells. Although the result was not consistent, PPBP may be involved in angiogenesis and vascular remodeling and less is known about its specific role in the process.

CD69 is located in human 12p13. This gene encodes a type II transmembrane receptor related to the calcium dependent lectin superfamily. CD69 is expressed following activation in most of bone marrow-derived cells including T and B lymphocytes, NK cells, monocytes and so on (Ramirez et al. 1996; Sancho et al. 2005). In circulating monocytes, the expression of CD69 is constitutive and the signal generated after CD69 crosslinking is a potent activator of monocyte functions (De Maria et al. 1994; Testi et al. 1994). Multiple studies showed that CD69 is persistently expressed in the infiltrates of leukocytes produced in chronic inflammatory diseases (e.g. rheumatoid arthritis and chronic viral hepatitis) and autoimmune disorders (e.g systemic lupus erythematosus) (Gomez et al. 2009). CD 69 is considered to exert not only a proinflammatory function but also modulate the inflammatory response. Furthermore, it may exert immunoregulatory functions following activation (Sancho et al. 2005). There is little data about CD69 expression of monocytes in the context of atherosclerotic lesions. Interestingly, our discovery data showed a significant up-regulation (relative fold change microarray/qRT-PCR is 5.77/8.12, respectively) of circulating monocytes in CAD patients versus healthy control. Although it was not confirmed in our replication set, it provided a novel clue about the potential molecules involved in the pathogenesis of CAD.

CCL2 also known as *MCP1* is located in human 17q11.2-q12, and *CCL5* also known as *RANTES* is located in human 17q12 too. The proteins encoded by the two genes are well-known involved in immunoregulatory and inflammatory processes. CCL2 have chemotactic activity for monocytes and basophils, which play crucial roles in the early accumulation of circulating monocytes in impaired endothelium. CCL5 has been shown to orchestrate the recruitment of circulating

monocytes, dentritic cells, neutrophil, memory T helper cells and eosinophils to inflamed tissues through the receptors of CCR1, CCR3, or CCR5 (Ferrandi et al. 2007; Houard et al. 2009; Suffee et al. 2012). It has been reported that CCL5 and CCL2 are increased in vascular wall cells and recruite leukocyte to the injured artery during vascular remodeling (Raines et al. 2005), and the CCL5 antagonist prevented progression of established atherosclerotic lesions in mice (Braunersreuther et al. 2008). Recent clinical data showed high CCL5 plaque levels were associated with an unstable plaque phenotype (Herder et al. 2011). More evidence support that CCL2 and CCL5 are implicated in the pathogenisis of CAD. As the receptor of CCL5, there is no doubt that CCR1 is involved in CAD, and the function of gene *CCR1* in host protection from inflammatory response has been confirmed in lots of knockout studies in mouse.

LDLR i.e low density lipoprotein receptor is located in human 19p13.2. This gene encodes a cell surface receptor that mediates the endocytosis of cholesterol-rich LDL. Mutations in this gene cause the autosomal dominant disorder, familial hypercholesterolemia. A recent study showed that LDLR were significantly up-regulated in atherosclerotic coronary artery tissue compared to non-atherosclerotic coronary artery tissue in humans (Blin et al. 2013). In general, high LDL levels are associated with the increased risk of CVD. Therefore, overexpression of *LDLR* gene (relative fold change microarray/qRT-PCR is 2.18/2.25, respectively) in our discovery set might indicate that not only deficiency or mutations of *LDLR* gene, but also the overexpression may be associated with CAD, which may depends on the stage and severity of the CAD. To gain a further insight into the role of LDLR more studies are needed.

MAPK14 also known as *p38-α* i.e. mitogen-activated protein kinase 14 is located in human 6p21.3-p21.2. The gene encodes an enzyme that is an isoform of the p38 MAPKs which are activated by stress, proinflammatory cytokines (e.g. TNF, IL-1) and endotoxin. p38 MAPKs are crucial for a wide range of biological processes such as cell differentiation, apoptosis and the expression of inflammatory cytokines (Ashwell. 2006). Moreover, p38 MAPKs have been implicated in the development

and progression of atherosclerosis via various aspects such as regulation of scavenger receptor expression (Zhao et al. 2002), control of ox-LDL-induced CXCR2 expression (Lei et al. 2002), proliferation and migration of endothelial cells (Denes et al. 2002; McMullen et al. 2005). However, some study indicates that deficiency of p38-α promotes apoptosis and plaque necrosis in advanced atherosclerotic lesions in mice (Seimon et al. 2009). p38-α was revealed a dual function in regulation of inflammation (Kim et al. 2008). In our discovery set, the expression of *MAPK14* gene (relative fold change microarray/qRT-PCR is -2.04/-2.71, respectively) was down-regulated in CAD patients versus healthy control, while it showed opposite regulation in discoversy set. The function of p38-α has not been clear thoroughly.

THBD also known as *THBD* or *CD141* i.e. thrombomodulin is located in human 20p11.2. Protein thrombomodulin is a glycoprotein membrane receptor on the surface of endothelial cells that modulated hemostasis and inflammation. It is also expressed on human mesothelial cells, monocytes and DC subset. The binding of thrombin and THBD results in the activation of protein C, conducting the important endogenous anticoagulant. Therefore, soluble THBD was thought to indicate endothelial injury and considered to be a protective factor for thrombus (Sadler. 1997; Weiler et al. 2003). The increased plasma soluble THBD was observed in cardioembolic stroke (Dharmasaroja et al. 2012), acute myocardial infarction and hypertension (Dohi et al. 2003). The association between circulating levels of THBD and CAD is controversial. The prospective ARIC study reported that high level soluble THBD was protective against CAD (OR 0.31, 95%CI=0.14-0.69), however, it tended to be positive relationship with carotid atherosclerosis especially among Caucasians (OR 2.94, 95%CI=1.15-7.51) (Salomaa et al. 1999). In contrast, the prospective PRIME study (Morange et al. 2004) and MONICA/KORA cohort study (Karakas et al. 2011) did not support a potential predictive role or protective role of high level soluble THBD against CAD. Moreover, it was reported that high glucose conditions induced *THBD* expression in human aortic endothelial cells (Wang et al. 2012). The increment of THBD across the coronary circulation was

significantly correlated with the severity of coronary atherosclerosis (Nakagawa et al. 2001). The explanation for these different results is not clear. In our discovery set, the expression of *THBD* showed significant up-regulation (relative fold change microarray/qRT-PCR is 4.17/5.94, respectively) in CAD patients versus healthy control. The controversial results and its important role in formation of thrombus make THBD deserve more attention.

SDC4 i.e. syndecan 4 is located in human 20q12. The protein encoded is a transmembrane heparan sulfate proteoglycan that functions as a receptor in intracellular signaling. Increased syndecan 4 levels are essential for myocardial remodeling (Matsui et al. 2011; Strand et al. 2013). The expression of *SDC4* gene (relative fold change microarray/qRT-PCR is 2.55/3.43, respectively) was up-regulated in CAD patients versus healthy control in our discovery set, it indicated that SDC4 maybe implicated in the process of CAD, especially the long-term cardiac remodeling after the initiation of the pathogenesis.

4.2.2.2. The second panel of candidate genes

EMP1 also known as *TMP* i.e. epithelial membrane protein 1 is located in human 12p12.3. The protein encoded by this gene is a transmembrane glycoprotein. EMP1 was found to regulate epithelial cell proliferation and differentiation. It has been reported to be implicated in epithelial cell proliferation and differentiation. Down-regulation of EMP1 has been observed in head and neck squamous cell carcinoma (Kuriakose et al. 2004), oral squamous cell carcinoma (Zhang et al. 2011), oesophageal cancer cells (Wang et al. 2003). A recent data indicates that the up-regulation of *EMP1* expression is associated with prednisolone-resistant in pediatric acute lymphoblastic leukemia patients and leads to a poorer 5-year event-free survival of patients (Aries et al. 2014). Little is known about the exact function. In our discovery set, CAD patients showed overt high expression of *EMP1* (relative fold change microarray/qRT-PCR is 5.20/5.70, respectively) compared to healthy control. It may provide a novel insight into the pathogenesis of CAD.

SMAD7 i.e. SMAD family member 7 is located in human 18q21.1. Protein SMAD7

is a nuclear protein, which belongs to the TGF β superfamily of ligands, involved in cell signalling. It is an inhibitory SMAD, which block the activation of receptor-regulated SMADs and common-mediator SMAD (Shi et al. 2003). SMAD7 is a general antagonist against TGF β signaling by blocking access to R-SMAD, degrading TGF β receptor type-1 (Hayashi et al. 1997; Shi et al. 2003). Expression of this gene is induced by TGF β and non-superfamily signals such as TNF-α and IFN-γ (Bitzer et al. 2000; Yan et al. 2011). SMAD appears to play important role in several disease processes such as colorectal cancer (Halder et al. 2008), pancreatic cancer (Arnold et al. 2004) and hypertensive cardiac remodeling (Wei et al. 2013), while the role in pancreatic cancer is still controversial. Data from our discovery set showed that up-regulation of *EMP1* (relative fold change microarray/qRT-PCR is 5.12/6.21, respectively) occurred in CAD patients compared to healthy control. However, a lot of animal studies showed SMAD7 has protection of cardiac funtion and remodeling (Chen et al. 2009; Wei et al. 2013; Wei et al. 2013). The SMAD7 function in human atherosclerosis is less illustrated.

