Atherosclerosis is aggravated by iron overload and ameliorated by dietary and pharmacological iron restriction

Francesca Vinchi[1,2,3,10], Graca Porto[4,5,6#], Andreas Simmelbauer[1,2#], Sandro Altamura[1,2,10], Sara T. Passos[3], Maciej Garbowski[7*], André M. N. Silva[8*], Sebastian Spaich[9], Svenja E. Seide[10], Richard Sparla[1], Matthias W. Hentze[2,11+] and Martina U. Muckenthaler [1,2,11+]

[1] Department of Pediatric Oncology, Hematology and Immunology, University of Heidelberg, Heidelberg, Germany

[2] Molecular Medicine Partnership Unit (MMPU), Heidelberg University & European Molecular Biology Laboratory (EMBL), Heidelberg, Germany

[3]New York Blood Center (NYBC), Iron Research Program, Lindsley F. Kimball Research Institute (LFKRI), New York, USA

[4] Centro Hospitalar do Porto-Hospital Santo António, Porto, Portugal

[5] Instituto de Biologia Molecular e Celular & Instituto de Investigação e Inovação em Saúde, University of Porto, Porto, Portugal

[6] Instituto de Ciências Biomédicas Abel Salazar, University of Porto, Porto, Portugal

[7] Hematology Department, University College London Cancer Institute, London, United Kingdom

[8] REQUIMTE-LAQV, Departamento de Química e Bioquímica, Faculdade de Ciências, University of Porto, Porto, Portugal

[9] Department of Cardiology, Angiology and Pneumonology, University of Heidelberg, Heidelberg, Germany

[10] Institute of Medical Biometry and Informatics (IMBI), University Hospital Heidelberg, Heidelberg, Germany

[11]European Molecular Biology Laboratory (EMBL), Heidelberg, Germany

[#]* Equal contribution; [+] Corresponding authors

Corresponding authors:
Martina U. Muckenthaler, PhD
Department of Pediatric Oncology, Hematology and Immunology, University of Heidelberg
Otto Meyerhof Zentrum, Im Neuenheimer Feld 350, 69120 Heidelberg, Germany
email: martina.muckenthaler@med.uni-heidelberg.de

Matthias W. Hentze, MD
European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, 69117 Heidelberg, Germany. email: hentze@embl.de

Abstract

Aims: Whether and how iron affects the progression of atherosclerosis remains highly debated. Here, we investigate susceptibility to atherosclerosis in a mouse model (ApoE-/- FPN$^{wt/C326S}$) which develops atherosclerosis in the context of elevated non-transferrin bound serum iron (NTBI).

Methods and Results: Compared to normo-ferremic ApoE-/- mice, atherosclerosis is profoundly aggravated in iron-loaded ApoE-/-FPN$^{wt/C326S}$ mice, suggesting a pro-atherogenic role for iron. Iron heavily deposits in the arterial media layer, which correlates with plaque formation, vascular oxidative stress and dysfunction. Atherosclerosis is exacerbated by iron-triggered lipid profile alterations, vascular permeabilization, sustained endothelial activation, elevated pro-atherogenic inflammatory mediators and reduced nitric oxide availability. NTBI causes iron overload, induces ROS production and apoptosis in cultured vascular cells, and stimulates massive MCP-1-mediated monocyte recruitment, well-established mechanisms contributing to atherosclerosis. NTBI-mediated toxicity is prevented by transferrin- or chelator-mediated iron scavenging. Consistently, a low-iron diet and iron chelation therapy strongly improved the course of the disease in ApoE-/-FPN$^{wt/C326S}$ mice. Our results are corroborated by analyses of serum samples of hemochromatosis patients, which show an inverse correlation between the degree of iron depletion and hallmarks of endothelial dysfunction and inflammation.

Conclusions: Our data demonstrate that NTBI-triggered iron overload aggravates atherosclerosis and unravel a causal link between NTBI and the progression of atherosclerotic lesions. Our findings support clinical applications of iron restriction in iron-loaded individuals to counteract iron-aggravated vascular dysfunction and atherosclerosis.

Keywords: iron overload; atherosclerosis; non-transferrin bound iron (NTBI); vascular iron deposition; oxidative stress; iron restriction.