TNFRSF12A also known as *TWEAKR* or *FN14* i.e. tumor necrosis factor receptor superfamily, member 12A is located in human 16p13.3. Generally, protein TWEAKR is expressed at low levels in many cells and highly regulated by cytokines (Munoz-Garcia et al. 2006) and growth factors (Feng et al. 2000; Wiley et al. 2001). It showed high expression after tissue injury (Feng et al. 2000; Wiley et al. 2001; Yepes et al. 2005). The interaction of TWEAKR and its ligand TWEAK enhances tissue factor and PAI-1 expression in atherosclerotic plaques and in cultured vascular smooth muscle cells (Munoz-Garcia et al. 2011). They are also involved in some processes of vascular cells including proliferation, migration (Lynch et al. 1999; Meighan-Mantha et al. 1999), monocyte cytotoxicity (Nakayama et al. 2000), apoptosis (Nakayama et al. 2002) and angiogenesis (Lynch et al. 1999; Wiley et al. 2001). Immunohistochemical staining of human atherosclerotic plaques showed high expression of TWEAKR in macrophage/foam cells. TWEAKR may destabilize atherosclerotic plaques by inducing MMPs (Kim et al. 2001). Our data showed that CAD patients had a significant up-regulation of *TNFRSF12A* (relative

fold change microarray/qRT-PCR is 3.45/8.32, respectively) compared to healthy controls, confirming the role of TNFRSF12A in the pathogenesis of atherosclerosis. *ID1* i.e. inhibitor of DNA binding 1, dominant negative helix-loop-helix protein is located in human 20q11. Protein ID1 is a helix-loop-helix (HLH) protein that can dimerize with basic HLH, but because ID1 has no DNA binding activity, it can inhibit the DNA binding and transcriptional activation ability of basic HLH. It is reported that ID1 is implicated in human cancers, and high levels of ID1 gene expression have been observed in many aggressive tumor cells compared to normal cells (Fong et al. 2004; Ling et al. 2006; Soroceanu et al. 2013) and rheumatoid arthritis (Sakurai et al. 2001). It may play a role in cell proliferation and the progression of cell cycle. Moreover, ID1 can induce activation and angiogenesis in cultured human vascular endothelial cells (Sakurai et al. 2004). The direct data regarding ID1 function in CAD is sparse. Our data firstly showed that patients with CAD had high expression (relative fold change microarray/qRT-PCR is 3.39/4.21, respectively) of ID1 compared to healthy controls. However, these data need further validation in replication sets.

OGT i.e. O-linked N-acetylglucosamine transferase is located in human Xq13. Protein OGT plays an important role in both O-glycosylation and transcription (Yang et al. 2002). It may be linked to chronic disease conditions such as T2DM, neurodegeneration and cancers (Bond et al. 2013). It is reported that diabetes inhibits OGT expression while insulin treatment can stimulates its expression in streptozotocin-induced diabetic rats (Majumdar et al. 2004). The down-regulation of *OGT* (relative fold change microarray/qRT-PCR is -3.13/-8.37, respectively) in CAD patients versus healthy control was shown in our discovery data. It provides a unique window for research on pathogenesis of CAD.

4.2.2.3. Other potential candidate gene observed-HMOX1

Recent data indicates that HMOX1 potently and independently drives metaflammation and IR in the hepatic and macrophage compartments. This gene was described as one of the top predictors of metabolic disease in humans and

mice (Jais et al. 2014). *HMOX1* also known as *HO-1* i.e. heme oxygenase-1 is located in human 22q13.1. This gene encodes heme oxygenase-1, an essential enzyme in heme catabolism, degrade heme to form biliverdin, iron and carbon monoxide. HMOX1 activity is induced by heme as well as various stressors including endotoxin, cytokines, and oxidants (Abraham et al. 2008). Several studies have shown that HMOX1 loss-of-function results in endothelial cell injury and early death in humans (Yachie et al. 1999), and deletion in mice leads to high perinatal mortality and increased susceptibility to inflammation (Kapturczak et al. 2004). In our microarray data, *HMOX1* is slightly up-regulated (relative fold change is 1.820 in combined analysis) in every subgroup of CAD patients versus healthy control. There is sparse evidence regarding *HMOX1* expression in CAD. Given CAD is a low-grade inflammation status, what we found is consistent with aforementioned studies although it needs further validation. These findings may suggest that HMOX1 inhibition may be a potential therapeutic strategy for metabolic diseases even for CAD.

In conclusion, no differentially expressed genes related to long-term outcomes, gender disparities and T2DM influences in CAD patients were observed in our study. However, upregulation of *CTSL1* in circulating monocytes of CAD patients versus healthy controls was identified. The result indicates that CTSL1 may be associated with pathogenesis of CAD. We also found a candidate gene panel containing several novel genes which may be indicated in the progress of coronary atherosclerosis and most of them are associated with inflammatory, although they were not confirmed in our replication set. Given to the small sample size in replication set, further work to confirm these finding is still needed.

5. Summary

Coronary artery disease (CAD) which is characterized by atherosclerosis in coronary artery becomes the single leading cause of death worldwide. Non-alcoholic fatty liver disease (NAFLD) has been identified as an independent risk factor for CAD and perceived as pre-status of CAD. CAD patients display different outcomes over long-term follow-up, and gender disparities in CAD prevalence and outcome do exist. Although CAD has been extensively studied, key pathways involved in the pathogenesis are not thoroughly understood. As main component of the innate immune system, monocytes play major roles in pathogenesis of NAFLD and CAD. Therefore, our study focused on the phenotyping of circulating monocytes.

In the first part of present study, the circulating monocyte phenome in NAFLD was conducted to investigate the association between NAFLD and alteration in circulating monocyte subsets as a cellular hallmark of the CAD state. It was demonstrated that total monocyte fraction and non-classical monocyte fraction were increased and defined independently risk for NAFLD. The non-classical monocyte fraction and classical monocyte fraction were strongly associated with waist-to-hip ratio (WHR). In the second part of this study, the whole genome expression profiling was performed to identify key genes expressed on monocytes associated with clinical outcomes and gender differences of CAD, as well as novel potential pathways related to the expression of CAD. Firstly, there were no signifiant differentially expressed genes on monocytes associated with clinical outcomes and gender differences of CAD. Secondly, 20 candidate gens including *PPBP, CD69, CCL2, EMP1, SMAD7, THBD, TNFRSF12A, ID1, CD226, CCL5, CTSL1, SDC4, NAB2, PIM1, FFAR3, LDLR, CCR1, MAPK14, UBR2, OGT* were found to be differently expressed in CAD patients compared to healthy controls. Most candidates identified were associated with inflammatory response and lipid metabolism. Finally, the up-regulation of *CTSL1* was confirmed using an independent replication set.

In conclusion, these data show that circulating monocytes are indicative a dysmetabolic and inflammatory state in humans, and inflammatory pathway is important for the pathogenesis of CAD.

6. References

1. Abraham NG, Kappas A: Pharmacological and clinical aspects of heme oxygenase. Pharmacol Rev 60: 79-127 (2008)

2. Allen N, Berry JD, Ning H, Van Horn L, Dyer A, Lloyd-Jones DM: Impact of blood pressure and blood pressure change during middle age on the remaining lifetime risk for cardiovascular disease: the cardiovascular lifetime risk pooling project. Circulation 125: 37-44 (2012)

3. Altieri DC, Morrissey JH, Edgington TS: Adhesive receptor Mac-1 coordinates the activation of factor X on stimulated cells of monocytic and myeloid differentiation: an alternative initiation of the coagulation protease cascade. Proc Natl Acad Sci U S A 85: 7462-7466 (1988)

4. American Diabetes Association: Executive summary: Standards of medical care in diabetes--2012. Diabetes Care 35 Suppl 1: S4-S10 (2012)

5. Ancuta P, Rao R, Moses A, Mehle A, Shaw SK, Luscinskas FW, Gabuzda D: Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. J Exp Med 197: 1701-1707 (2003)

6. Anstee QM, McPherson S, Day CP: How big a problem is non-alcoholic fatty liver disease? BMJ 343: d3897 (2011)

7. Anstee QM, Targher G, Day CP: Progression of NAFLD to diabetes mellitus, cardiovascular disease or cirrhosis. Nat Rev Gastroenterol Hepatol 10: 330-344 (2013)

8. Aries IM, Jerchel IS, van den Dungen RE, van den Berk LC, Boer JM, Horstmann MA, Escherich G, Pieters R, den Boer ML: EMP1, a novel poor prognostic factor in pediatric leukemia regulates prednisolone resistance, cell proliferation, migration and adhesion. Leukemia:(2014)

9. Armutcu F, Akyol S, Ucar F, Erdogan S, Akyol O: Markers in nonalcoholic steatohepatitis. Adv Clin Chem 61: 67-125 (2013)

10. Arnold NB, Ketterer K, Kleeff J, Friess H, Buchler MW, Korc M: Thioredoxin is downstream of Smad7 in a pathway that promotes growth and suppresses

cisplatin-induced apoptosis in pancreatic cancer. Cancer Res 64: 3599-3606 (2004)

11. Ashwell JD: The many paths to p38 mitogen-activated protein kinase activation in the immune system. Nat Rev Immunol 6: 532-540 (2006)

12. Assy N, Djibre A, Farah R, Grosovski M, Marmor A: Presence of coronary plaques in patients with nonalcoholic fatty liver disease. Radiology 254: 393-400 (2010)

13. A WHO Expert Committee: Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee. World Health Organ Tech Rep Ser 854: 1-452 (1995)

14. Badimon L, Storey RF, Vilahur G: Update on lipids, inflammation and atherothrombosis. Thromb Haemost 105 Suppl 1: S34-S42 (2011)

15. Badman MK, Flier JS: The adipocyte as an active participant in energy balance and metabolism. Gastroenterology 132: 2103-2115 (2007)

16. Bairey MC, Shaw LJ, Reis SE, Bittner V, Kelsey SF, Olson M, Johnson BD, Pepine CJ, Mankad S, Sharaf BL, Rogers WJ, Pohost GM, Lerman A, Quyyumi AA, Sopko G: Insights from the NHLBI-Sponsored Women's Ischemia Syndrome Evaluation (WISE) Study: Part II: gender differences in presentation, diagnosis, and outcome with regard to gender-based pathophysiology of atherosclerosis and macrovascular and microvascular coronary disease. J Am Coll Cardiol 47: S21-S29 (2006)

17. Bedogni G, Bellentani S, Miglioli L, Masutti F, Passalacqua M, Castiglione A, Tiribelli C: The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. BMC Gastroenterol 6: 33 (2006)

18. Belge KU, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, Espevik T, Ziegler-Heitbrock L: The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. J Immunol 168: 3536-3542 (2002)

19. Beyan H, Drexhage RC, van der Heul NL, de Wit H, Padmos RC, Schloot NC, Drexhage HA, Leslie RD: Monocyte gene-expression profiles associated with childhood-onset type 1 diabetes and disease risk: a study of identical twins.

Diabetes 59: 1751-1755 (2010)

20. Bitzer M, von Gersdorff G, Liang D, Dominguez-Rosales A, Beg AA, Rojkind M, Bottinger EP: A mechanism of suppression of TGF-beta/SMAD signaling by NF-kappa B/RelA. Genes Dev 14: 187-197 (2000)

21. Blin J, Ahmad Z, Rampal LR, Mohtarrudin N, Tajudin AK, Adnan RS: Preliminary assessment of differential expression of candidate genes associated with atherosclerosis. Genes Genet Syst 88: 199-209 (2013)

22. Bond MR, Hanover JA: O-GlcNAc cycling: a link between metabolism and chronic disease. Annu Rev Nutr 33: 205-229 (2013)

23. Braunersreuther V, Steffens S, Arnaud C, Pelli G, Burger F, Proudfoot A, Mach F: A novel RANTES antagonist prevents progression of established atherosclerotic lesions in mice. Arterioscler Thromb Vasc Biol 28: 1090-1096 (2008)

24. Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH: Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology 40: 1387-1395 (2004)

25. Brun P, Castagliuolo I, Di Leo V, Buda A, Pinzani M, Palu G, Martines D: Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. Am J Physiol Gastrointest Liver Physiol 292: G518-G525 (2007)

26. Burke AP, Farb A, Malcom G, Virmani R: Effect of menopause on plaque morphologic characteristics in coronary atherosclerosis. Am Heart J 141: S58-S62 (2001)

27. Burke AP, Farb A, Malcom GT, Liang Y, Smialek J, Virmani R: Effect of risk factors on the mechanism of acute thrombosis and sudden coronary death in women. Circulation 97: 2110-2116 (1998)

28. Canto JG, Goldberg RJ, Hand MM, Bonow RO, Sopko G, Pepine CJ, Long T: Symptom presentation of women with acute coronary syndromes: myth vs reality. Arch Intern Med 167: 2405-2413 (2007)

29. Chen Q, Chen H, Zheng D, Kuang C, Fang H, Zou B, Zhu W, Bu G, Jin T, Wang Z, Zhang X, Chen J, Field LJ, Rubart M, Shou W, Chen Y: Smad7 is required

for the development and function of the heart. J Biol Chem 284: 292-300 (2009)

30. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JJ, Jones DW, Materson BJ, Oparil S, Wright JJ, Roccella EJ: The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. JAMA 289: 2560-2572 (2003)

31. Cobbold JF, Anstee QM, Taylor-Robinson SD: The importance of fatty liver disease in clinical practice. Proc Nutr Soc 69: 518-527 (2010)

32. Collot-Teixeira S, Martin J, McDermott-Roe C, Poston R, McGregor JL: CD36 and macrophages in atherosclerosis. Cardiovasc Res 75: 468-477 (2007)

33. Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, Puel A, Biswas SK, Moshous D, Picard C, Jais JP, D'Cruz D, Casanova JL, Trouillet C, Geissmann F: Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. Immunity 33: 375-386 (2010)

34. De Maria R, Cifone MG, Trotta R, Rippo MR, Festuccia C, Santoni A, Testi R: Triggering of human monocyte activation through CD69, a member of the natural killer cell gene complex family of signal transducing receptors. J Exp Med 180: 1999-2004 (1994)

35. Denes L, Jednakovits A, Hargitai J, Penzes Z, Balla A, Talosi L, Krajcsi P, Csermely P: Pharmacologically activated migration of aortic endothelial cells is mediated through p38 SAPK. Br J Pharmacol 136: 597-603 (2002)

36. Dey S, Flather MD, Devlin G, Brieger D, Gurfinkel EP, Steg PG, Fitzgerald G, Jackson EA, Eagle KA: Sex-related differences in the presentation, treatment and outcomes among patients with acute coronary syndromes: the Global Registry of Acute Coronary Events. Heart 95: 20-26 (2009)

37. Dharmasaroja P, Dharmasaroja PA, Sobhon P: Increased plasma soluble thrombomodulin levels in cardioembolic stroke. Clin Appl Thromb Hemost 18: 289-293 (2012)

38. Dohi Y, Ohashi M, Sugiyama M, Takase H, Sato K, Ueda R: Circulating thrombomodulin levels are related to latent progression of atherosclerosis in hypertensive patients. Hypertens Res 26: 479-483 (2003)

39. Erbilgin A, Siemers N, Kayne P, Yang WP, Berliner J, Lusis AJ: Gene expression analyses of mouse aortic endothelium in response to atherogenic stimuli. Arterioscler Thromb Vasc Biol 33: 2509-2517 (2013)

40. Fabbrini E, Sullivan S, Klein S: Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications. Hepatology 51: 679-689 (2010)

41. Feng SL, Guo Y, Factor VM, Thorgeirsson SS, Bell DW, Testa JR, Peifley KA, Winkles JA: The Fn14 immediate-early response gene is induced during liver regeneration and highly expressed in both human and murine hepatocellular carcinomas. Am J Pathol 156: 1253-1261 (2000)

42. Ferrandi C, Ardissone V, Ferro P, Ruckle T, Zaratin P, Ammannati E, Hauben E, Rommel C, Cirillo R: Phosphoinositide 3-kinase gamma inhibition plays a crucial role in early steps of inflammation by blocking neutrophil recruitment. J Pharmacol Exp Ther 322: 923-930 (2007)

43. Finegold JA, Asaria P, Francis DP: Mortality from ischaemic heart disease by country, region, and age: Statistics from World Health Organisation and United Nations. Int J Cardiol 168: 934-945 (2013)

44. Fong S, Debs RJ, Desprez PY: Id genes and proteins as promising targets in cancer therapy. Trends Mol Med 10: 387-392 (2004)

45. Frink RJ: Gender gap, inflammation and acute coronary disease: are women resistant to atheroma growth? Observations at autopsy. J Invasive Cardiol 21: 270-277 (2009)

46. Gallardo E, de Andres I, Illa I: Cathepsins are upregulated by IFN-gamma/STAT1 in human muscle culture: a possible active factor in dermatomyositis. J Neuropathol Exp Neurol 60: 847-855 (2001)

47. Gastaldelli A, Kozakova M, Hojlund K, Flyvbjerg A, Favuzzi A, Mitrakou A, Balkau B: Fatty liver is associated with insulin resistance, risk of coronary heart disease, and early atherosclerosis in a large European population. Hepatology 49: 1537-1544 (2009)

48. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K: Development of monocytes, macrophages, and dendritic cells. Science 327: 656-661 (2010)

49. Gerber A, Wille A, Welte T, Ansorge S, Buhling F: Interleukin-6 and transforming growth factor-beta 1 control expression of cathepsins B and L in human lung epithelial cells. J Interferon Cytokine Res 21: 11-19 (2001)

50. Ghattas A, Griffiths HR, Devitt A, Lip GY, Shantsila E: Monocytes in coronary artery disease and atherosclerosis: where are we now? J Am Coll Cardiol 62: 1541-1551 (2013)

51. Gleissner CA, von Hundelshausen P, Ley K: Platelet chemokines in vascular disease. Arterioscler Thromb Vasc Biol 28: 1920-1927 (2008)

52. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Wong ND, Woo D, Turner MB: Executive summary: heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation 129: 399-410 (2014)

53. Gomez M, Sanz-Gonzalez SM, Abu NY, Lamana A, Sanchez-Madrid F, Andres V: Atherosclerosis development in apolipoprotein E-null mice deficient for CD69. Cardiovasc Res 81: 197-205 (2009)

54. Grip O, Bredberg A, Lindgren S, Henriksson G: Increased subpopulations of CD16(+) and CD56(+) blood monocytes in patients with active Crohn's disease. Inflamm Bowel Dis 13: 566-572 (2007)