Introduction

Oxidative stress is thought to augment the development of atherosclerosis, since free radicals promote the onset and accelerate the progression of the disease by triggering lipid and protein oxidation, inflammation, endothelial cell (EC) activation and immune cell recruitment. In this context, iron is potentially detrimental due to its ability to catalyze the formation of reactive oxygen species (ROS)[1]. Iron is absorbed by duodenal enterocytes and recycled by reticulo-endothelial macrophages from aging red blood cells (RBC) via the only known iron exporter ferroportin (FPN)[2, 3]. If iron supplies are sufficient, FPN-mediated iron export is reduced by the hepatic iron hormone hepcidin, which binds FPN and induces its internalization and degradation. If body iron levels are low, hepcidin expression is decreased, thus promoting dietary iron influx through FPN. Once released systemically, iron is bound to transferrin, which is mainly utilized by erythroblasts for hemoglobin synthesis. In hereditary hemochromatosis (HH), the hepcidin/ferroportin regulatory axis is disrupted due to mutations in hepcidin activators (HFE, TfR2, HJV), in hepcidin itself or ferroportin. Iron overload conditions, including HH, are hallmarked by elevated transferrin saturation and NTBI formation. Within the NTBI pool, the labile plasma iron (LPI) represents the reactive fraction. LPI is loosely bound to plasma proteins and pro-oxidant. Iron accumulates in tissues with high expression of the NTBI importer ZIP14 and/or low FPN levels[3]. Iron accumulation predisposes to oxidative stress, organ dysfunction and eventually failure.

In 1981, Jerome Sullivan linked iron to CVD by proposing the 'iron hypothesis' to explain sex differences in CVD and the increased incidence among women after menopause[1, 4-6]. Several

follow up studies in humans and animals indeed found a correlation between iron content and disease severity [1, 7, 8], whereas others failed to establish a link between body iron stores, monitored via serum ferritin or transferrin saturation, and CVD [1, 9, 10]. The Brunick study was one of the few to indicate serum ferritin as one of the strongest predictors of carotid artery disease [11]. The FeAST trial instead failed to demonstrate a beneficial effect of reduced body iron stores on PAD (peripheral arterial disease)-related mortality and myocardial infarction/stroke[12, 13]. The use of markers of iron levels (e.g.serum ferritin) which poorly reflect the systemic and tissue iron burden, may have biased the conclusions of those studies. Thus, it remains to be answered whether iron exerts a pro-atherogenic role, and in particular, whether NTBI or tissue iron stores affect atherogenesis. Additional markers may be required, such as NTBI or tissue iron levels to adequately predict predisposition to CVD. In addition, the incidence of atherosclerosis in hereditary hemochromatosis (HH) patients with life-long iron overload has remained controversial [1, 14, 15].

Surprisingly, atherosclerosis is understudied in animal models of genetic iron overload (e.g. HH mice). Here we aimed to clarify whether and how iron overload affects the development of atherosclerosis by analyzing a mouse model of type IV hereditary hemochromatosis that we recently established, the $FPN^{wt/C326S}$ knock-in mouse [2]. The $FPN^{wt/C326S}$ model carries a mutation in FPN which disrupts the hepcidin/FPN interaction, causing a constitutive efflux of iron into the blood stream. As a consequence, serum and tissue iron overload ensues. Although this mutation is rare in patients and causes more severe iron overload than the more frequent hemochromatosis mutations (e.g. HFE^{C282Y}), this mouse allows to analyze the contribution of elevated systemic iron levels and NTBI to atherosclerosis in a defined genetic model. The $FPN^{wt/C326S}$ knock-in mouse was crossed with the ApoE-deficient mouse, which is a well-studied model for

hypercholesterolemia that spontaneously develops atherosclerosis[16]. We found that NTBI profoundly aggravates atherosclerosis, by triggering oxidative stress, inflammation and vascular iron deposition. Analysis of serum samples from a cohort of HH patients corroborate these findings and support their clinical relevance. Our data show that iron enhances the severity and progression of atherosclerosis, and demonstrate the preventive benefits of iron restriction.

Methods

Mouse and animal studies

Mice used in this study were ApoE-/- and ApoE-/- $FPN^{wt/C326S}$ littermates on a pure C57/BL6N genetic background. Mouse experiments were approved by and conducted in compliance with the guidelines of Regierungspräsidium Karlsruhe (Germany) (Project Nr.G-193/14) and LARS facility (Laboratory Animals Research Services) at LFKRI, New York Blood Center (USA). Data from a total of 69 patients with hereditary hemochromatosis (HH), treated in the Centro Hospitalar do Porto, were analyzed for this study. All patient data were anonymized and confidentiality was maintained during the study process according to the Helsinki Declaration and Portuguese Law.

Additional details on material and methods and statistical analyses are included in the supplemental data.