55. Haenle MM, Brockmann SO, Kron M, Bertling U, Mason RA, Steinbach G, Boehm BO, Koenig W, Kern P, Piechotowski I, Kratzer W: Overweight, physical activity, tobacco and alcohol consumption in a cross-sectional random sample of German adults. BMC Public Health 6: 233 (2006)

56. Halder SK, Rachakonda G, Deane NG, Datta PK: Smad7 induces hepatic metastasis in colorectal cancer. Br J Cancer 99: 957-965 (2008)

57. Hamaguchi M, Kojima T, Itoh Y, Harano Y, Fujii K, Nakajima T, Kato T, Takeda

N, Okuda J, Ida K, Kawahito Y, Yoshikawa T, Okanoue T: The severity of ultrasonographic findings in nonalcoholic fatty liver disease reflects the metabolic syndrome and visceral fat accumulation. Am J Gastroenterol 102: 2708-2715 (2007)

58. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MJ, Wrana JL, Falb D: The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. Cell 89: 1165-1173 (1997)

59. Healy B: The Yentl syndrome. N Engl J Med 325: 274-276 (1991)

60. Heimbeck I, Hofer TP, Eder C, Wright AK, Frankenberger M, Marei A, Boghdadi G, Scherberich J, Ziegler-Heitbrock L: Standardized single-platform assay for human monocyte subpopulations: Lower CD14+CD16++ monocytes in females. Cytometry A 77: 823-830 (2010)

61. Heine GH, Ulrich C, Seibert E, Seiler S, Marell J, Reichart B, Krause M, Schlitt A, Koehler H, Girndt M: CD14(++)CD16+ monocytes but not total monocyte numbers predict cardiovascular events in dialysis patients. Kidney Int 73: 622-629 (2008)

62. Herder C, Peeters W, Illig T, Baumert J, de Kleijn DP, Moll FL, Poschen U, Klopp N, Muller-Nurasyid M, Roden M, Preuss M, Karakas M, Meisinger C, Thorand B, Pasterkamp G, Koenig W, Assimes TL, Deloukas P, Erdmann J, Holm H, Kathiresan S, Konig IR, McPherson R, Reilly MP, Roberts R, Samani NJ, Schunkert H, Stewart AF: RANTES/CCL5 and risk for coronary events: results from the MONICA/KORA Augsburg case-cohort, Athero-Express and CARDIoGRAM studies. PLoS One 6: e25734 (2011)

63. Horelt A, Belge KU, Steppich B, Prinz J, Ziegler-Heitbrock L: The CD14+CD16+ monocytes in erysipelas are expanded and show reduced cytokine production. Eur J Immunol 32: 1319-1327 (2002)

64. Houard X, Touat Z, Ollivier V, Louedec L, Philippe M, Sebbag U, Meilhac O, Rossignol P, Michel JB: Mediators of neutrophil recruitment in human abdominal aortic aneurysms. Cardiovasc Res 82: 532-541 (2009)

65. Howard BV, Rodriguez BL, Bennett PH, Harris MI, Hamman R, Kuller LH, Pearson TA, Wylie-Rosett J: Prevention Conference VI: Diabetes and Cardiovascular disease: Writing Group I: epidemiology. Circulation 105: e132-e137 (2002)

66. Hristov M, Zernecke A, Bidzhekov K, Liehn EA, Shagdarsuren E, Ludwig A, Weber C: Importance of CXC chemokine receptor 2 in the homing of human peripheral blood endothelial progenitor cells to sites of arterial injury. Circ Res 100: 590-597 (2007)

67. Jais A, Einwallner E, Sharif O, Gossens K, Lu TT, Soyal SM, Medgyesi D, Neureiter D, Paier-Pourani J, Dalgaard K, Duvigneau JC, Lindroos-Christensen J, Zapf TC, Amann S, Saluzzo S, Jantscher F, Stiedl P, Todoric J, Martins R, Oberkofler H, Muller S, Hauser-Kronberger C, Kenner L, Casanova E, Sutterluty-Fall H, Bilban M, Miller K, Kozlov AV, Krempler F, Knapp S, Lumeng CN, Patsch W, Wagner O, Pospisilik JA, Esterbauer H: Heme oxygenase-1 drives metaflammation and insulin resistance in mouse and man. Cell 158: 25-40 (2014)

68. Kaptoge S, Seshasai SR, Gao P, Freitag DF, Butterworth AS, Borglykke A, Di Angelantonio E, Gudnason V, Rumley A, Lowe GD, Jorgensen T, Danesh J: Inflammatory cytokines and risk of coronary heart disease: new prospective study and updated meta-analysis. Eur Heart J 35: 578-589 (2014)

69. Kapturczak MH, Wasserfall C, Brusko T, Campbell-Thompson M, Ellis TM, Atkinson MA, Agarwal A: Heme oxygenase-1 modulates early inflammatory responses: evidence from the heme oxygenase-1-deficient mouse. Am J Pathol 165: 1045-1053 (2004)

70. Karakas M, Baumert J, Herder C, Rottbauer W, Meisinger C, Koenig W, Thorand B: Soluble thrombomodulin in coronary heart disease: lack of an association in the MONICA/KORA case-cohort study. J Thromb Haemost 9: 1078-1080 (2011)

71. Kashiwagi M, Imanishi T, Tsujioka H, Ikejima H, Kuroi A, Ozaki Y, Ishibashi K, Komukai K, Tanimoto T, Ino Y, Kitabata H, Hirata K, Akasaka T: Association of monocyte subsets with vulnerability characteristics of coronary plaques as

assessed by 64-slice multidetector computed tomography in patients with stable angina pectoris. Atherosclerosis 212: 171-176 (2010)

72. Kawanaka N, Yamamura M, Aita T, Morita Y, Okamoto A, Kawashima M, Iwahashi M, Ueno A, Ohmoto Y, Makino H: CD14+,CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. Arthritis Rheum 46: 2578-2586 (2002)

73. Kim C, Sano Y, Todorova K, Carlson BA, Arpa L, Celada A, Lawrence T, Otsu K, Brissette JL, Arthur JS, Park JM: The kinase p38 alpha serves cell type-specific inflammatory functions in skin injury and coordinates pro- and anti-inflammatory gene expression. Nat Immunol 9: 1019-1027 (2008)

74. Kim HL, Chung GE, Park IY, Choi JM, Hwang SM, Lee JH, Kim D: Elevated peripheral blood monocyte fraction in nonalcoholic fatty liver disease. Tohoku J Exp Med 223: 227-233 (2011)

75. Kim SH, Lee WH, Kwon BS, Oh GT, Choi YH, Park JE: Tumor necrosis factor receptor superfamily 12 may destabilize atherosclerotic plaques by inducing matrix metalloproteinases. Jpn Circ J 65: 136-138 (2001)

76. Kitamoto S, Sukhova GK, Sun J, Yang M, Libby P, Love V, Duramad P, Sun C, Zhang Y, Yang X, Peters C, Shi GP: Cathepsin L deficiency reduces diet-induced atherosclerosis in low-density lipoprotein receptor-knockout mice. Circulation 115: 2065-2075 (2007)

77. Ko EM, Lee IY, Cheon IS, Kim J, Choi JS, Hwang JY, Cho JS, Lee DH, Kang D, Kim SH, Choe J: Monoclonal antibody to CD9 inhibits platelet-induced human endothelial cell proliferation. Mol Cells 22: 70-77 (2006)

78. Kuriakose MA, Chen WT, He ZM, Sikora AG, Zhang P, Zhang ZY, Qiu WL, Hsu DF, McMunn-Coffran C, Brown SM, Elango EM, Delacure MD, Chen FA: Selection and validation of differentially expressed genes in head and neck cancer. Cell Mol Life Sci 61: 1372-1383 (2004)

79. Lah TT, Hawley M, Rock KL, Goldberg AL: Gamma-interferon causes a selective induction of the lysosomal proteases, cathepsins B and L, in macrophages. Febs Lett 363: 85-89 (1995)

80. Laman JD, Schoneveld AH, Moll FL, van Meurs M, Pasterkamp G: Significance

of peptidoglycan, a proinflammatory bacterial antigen in atherosclerotic arteries and its association with vulnerable plaques. Am J Cardiol 90: 119-123 (2002)

81. Lambert JM, Lopez EF, Lindsey ML: Macrophage roles following myocardial infarction. Int J Cardiol 130: 147-158 (2008)

82. LaRosa JC, Grundy SM, Waters DD, Shear C, Barter P, Fruchart JC, Gotto AM, Greten H, Kastelein JJ, Shepherd J, Wenger NK: Intensive lipid lowering with atorvastatin in patients with stable coronary disease. N Engl J Med 352: 1425-1435 (2005)

83. Lei ZB, Zhang Z, Jing Q, Qin YW, Pei G, Cao BZ, Li XY: OxLDL upregulates CXCR2 expression in monocytes via scavenger receptors and activation of p38 mitogen-activated protein kinase. Cardiovasc Res 53: 524-532 (2002)

84. Li W, Dalen H, Eaton JW, Yuan XM: Apoptotic death of inflammatory cells in human atheroma. Arterioscler Thromb Vasc Biol 21: 1124-1130 (2001)

85. Li W, Kornmark L, Jonasson L, Forssell C, Yuan XM: Cathepsin L is significantly associated with apoptosis and plaque destabilization in human atherosclerosis. Atherosclerosis 202: 92-102 (2009)