Results

Iron overload aggravates atherosclerosis

To study the role of iron in atherosclerosis, we analyzed adult homozygous ApoE-null (ApoE-/-) mice crossed with heterozygous $FPN^{wt/C326S}$ mice (ApoE-/- $FPN^{wt/C326S}$)[2]. ApoE-/- $FPN^{wt/C326S}$ mice display severely aggravated atherosclerosis with enhanced aortic lesion area and increased lesion numbers compared to aged-matched ApoE-/- mice (Fig.1A). Six and twelve months-old ApoE-/- $FPN^{wt/C326S}$ mice display a 3.5- and 2-fold increase in lesion area, respectively, and 1.6-fold higher lesion number (Fig.1A,S1). The difference in atherosclerosis severity was further confirmed by the analysis of cardiac aortic sinuses (Fig.S2). Iron deposited in aortae of ApoE-/- $FPN^{wt/C326S}$ mice in contrast to ApoE-/- mice (Fig.1B), highlighting a positive correlation between vascular iron deposition and disease severity. Consistent with vessel iron accumulation, ApoE-/- $FPN^{wt/C326S}$ mice exhibited increased ferritin as well as FPN expression compared to ApoE-/- mice (Fig.1C-D,S3). Elevated aortic expression of anti-oxidant enzymes (SOD1, HO-1) (Fig.1E-F,S4), and marked lipid peroxidation, indicated by high aortic malondyaldheide (MDA) levels (Fig.1G), suggested that vascular iron loading was associated with altered vascular oxidative status in ApoE-/- $FPN^{wt/C326S}$ mice. Taken together, these data demonstrate that iron accumulation associated with the FPN^{C326S} mutation strongly aggravates the development of atherosclerotic lesions of ApoE-/- mice.

NTBI formation and elevated pro-inflammatory mediators promote severe atherosclerosis

To understand how iron accelerates atherosclerosis progression, we analyzed serum and vascular markers and inflammatory mediators in iron-loaded mice. ApoE-/- $FPN^{wt/C326S}$ mice showed elevated systemic iron levels and highly saturated transferrin (Tf) from 3 months of age

(Fig.2A,S4). Increased Tf saturation lead to the generation of NTBI and LPI (Fig.2B). Both, vascular iron deposition as well as NTBI and LPI levels show a significant direct correlation with aortic lesion area (Fig.2C). In addition, NTBI and LPI levels correlate best with the degree of atherosclerosis progression in ApoE-/- $FPN^{wt/C326S}$ mice (TableS1). Serum analysis showed slightly increased levels of total cholesterol, LDL and triglycerides in ApoE-/- and ApoE-/- $FPN^{wt/C326S}$ mice at 6 months of age (Fig.2D, S5). Importantly, oxidized LDLs were higher in ApoE-/- $FPN^{wt/C326S}$ mice from 3 months of age (Fig.2E). Consistently, lipid peroxidation (TBARS), protein oxidation (AOPP) and carbonylation increased in the serum of 12 months-old ApoE-/- $FPN^{wt/C326S}$ mice compared to age-matched ApoE-/- animals (Fig.2F-G). These results suggest that both, alterations in LDL levels as well as in the oxidative status of serum lipids and proteins, can contribute to iron-exacerbated atherosclerosis. Pro-atherosclerotic chemokines/cytokines (VEGF1, MCP-1, IL-6 and TNFα) were elevated in the aortae and sera of ApoE-/- $FPN^{wt/C326S}$ mice (Fig.2H-I, S8). These results indicate that vascular production of these mediators may induce a local pro-atherogenic and pro-inflammatory microenvironment, as NTBI and LPI critically contribute to vessel iron loading. Thus, our findings suggest that iron overload alters serum composition, including lipid profile, molecule oxidation and inflammatory mediator release, which aggravates atherosclerosis progression.

Iron-aggravated atherosclerosis features enhanced endothelial activation and dysfunction