86. Li Y, Xu C, Yu C, Xu L, Miao M: Association of serum uric acid level with non-alcoholic fatty liver disease: a cross-sectional study. J Hepatol 50: 1029-1034 (2009)

87. Ling MT, Wang X, Zhang X, Wong YC: The multiple roles of Id-1 in cancer progression. Differentiation 74: 481-487 (2006)

88. Liu J, Sukhova GK, Yang JT, Sun J, Ma L, Ren A, Xu WH, Fu H, Dolganov GM, Hu C, Libby P, Shi GP: Cathepsin L expression and regulation in human abdominal aortic aneurysm, atherosclerosis, and vascular cells. Atherosclerosis 184: 302-311 (2006)

89. Lynch CN, Wang YC, Lund JK, Chen YW, Leal JA, Wiley SR: TWEAK induces angiogenesis and proliferation of endothelial cells. J Biol Chem 274: 8455-8459 (1999)

90. Maas AH, van der Schouw YT, Regitz-Zagrosek V, Swahn E, Appelman YE, Pasterkamp G, Ten CH, Nilsson PM, Huisman MV, Stam HC, Eizema K,

Stramba-Badiale M: Red alert for women's heart: the urgent need for more research and knowledge on cardiovascular disease in women: proceedings of the workshop held in Brussels on gender differences in cardiovascular disease, 29 September 2010. Eur Heart J 32: 1362-1368 (2011)

91. Maecker HT, Todd SC, Levy S: The tetraspanin superfamily: molecular facilitators. Faseb J 11: 428-442 (1997)

92. Majumdar G, Wright J, Markowitz P, Martinez-Hernandez A, Raghow R, Solomon SS: Insulin stimulates and diabetes inhibits O-linked N-acetylglucosamine transferase and O-glycosylation of Sp1. Diabetes 53: 3184-3192 (2004)

93. Mangin PH, Kleitz L, Boucheix C, Gachet C, Lanza F: CD9 negatively regulates integrin alphaIIbbeta3 activation and could thus prevent excessive platelet recruitment at sites of vascular injury. J Thromb Haemost 7: 900-902 (2009)

94. Matsui Y, Ikesue M, Danzaki K, Morimoto J, Sato M, Tanaka S, Kojima T, Tsutsui H, Uede T: Syndecan-4 prevents cardiac rupture and dysfunction after myocardial infarction. Circ Res 108: 1328-1339 (2011)

95. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28: 412-419 (1985)

96. McMullen ME, Bryant PW, Glembotski CC, Vincent PA, Pumiglia KM: Activation of p38 has opposing effects on the proliferation and migration of endothelial cells. J Biol Chem 280: 20995-21003 (2005)

97. Meadows TA, Bhatt DL, Cannon CP, Gersh BJ, Rother J, Goto S, Liau CS, Wilson PW, Salette G, Smith SC, Steg PG: Ethnic differences in cardiovascular risks and mortality in atherothrombotic disease: insights from the Reduction of Atherothrombosis for Continued Health (REACH) registry. Mayo Clin Proc 86: 960-967 (2011)

98. Meighan-Mantha RL, Hsu DK, Guo Y, Brown SA, Feng SL, Peifley KA, Alberts GF, Copeland NG, Gilbert DJ, Jenkins NA, Richards CM, Winkles JA: The mitogen-inducible Fn14 gene encodes a type I transmembrane protein that

modulates fibroblast adhesion and migration. J Biol Chem 274: 33166-33176 (1999)

99. Mikita J, Dubourdieu-Cassagno N, Deloire MS, Vekris A, Biran M, Raffard G, Brochet B, Canron MH, Franconi JM, Boiziau C, Petry KG: Altered M1/M2 activation patterns of monocytes in severe relapsing experimental rat model of multiple sclerosis. Amelioration of clinical status by M2 activated monocyte administration. Mult Scler 17: 2-15 (2011)

100. Miura K, Yang L, van Rooijen N, Ohnishi H, Seki E: Hepatic recruitment of macrophages promotes nonalcoholic steatohepatitis through CCR2. Am J Physiol Gastrointest Liver Physiol 302: G1310-G1321 (2012)

101. Moore KJ, Sheedy FJ, Fisher EA: Macrophages in atherosclerosis: a dynamic balance. Nat Rev Immunol 13: 709-721 (2013)

102. Morange PE, Simon C, Alessi MC, Luc G, Arveiler D, Ferrieres J, Amouyel P, Evans A, Ducimetiere P, Juhan-Vague I: Endothelial cell markers and the risk of coronary heart disease: the Prospective Epidemiological Study of Myocardial Infarction (PRIME) study. Circulation 109: 1343-1348 (2004)

103. More Evidence That Non-Alcoholic Fatty Liver Disease is an Independent Cardiovascular Risk Factor. The International Liver Congress. London (2014)

104. Mosig S, Rennert K, Krause S, Kzhyshkowska J, Neunubel K, Heller R, Funke H: Different functions of monocyte subsets in familial hypercholesterolemia: potential function of CD14+ CD16+ monocytes in detoxification of oxidized LDL. Faseb J 23: 866-874 (2009)

105. Munoz-Garcia B, Madrigal-Matute J, Moreno JA, Martin-Ventura JL, Lopez-Franco O, Sastre C, Ortega L, Burkly LC, Egido J, Blanco-Colio LM: TWEAK-Fn14 interaction enhances plasminogen activator inhibitor 1 and tissue factor expression in atherosclerotic plaques and in cultured vascular smooth muscle cells. Cardiovasc Res 89: 225-233 (2011)

106. Munoz-Garcia B, Martin-Ventura JL, Martinez E, Sanchez S, Hernandez G, Ortega L, Ortiz A, Egido J, Blanco-Colio LM: Fn14 is upregulated in cytokine-stimulated vascular smooth muscle cells and is expressed in human

carotid atherosclerotic plaques: modulation by atorvastatin. Stroke 37: 2044-2053 (2006)

107. Murdoch C, Tazzyman S, Webster S, Lewis CE: Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. J Immunol 178: 7405-7411 (2007)

108. Musso G, Gambino R, Cassader M, Pagano G: Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. Ann Med 43: 617-649 (2011)

109. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, Libby P, Weissleder R, Pittet MJ: The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med 204: 3037-3047 (2007)

110. Naito M, Hasegawa G, Takahashi K: Development, differentiation, and maturation of Kupffer cells. Microsc Res Tech 39: 350-364 (1997)

111. Nakagawa I, Matsubara T, Hori T, Imai S, Ozaki K, Mezaki T, Nasuno A, Kubota K, Nakano M, Yamazoe M, Aizawa Y: [Significance of soluble thrombomodulin in the coronary circulation of patients with coronary artery disease]. J Cardiol 38: 145-152 (2001)

112. Nakayama M, Ishidoh K, Kayagaki N, Kojima Y, Yamaguchi N, Nakano H, Kominami E, Okumura K, Yagita H: Multiple pathways of TWEAK-induced cell death. J Immunol 168: 734-743 (2002)

113. Nakayama M, Kayagaki N, Yamaguchi N, Okumura K, Yagita H: Involvement of TWEAK in interferon gamma-stimulated monocyte cytotoxicity. J Exp Med 192: 1373-1380 (2000)

114. Nakazawa Y, Sato S, Naito M, Kato Y, Mishima K, Arai H, Tsuruo T, Fujita N: Tetraspanin family member CD9 inhibits Aggrus/podoplanin-induced platelet aggregation and suppresses pulmonary metastasis. Blood 112: 1730-1739 (2008)

115. Newby AC, George SJ, Ismail Y, Johnson JL, Sala-Newby GB, Thomas AC: Vulnerable atherosclerotic plaque metalloproteinases and foam cell phenotypes. Thromb Haemost 101: 1006-1011 (2009)

116. Ott SJ, El MN, Musfeldt M, Hellmig S, Freitag S, Rehman A, Kuhbacher T, Nikolaus S, Namsolleck P, Blaut M, Hampe J, Sahly H, Reinecke A, Haake N, Gunther R, Kruger D, Lins M, Herrmann G, Folsch UR, Simon R, Schreiber S: Detection of diverse bacterial signatures in atherosclerotic lesions of patients with coronary heart disease. Circulation 113: 929-937 (2006)

117. Paynter NP, Mazer NA, Pradhan AD, Gaziano JM, Ridker PM, Cook NR: Cardiovascular risk prediction in diabetic men and women using hemoglobin A1c vs diabetes as a high-risk equivalent. Arch Intern Med 171: 1712-1718 (2011)

118. Pope JH, Aufderheide TP, Ruthazer R, Woolard RH, Feldman JA, Beshansky JR, Griffith JL, Selker HP: Missed diagnoses of acute cardiac ischemia in the emergency department. N Engl J Med 342: 1163-1170 (2000)

119. Price GM, Uauy R, Breeze E, Bulpitt CJ, Fletcher AE: Weight, shape, and mortality risk in older persons: elevated waist-hip ratio, not high body mass index, is associated with a greater risk of death. Am J Clin Nutr 84: 449-460 (2006)

120. Raines EW, Ferri N: Thematic review series: The immune system and atherogenesis. Cytokines affecting endothelial and smooth muscle cells in vascular disease. J Lipid Res 46: 1081-1092 (2005)

121. Ramirez R, Carracedo J, Castedo M, Zamzami N, Kroemer G: CD69-induced monocyte apoptosis involves multiple nonredundant signaling pathways. Cell Immunol 172: 192-199 (1996)

122. Ratziu V, Bellentani S, Cortez-Pinto H, Day C, Marchesini G: A position statement on NAFLD/NASH based on the EASL 2009 special conference. J Hepatol 53: 372-384 (2010)

123. Rea TD, Heckbert SR, Kaplan RC, Smith NL, Lemaitre RN, Psaty BM: Smoking status and risk for recurrent coronary events after myocardial infarction. Ann Intern Med 137: 494-500 (2002)

124. Rexrode KM, Carey VJ, Hennekens CH, Walters EE, Colditz GA, Stampfer MJ, Willett WC, Manson JE: Abdominal adiposity and coronary heart disease in women. JAMA 280: 1843-1848 (1998)

125. Rivera CA, Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M:

Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. J Hepatol 47: 571-579 (2007)

126. Rogacev KS, Cremers B, Zawada AM, Seiler S, Binder N, Ege P, Grosse-Dunker G, Heisel I, Hornof F, Jeken J, Rebling NM, Ulrich C, Scheller B, Bohm M, Fliser D, Heine GH: CD14++CD16+ monocytes independently predict cardiovascular events: a cohort study of 951 patients referred for elective coronary angiography. J Am Coll Cardiol 60: 1512-1520 (2012)

127. Rogacev KS, Seiler S, Zawada AM, Reichart B, Herath E, Roth D, Ulrich C, Fliser D, Heine GH: CD14++CD16+ monocytes and cardiovascular outcome in patients with chronic kidney disease. Eur Heart J 32: 84-92 (2011)

128. Rogacev KS, Ulrich C, Bloemer L, Hornof F, Oster K, Ziegelin M, Cremers B, Grenner Y, Geisel J, Schlitt A, Koehler H, Fliser D, Girndt M, Heine GH: Monocyte heterogeneity in obesity and subclinical atherosclerosis. Eur Heart J 31: 369-376 (2010)

129. Sabatine MS, Morrow DA, Jablonski KA, Rice MM, Warnica JW, Domanski MJ, Hsia J, Gersh BJ, Rifai N, Ridker PM, Pfeffer MA, Braunwald E: Prognostic significance of the Centers for Disease Control/American Heart Association high-sensitivity C-reactive protein cut points for cardiovascular and other outcomes in patients with stable coronary artery disease. Circulation 115: 1528-1536 (2007)

130. Sadler JE: Thrombomodulin structure and function. Thromb Haemost 78: 392-395 (1997)

131. Sakaguchi S, Takahashi S, Sasaki T, Kumagai T, Nagata K: Progression of alcoholic and non-alcoholic steatohepatitis: common metabolic aspects of innate immune system and oxidative stress. Drug Metab Pharmacokinet 26: 30-46 (2011)

132. Sakurai D, Tsuchiya N, Yamaguchi A, Okaji Y, Tsuno NH, Kobata T, Takahashi K, Tokunaga K: Crucial role of inhibitor of DNA binding/differentiation in the vascular endothelial growth factor-induced activation and angiogenic processes of human endothelial cells. J Immunol 173: 5801-5809 (2004)

133. Sakurai D, Yamaguchi A, Tsuchiya N, Yamamoto K, Tokunaga K: Expression of ID family genes in the synovia from patients with rheumatoid arthritis. Biochem

Biophys Res Commun 284: 436-442 (2001)

134. Salomaa V, Matei C, Aleksic N, Sansores-Garcia L, Folsom AR, Juneja H, Chambless LE, Wu KK: Soluble thrombomodulin as a predictor of incident coronary heart disease and symptomless carotid artery atherosclerosis in the Atherosclerosis Risk in Communities (ARIC) Study: a case-cohort study. Lancet 353: 1729-1734 (1999)

135. Sancho D, Gomez M, Sanchez-Madrid F: CD69 is an immunoregulatory molecule induced following activation. Trends Immunol 26: 136-140 (2005)

136. Satoh D, Yagi T, Nagasaka T, Shinoura S, Umeda Y, Yoshida R, Utsumi M, Tanaka T, Sadamori H, Fujiwara T: CD14 upregulation as a distinct feature of non-alcoholic fatty liver disease after pancreatoduodenectomy. World J Hepatol 5: 189-195 (2013)

137. Seimon TA, Wang Y, Han S, Senokuchi T, Schrijvers DM, Kuriakose G, Tall AR, Tabas IA: Macrophage deficiency of p38alpha MAPK promotes apoptosis and plaque necrosis in advanced atherosclerotic lesions in mice. J Clin Invest 119: 886-898 (2009)

138. Shantsila E, Wrigley B, Tapp L, Apostolakis S, Montoro-Garcia S, Drayson MT, Lip GY: Immunophenotypic characterization of human monocyte subsets: possible implications for cardiovascular disease pathophysiology. J Thromb Haemost 9: 1056-1066 (2011)

139. Shaw LJ, Bugiardini R, Merz CN: Women and ischemic heart disease: evolving knowledge. J Am Coll Cardiol 54: 1561-1575 (2009)

140. Shi Y, Massague J: Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113: 685-700 (2003)

141. Shoelson SE, Herrero L, Naaz A: Obesity, inflammation, and insulin resistance. Gastroenterology 132: 2169-2180 (2007)

142. Shulman GI: Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease. N Engl J Med 371: 1131-1141 (2014)

143. Sirota JC, McFann K, Targher G, Johnson RJ, Chonchol M, Jalal DI: Elevated serum uric acid levels are associated with non-alcoholic fatty liver disease

independently of metabolic syndrome features in the United States: Liver ultrasound data from the National Health and Nutrition Examination Survey. Metabolism 62: 392-399 (2013)

144. Smith K: Liver disease: Kupffer cells regulate the progression of ALD and NAFLD. Nat Rev Gastroenterol Hepatol 10: 503 (2013)

145. Soares G, Barral A, Costa JM, Barral-Netto M, Van Weyenbergh J: CD16+ monocytes in human cutaneous leishmaniasis: increased ex vivo levels and correlation with clinical data. J Leukoc Biol 79: 36-39 (2006)

146. Sookoian S, Pirola CJ: Non-alcoholic fatty liver disease is strongly associated with carotid atherosclerosis: a systematic review. J Hepatol 49: 600-607 (2008)

147. Soroceanu L, Murase R, Limbad C, Singer E, Allison J, Adrados I, Kawamura R, Pakdel A, Fukuyo Y, Nguyen D, Khan S, Arauz R, Yount GL, Moore DH, Desprez PY, McAllister SD: Id-1 is a key transcriptional regulator of glioblastoma aggressiveness and a novel therapeutic target. Cancer Res 73: 1559-1569 (2013)

148. Stefan N, Kantartzis K, Haring HU: Causes and metabolic consequences of Fatty liver. Endocr Rev 29: 939-960 (2008)

149. Strand ME, Herum KM, Rana ZA, Skrbic B, Askevold ET, Dahl CP, Vistnes M, Hasic A, Kvaloy H, Sjaastad I, Carlson CR, Tonnessen T, Gullestad L, Christensen G, Lunde IG: Innate immune signaling induces expression and shedding of the heparan sulfate proteoglycan syndecan-4 in cardiac fibroblasts and myocytes, affecting inflammation in the pressure-overloaded heart. FEBS J 280: 2228-2247 (2013)

150. Suffee N, Hlawaty H, Meddahi-Pelle A, Maillard L, Louedec L, Haddad O, Martin L, Laguillier C, Richard B, Oudar O, Letourneur D, Charnaux N, Sutton A: RANTES/CCL5-induced pro-angiogenic effects depend on CCR1, CCR5 and glycosaminoglycans. Angiogenesis 15: 727-744 (2012)

151. Sung KC, Wild SH, Kwag HJ, Byrne CD: Fatty liver, insulin resistance, and features of metabolic syndrome: relationships with coronary artery calcium in 10,153 people. Diabetes Care 35: 2359-2364 (2012)

152. Tapp LD, Shantsila E, Wrigley BJ, Pamukcu B, Lip GY: The CD14++CD16+

monocyte subset and monocyte-platelet interactions in patients with ST-elevation myocardial infarction. J Thromb Haemost 10: 1231-1241 (2012)

153. Targher G, Bertolini L, Rodella S, Zoppini G, Scala L, Zenari L, Falezza G: Associations between plasma adiponectin concentrations and liver histology in patients with nonalcoholic fatty liver disease. Clin Endocrinol (Oxf) 64: 679-683 (2006)

154. Testi R, D'Ambrosio D, De Maria R, Santoni A: The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. Immunol Today 15: 479-483 (1994)

155. Tilg H, Moschen AR: Insulin resistance, inflammation, and non-alcoholic fatty liver disease. Trends Endocrinol Metab 19: 371-379 (2008)

156. Tonan T, Fujimoto K, Qayyum A, Morita Y, Nakashima O, Ono N, Kawahara A, Kage M, Hayabuchi N, Ueno T: CD14 expression and Kupffer cell dysfunction in non-alcoholic steatohepatitis: superparamagnetic iron oxide-magnetic resonance image and pathologic correlation. J Gastroenterol Hepatol 27: 789-796 (2012)