Endothelial permeabilization and activation are early events in the onset of atherosclerosis[17], which promote LDL infiltration and monocyte recruitment in the sub-endothelial space to stimulate fatty streak formation[18, 19]. We found significantly increased vaso-permeabilization in ApoE-/- $FPN^{wt/C326S}$ mice, indicated by enhanced Evans Blue dye extravasation in vascularized

tissues (e.g. aorta, heart, liver, kidney, duodenum, lung), compared to ApoE-/- mice (Fig.3A,S6). The expression of adhesion molecules, including ICAM-1, VCAM-1, P-selectin and E-selectin, was already upregulated in the aorta of 3 month-old ApoE-/- $FPN^{wt/C326S}$ mice (Fig.3B) and maintained in older animals (6 month-old:not shown; 12 month-old:Fig.3C). In addition, the soluble fraction of these molecules was higher in the serum of ApoE-/- $FPN^{wt/C326S}$ mice (Fig.3D). The reduced aortic expression of eNOS and increased levels of serum nitrosylated proteins in ApoE-/- $FPN^{wt/C326S}$ mice suggest reduced NO production coupled with an increased NO oxidative consumption (Fig.3E-F). These results indicate decreased NO bioavailability, which could significantly contribute to endothelial dysfunction. Collectively, these data show that elevated systemic iron levels induce vascular permeabilization and endothelial activation and dysfunction, which eventually promote the onset and progression of atherosclerosis.

Iron deposits in the VSMC media layer induce highly vulnerable plaques

DAB-enhanced Perls´ staining indicates that iron mainly deposits in the media layer of the arteries in ApoE-/- $FPN^{wt/C326S}$ mice (Fig.4A,S7). Immunohistochemistry for α-smooth muscle actin (α-SMA), a marker of VSMCs, largely mirrors the pattern of iron staining, indicating that VSMCs accumulate iron within the arterial media (Fig.4B). FPN expression was minimal in the media, which explains the iron accumulation in VSMCs, and detectable in cells within atherosclerotic plaques, which correlated with almost complete iron depletion (Fig.4A,C). The expression of HO-1 and SOD-1 was elevated in iron-loaded VSMCs and some cells within the highly oxidized environment of the plaque (Fig.4D,S7).

To evaluate plaque progression and predisposition to rupture[20], we analyzed plaque composition (Fig.4E-J). Masson´s trichrome staining revealed reduced amounts of collagen and increased

numbers of lipid droplets within plaques from ApoE-/- $FPN^{wt/C326S}$ mice (Fig.4E). Staining with the macrophage marker MAC2 indicated that foam cells were significantly more abundant in plaques of ApoE-/- $FPN^{wt/C326S}$ mice (Fig.4F). Interestingly, MAC2 staining partially reflected α-SMA staining, suggesting that intra-plaque VSMCs can acquire a macrophage-like phenotype (Fig.4G). Migration of iron-loaded VSMCs can be appreciated in Perls' stained IA sections (Fig.4A). Reduced collagen and increased foam cells and lipid content are hallmarks of unstable plaques, potentially prone to rupture, which was confirmed by the higher vulnerability index of plaques from ApoE-/- $FPN^{wt/C326S}$ mice (Fig.4I). The presence of frequent and bigger vessel calcification deposits as well as apoptotic areas (within plaque and media layer) highlighted a more advanced stage of atherosclerosis in ApoE-/- $FPN^{wt/C326S}$ mice (Fig.4H,I,J).

The impact of reduced NO, oxidative stress and advanced plaque formation on heart function/remodeling[17, 21, 22] was evaluated by echocardiography. 12 months-old ApoE-/- $FPN^{wt/C326S}$ mice exhibited increased left ventricular mass, systolic and diastolic volume and area, indicating significant left ventricle hypertrophy (Fig.4K). Unaltered ejection fraction, fractional shortening and cardiac output (Fig.S8) suggest compensatory cardiac hypertrophy. Consistently, blood pressure is almost unchanged in ApoE-/- $FPN^{wt/C326S}$ mice (Fig.S9). Overall, these data demonstrate that vessels accumulate NTBI in the VSMCs of the tunica media, which contributes to the development of an oxidized, vascular microenvironment prone to foam cell development and unstable plaque formation.

Iron-induced apoptosis of vascular cells and increased monocyte recruitment contribute to the pro-atherosclerotic action of iron