157. Tsujioka H, Imanishi T, Ikejima H, Kuroi A, Takarada S, Tanimoto T, Kitabata H, Okochi K, Arita Y, Ishibashi K, Komukai K, Kataiwa H, Nakamura N, Hirata K, Tanaka A, Akasaka T: Impact of heterogeneity of human peripheral blood monocyte subsets on myocardial salvage in patients with primary acute myocardial infarction. J Am Coll Cardiol 54: 130-138 (2009)

158. Urra X, Villamor N, Amaro S, Gomez-Choco M, Obach V, Oleaga L, Planas AM, Chamorro A: Monocyte subtypes predict clinical course and prognosis in human stroke. J Cereb Blood Flow Metab 29: 994-1002 (2009)

159. Vaccarino V, Parsons L, Every NR, Barron HV, Krumholz HM: Sex-based differences in early mortality after myocardial infarction. National Registry of Myocardial Infarction 2 Participants. N Engl J Med 341: 217-225 (1999)

160. Vaccarino V, Parsons L, Peterson ED, Rogers WJ, Kiefe CI, Canto J: Sex differences in mortality after acute myocardial infarction: changes from 1994 to 2006. Arch Intern Med 169: 1767-1774 (2009)

161. Vasan RS, Larson MG, Leip EP, Evans JC, O'Donnell CJ, Kannel WB, Levy D:

Impact of high-normal blood pressure on the risk of cardiovascular disease. N Engl J Med 345: 1291-1297 (2001)

162. Wan J, Benkdane M, Teixeira-Clerc F, Bonnafous S, Louvet A, Lafdil F, Pecker F, Tran A, Gual P, Mallat A, Lotersztajn S, Pavoine C: M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. Hepatology 59: 130-142 (2014)

163. Wang HJ, Huang HC, Chuang YC, Liao PJ, Yang DM, Yang WK, Huang H: Modulation of tissue factor and thrombomodulin expression in human aortic endothelial cells incubated with high glucose. Acta Diabetol 49: 125-130 (2012)

164. Wang HT, Kong JP, Ding F, Wang XQ, Wang MR, Liu LX, Wu M, Liu ZH: Analysis of gene expression profile induced by EMP-1 in esophageal cancer cells using cDNA Microarray. World J Gastroenterol 9: 392-398 (2003)

165. Weber C, Belge KU, von Hundelshausen P, Draude G, Steppich B, Mack M, Frankenberger M, Weber KS, Ziegler-Heitbrock HW: Differential chemokine receptor expression and function in human monocyte subpopulations. J Leukoc Biol 67: 699-704 (2000)

166. Wei LH, Huang XR, Zhang Y, Li YQ, Chen HY, Heuchel R, Yan BP, Yu CM, Lan HY: Deficiency of Smad7 enhances cardiac remodeling induced by angiotensin II infusion in a mouse model of hypertension. PLoS One 8: e70195 (2013)

167. Wei LH, Huang XR, Zhang Y, Li YQ, Chen HY, Yan BP, Yu CM, Lan HY: Smad7 inhibits angiotensin II-induced hypertensive cardiac remodelling. Cardiovasc Res 99: 665-673 (2013)

168. Weiler H, Isermann BH: Thrombomodulin. J Thromb Haemost 1: 1515-1524 (2003)

169. Wenger NK: Women and coronary heart disease: a century after Herrick: understudied, underdiagnosed, and undertreated. Circulation 126: 604-611 (2012)

170. Wenger NK: Coronary heart disease: the female heart is vulnerable. Prog Cardiovasc Dis 46: 199-229 (2003)

171. Wildgruber M, Lee H, Chudnovskiy A, Yoon TJ, Etzrodt M, Pittet MJ,

Nahrendorf M, Croce K, Libby P, Weissleder R, Swirski FK: Monocyte subset dynamics in human atherosclerosis can be profiled with magnetic nano-sensors. PLoS One 4: e5663 (2009)

172. Wiley SR, Cassiano L, Lofton T, Davis-Smith T, Winkles JA, Lindner V, Liu H, Daniel TO, Smith CA, Fanslow WC: A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis. Immunity 15: 837-846 (2001)

173. Winkelmann BR, Marz W, Boehm BO, Zotz R, Hager J, Hellstern P, Senges J: Rationale and design of the LURIC study--a resource for functional genomics, pharmacogenomics and long-term prognosis of cardiovascular disease. Pharmacogenomics 2: S1-S73 (2001)

174. Wong KL, Tai JJ, Wong WC, Han H, Sem X, Yeap WH, Kourilsky P, Wong SC: Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood 118: e16-e31 (2011)

175. Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y, Koizumi S: Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. J Clin Invest 103: 129-135 (1999)

176. Yan X, Chen YG: Smad7: not only a regulator, but also a cross-talk mediator of TGF-beta signalling. Biochem J 434: 1-10 (2011)

177. Yang J, Zhang L, Yu C, Yang XF, Wang H: Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. Biomark Res 2: 1 (2014)

178. Yang X, Zhang F, Kudlow JE: Recruitment of O-GlcNAc transferase to promoters by corepressor mSin3A: coupling protein O-GlcNAcylation to transcriptional repression. Cell 110: 69-80 (2002)

179. Yepes M, Brown SA, Moore EG, Smith EP, Lawrence DA, Winkles JA: A soluble Fn14-Fc decoy receptor reduces infarct volume in a murine model of cerebral ischemia. Am J Pathol 166: 511-520 (2005)

180. Yki-Jarvinen H: Liver fat in the pathogenesis of insulin resistance and type 2 diabetes. Dig Dis 28: 203-209 (2010)

181. Zhang J, Cao W, Xu Q, Chen WT: The expression of EMP1 is downregulated in oral squamous cell carcinoma and possibly associated with tumour metastasis. J Clin Pathol 64: 25-29 (2011)

182. Zhao M, Liu Y, Wang X, New L, Han J, Brunk UT: Activation of the p38 MAP kinase pathway is required for foam cell formation from macrophages exposed to oxidized LDL. Apmis 110: 458-468 (2002)

183. Ziegler-Heitbrock HW, Fingerle G, Strobel M, Schraut W, Stelter F, Schutt C, Passlick B, Pforte A: The novel subset of CD14+/CD16+ blood monocytes exhibits features of tissue macrophages. Eur J Immunol 23: 2053-2058 (1993)

184. Ziegler-Heitbrock L: The CD14+ CD16+ blood monocytes: their role in infection and inflammation. J Leukoc Biol 81: 584-592 (2007)

Appendix

Supplement Table 1 Follow-up characteristics of enrolled subjects with reversed phenotype in present study

Characteristics	No FL to NAFLD (n=3)	NAFLD to No FL (n=8)
Age (years)	50.7 (31.6 -67.0)	62.4 (47.5 -65.5)
Male gender, n(%)	0 (0)	3(37.5%)
Degree of hepatic steatosis .Grade 1(n)	2	-
Grade 2(n)	1	-
Grade 3(n)	0	-
Body mass index (kg/m^2)	31.1 (28.1 -31.7)	27.3 (24.0 -30.2)
Normal (<25) n(%)	0 (0)	2 (25.0%)
Overweight (25~30) n(%)	1 (33.3%)	4 (50.0%)
Obese (>30) n(%)	2 (66.7%)	2 (25.0%)
Waist circumstance (cm)	101.0 (98.0 -104.0)	100.3 (75.9 -103.9)
Waist-to-hip ratio	0.86 (0.83 -0.93)	0.95 (0.79 -1.00)
Liver function panel		
Aspartate transaminase (U/L)	21 (20 -23)	22 (18 -27)
Alanine transaminase (U/L)	23 (19 -24)	19 (15 -23)
γ-glutamyl transferase (U/L)	20 (15 -33)	26 (19 -28)
Alkaline phosphatase (U/L)	49 (45 -66)	49 (46 -53)
Fatty liver index	2.75 (1.62 -4.98)	1.33 (0.19 -3.85)
Metabolism panel		
Cholesterol (mmol/L)	5.1 (5.1 -6.5)	5.5 (5.2 -6.2)
LDL-C (mmol/L)	3.1 (2.9 -4.1)	3.7 (3.4 -3.9)
HDL-C (mmol/L)	1.7 (1.3 -1.8)	1.4 (1.2 -2.0)
Triglycerides (mmol/L)	1.5 (1.4 -1.9)	1.2 (1.0 -1.3)
Insulin (mU/L)	5.1 (5.1 -6.5)	5.5 (5.2 -6.2)
Glucose (mg/dL)	95 (93 -97)	97 (85 -101)
HOMA-IR	2.39 (1.77 -2.84)	2.02 (1.02 -3.19)
Sex hormone binding globulin(nmol/L)	65.91 (46.78 -180.00)	53.36 (41.19 -74.02)
Inflammatory panel		
High sensitive c-reactive protein(mg/L)	0.60 (0.47 -8.56)	1.04 (0.49 -1.66)
Interleukin-6 (pg/mL)	0.68 (0.50 -1.07)	1.05 (0.62 -1.51)
Leukocyte count (10^9/L)	5.6 (4.9 -7.5)	6.2 (5.8 -8.1)
Leukocyte subtype		
Lymphocyte (%)	35.2 (31.0 -38.4)	28.2 (21.0 -36.9)
Neutrophil (%)	52.7 (52.6 -57.7)	61.7 (51.8 -69.5)
Monocyte (%)	8.1 (7.1 -8.3)	6.9 (5.8 -8.0)
Eosinophil (%)	2.7 (0.7 -4.7)	2.0 (0.9 -2.4)
Basophil (%)	0.3 (0.3 -0.5)	0.8 (0.4 -1.0)
Lymphocyte count (10^9/L)	1.9 (1.7 -2.6)	1.7 (1.3 -2.6)
Neutrophil count (10^9/L)	3.2 (2.6 -4.0)	4.1 (2.8 -4.9)

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Characteristics	No FL to NAFLD (n=3)	NAFLD to No FL (n=8)
Monocyte count (10^9/L)	0.5 (0.4 -0.5)	0.5 (0.3 -0.5)
Eosinophil count (10^9/L)	0.2 (0.0 -0.4)	0.1 (0.0 -0.2)
Basophil count (10^9/L)	0.0 (0.0 -0.0)	0.1 (0.0 -0.1)
Neutrophil/ Lymphocyte ratio	1.49 (1.37 -1.86)	2.25 (1.42 -3.33)
Monocyte subtype		
non-classical monocyte (%)	17.4 (8.0 -19.9)	13.5 (11.4 -14.2)
Intermediate monocyte (%)	9.8 (7.7 -10.1)	7.7 (6.2 -10.2)
Classical monocyte (%)	72.8 (70.3 -84.4)	78.5 (77.1 -79.8)

Note: Values were expressed as medians with interquartile ranges for countinous variables (the values of group “No FL to NAFLD” were expressed as medians with range because of 3 samples) and number of subjects (percentage) for categorical variables. FL, fatty liver disease; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment-estimated insulin resistance; LDL-C, low-density lipoprotein cholesterol; NAFLD, non-alcoholic fatty liver disease.

Supplement Table 2 Comparison of inflammatory parameters between different phenotype groups in follow-up study

Inflammatory parameter	Reversed phenotype		Stable phenotype		*P*
	No FL to NAFLD n=3	NAFLD to No FL n=8	NAFLD n=17	No FL n=26	
Leukocyte count (10^9/L)	5.6 (4.9 -7.5)	6.2 (5.8 -8.1)	6.3 (5.2 -7.9)	6.0 (5.2 -7.2)	*0.503*
Neutrophil (%)	52.7 (52.6 -57.7)	61.7 (51.8 -69.5)	52.5 ±11.5	58.9 ±7.2	*0.542*
Monocyte (%)	8.1 (7.1 -8.3)	6.9 (5.8 -8.0)	8.7 ±2.8	7.0 ±1.5	*0.968*
Monocyte subtype					
non-classical monocyte (%)	17.4 (8.0 -19.9)	13.5 (11.4 -14.2)	16.3±7.4	12.5±4.9	*0.516*
Intermediate monocyte (%)	9.8 (7.7 -10.1)	7.7 (6.2 -10.2)	10.3±4.6	8.0±3.4	*0.968*
Classical monocyte (%)	72.8 (70.3 -84.4)	78.5 (77.1 -79.8)	73.4±10.1	79.5±6.4	*0.745*
High sensitive C-reactive protein(mg/L)	0.60 (0.47 -8.56)	1.04 (0.49 -1.66)	3.11 (1.11 -5.85)	1.93 (0.72 -5.27)	*0.180*
Interleukin-6 (pg/mL)	0.68 (0.50 -1.07)	1.05 (0.62 -1.51)	1.73 (0.90 -3.09)	1.13 (0.83 -1.72)	*0.570*

Note: Values were expressed as means with standard ddeviation (normal distribution) or as medians with interquartile ranges (skewed distribution), the values of group “No FL to NAFLD” were expressed as medians with range because of 3 samples. P represented the p value of comparison between subjects with reversed phenotype from NAFLD to No FL and constant No FL. FL, fatty liver disease; NAFLD, non-alcoholic fatty liver disease.

Supplement Table 3 Comparison of parameters between baseline and the follow-up in all subjects

Pehotype status	Reversed phenotype						Stable phenotype					
	No fatty liver to NAFLD n=3			NAFLD to no fatty liver n=8			NAFLD n=17			No fatty liver n=26		
	2002	2013	*P*	2002	2013	*P*	2002	2013	*P*	2002	2013	*P*
BMI(kg/m^2)	27.4 (25.8 -30.5)	31.1 (28.1 -31.7)	-	29.2 (27.1 -33.4)	27.3 (24.0 -30.2)	*0.263*	27.5 (25.8 -30.7)	30.7 (27.1 -32.3)	*0.003*	27.0 (25.9 -31.1)	28.5 (26.6 -32.5)	*0.014*
WC(cm)	88.0 (84.0 -93.0)	101.0 (98.0 -104.0)	-	95.5 (89.3 -104.8)	100.3 (75.9 -103.9)	*0.441*	96.0 (91.5 -108.0)	104.0 (98.3 -108.5)	*0.011*	91.5 (83.5 -96.3)	94.0 (85.4 -98.3)	*0.135*
WHR	0.80 (0.80 -0.82)	0.86 (0.83 -0.93)	-	0.88 (0.80 -0.95)	0.95 (0.79 -1.00)	*0.208*	0.92±0.06	0.96 ±0.08	*0.005*	0.83±0.07	0.84 ±0.08	*0.761*
hs-CRP(mg/L)	0.93 (0.31 -8.79)	0.60 (0.47 -8.56)	-	1.56 (0.74 -4.83)	1.04 (0.49 -1.66)	*0.176*	1.72 (0.85 -3.93)	3.11 (1.11 -5.85)	*0.177*	1.48 (0.59 -3.18)	1.93 (0.72 -5.27)	*0.253*
IL-6(pg/mL)	1.23 (0.99 -3.94)	0.68 (0.50 -1.07)	-	1.55 (1.11 -2.01)	1.05 (0.62 -1.51)	*0.025*	2.04 (1.03 -3.15)	1.73 (0.90 -3.09)	*0.795*	1.43 (0.88 -2.03)	1.13 (0.83 -1.72)	*0.170*

Values were expressed as medians with interquartile ranges or mean with standard deviation, the values of group “No FL to NAFLD” were expressed as medians with range because of 3 samples. The comparisons of subjects with reversed phenotype from No fatty liver to NAFLD was not performed because of the insufficient samples. BMI, body mass index; hs-CRP, high sensitive C-reactive protein; IL-6, interleukin-6; NAFLD, non-alcoholic fatty liver disease; WC, waist circumstance; WHR, waist-to-hip ratio.

Supplement Table 4 Overview of analysis results in CAD with death (cardiac causes) versus survivor

Subjects	Coronary artery disease **Death (cardiac causes)** n	Coronary artery disease **Survivor** n	Differential genes
Integral analysis	30	41	2
Stratification analysis			
Female	8	19	11
Male	22	22	3
Type 2 Diabetes	24	34	5
No Diabetes	6	7	21
Female with type2 diabeties	6	16	17
Female without diabetes	2	3	Not available
Male with type 2 diateties	18	18	19
Male without diabetes	4	4	37

Note: Death (cardiac causes) represented patients died due to cardiac causes. The differential genes amount presented here was the original data after microarray analysis (*p<0.05*, fold change >1.5 or < -1.5). It contained several genes with high false discovery rate (>0.05), some small nucleolar RNA and replicated genes with different probes. The comparison of subgroup "Female without diabetes" was not available because of insufficient sample size (less than 3 samples of death (cardiac causes)). CAD, coronary artery disease.

Supplement Table 5 Overview of analysis results in CAD Female versus Male

Groups	Coronary artery disease **Female** n	Coronary artery disease **Male** n	Differential genes
Death (cardiac causes)	8	22	25
Survivor	19	22	25
Type 2 diabetes	22	36	23
No diabetes	3	4	45

Note: Death (cardiac causes) represented patients died due to cardiac causes. The differential genes amount presented here was the original data after microarray analysis (*p<0.05*, fold change >1.5 or < -1.5). It contained several genes with high false discovery rate (>0.05), some small nucleolar RNA and replicated genes with different probes. CAD, coronary artery disease.

Supplement Table 6 Overview of analysis results in CAD with type 2 diabetes versus without diabetes

Subjects	Coronary artery disease **with type 2 diabetes** n	Coronary artery disease **without diabetes** n	Differential genes
Integral analysis	58	13	55
Stratification analysis			
Female	36	8	63
Male	22	5	121
Death (cardiac causes)	24	6	39
Survivor	34	7	121

Note: Death (cardiac causes) represented patients died due to cardiac causes. The differential genes amount presented here was the original data after microarray analysis (*p<0.05*, fold change >1.5 or < -1.5). It contained several genes with high false discovery rate (>0.05), some small nucleolar RNA and replicated genes with different probes. CAD, coronary artery disease.

Supplement Table 7 Overview of analysis results in CAD versus healthy control

Subjects	Coronary artery disease n	Healthy control n	Differential genes
Integral analysis	71	10	456
Stratification analysis			
Female	27	5	465
Male	44	5	430
Type 2 diabetes	58	-	1124
No Diabetes	13	10	1349
Female with type 2 diabetes	22	-	1252
Female without diabetes	5	5	1510
Male with type 2 diabetes	36	-	1199
Male without diabetes	8	5	1309

Note: The differential genes amount presented here was the original data after microarray analysis (p<0.05, fold change >1.5 or < -1.5). It contained several genes with high false discovery rate (FDR, >0.05), some small nucleolar RNA and replicated genes with different probes. CAD, coronary artery disease.

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