To assess the impact of iron overload on vascular cells and its relevance for human disease, we explored the consequences of iron treatment in human aortic ECs (HAEC) and VSMCs (HAVSMC). Cells were treated with the NTBI-like iron source ferric ammonium citrate (FAC, 100μM) either alone to mimic NTBI, or together with Tf or deferoxamine (DFO), to mimic Tf-bound iron or chelator-mediated iron scavenging. HAECs and HAVSMCs accumulated more intracellular iron and produced more ROS when exposed to FAC alone compared to FAC-Tf and FAC-DFO (Fig.5A,B). Remarkably, both cell lines underwent severe apoptosis following treatment with FAC, which was prevented by the addition of FAC-Tf and FAC-DFO (Fig.5C). Importantly, ROS production and apoptosis, which are well known contributors to atherosclerosis progression, directly correlate with cellular iron accumulation, suggesting that these events depend on NTBI-induced cellular iron overload. The expression of adhesion molecules was significantly induced by FAC treatment and almost unaltered by FAC-Tf and FAC-DFO treatment (Fig.5D,S10-11). FAC induced a significant cell release of MCP-1, which was prevented by Tf and DFO. By contrast, VEGF levels were rather induced by FAC-Tf and FAC-DFO, suggesting that MCP-1 is regulated by NTBI, whereas VEGF is responsive to Tf-bound iron and/or hypoxia. Indeed a 50% Tf saturation induced lower levels of VEGF compared to 100% Tf saturation. Despite different responses to different iron sources, MCP-1 and VEGF are elevated by supra-physiologic systemic iron levels (Fig.5E,S12-13). Endothelial layer integrity was significantly impaired by FAC treatment, as suggested by the increased permeability of the HAEC layer to the small FITC-dextran molecule (Fig.5F). Importantly, the adhesion of THP-1 monocyte-like cells to FAC-treated HAECs was increased, a response

prevented by Tf or DFO treatment (Fig.5G). Furthermore, conditioned medium from FAC-treated HAVSMCs promoted the migration of a higher number of monocytes (Fig.5H). To assess the in vivo relevance of these findings, we injected FAC-treated HAVSMCs into the peritoneum of wild-type mice and studied the ability of these iron-loaded apoptotic cells to induce intraperitoneal immune cell recruitment, similarly to what expected in atherosclerotic plaques. Consistent with the data in cell-based assays, FAC-treated HAVSMCs stimulated the recruitment of a significantly higher number of macrophages, neutrophils and lymphocytes compared to FAC-Tf and FAC-DFO-treated cells (Fig.5I,S14). These data suggest that NTBI exacerbates atherosclerosis by impairing EC and VSMC vitality and functionality and stimulating immune cell recruitment. This effect is likely mediated by NTBI-induced cellular iron overload and its sequelae (e.g. ROS formation, apoptosis, cell damage, immune cell recruitment).

Iron restriction and anti-inflammatory therapy attenuate iron-aggravated atherosclerosis in mice

To formally test whether iron overload is responsible for the aggravation of atherosclerosis of ApoE-/- $FPN^{wt/C326S}$ mice, we evaluated whether iron restriction has a preventive benefit. By applying two independent approaches, ApoE-/- $FPN^{wt/C326S}$ mice received either a low-iron diet or iron chelation therapy. Unlike mice on a normal diet, ApoE-/- $FPN^{wt/C326S}$ mice fed a low-iron diet or a chelator-substituted diet from 2 to 6/10 months of age showed significantly reduced aortic plaque area and numbers (Fig.6A,E). The low-iron diet and chelation therapy decreased Tf saturation and efficiently prevented aortic iron loading (Fig.6B,F,S15) in ApoE-/- $FPN^{wt/C326S}$ mice. Importantly, both iron reduction strategies minimally altered most functional parameters, including serum AOPP, nitrotyrosine, soluble adhesion molecules and inflammatory mediators,

compared to ApoE-/- mice (Fig.6C-D,G-I,S16). Only VEGF was enhanced, as a result of the hypoxic effect induced by deferasirox. Chelation treatment partially protected from atherosclerosis, perhaps due to incomplete vessel iron removal and elevated VEGF. This suggests that vascular iron accumulation is a crucial pro-atherogenic mechanism. Considering the inflammatory status of ApoE-/-FPN$^{wt/C326S}$ mice, we next tested the effect of an anti-inflammatory therapy on atherosclerosis. Dexamethasone was applied to 4 month-old ApoE-/-FPN$^{wt/C326S}$ mice for two months and shown to significantly attenuate plaque formation by 60% (Fig.7A). Whereas serum and aortic iron levels were not decreased, inflammatory mediators and soluble adhesion molecules were significantly reduced by dexamethasone treatment (Fig.7B-D). These results are in agreement with our findings in cell-based assays and support the concept that the pro-atherogenic action of iron is partially inflammation-mediated.

Iron management reduces parameters of atherosclerosis in hemochromatosis patients

To complement the analyses of genetic mouse models, we investigated a patient cohort affected by classical HFE^{C282Y} hemochromatosis. We classified patients into 4 groups: healthy control subjects (Ctrls), untreated HH patients (HH T0), HH patients undergoing intensive (1 phlebotomy/week for at least 4 weeks; HH T1), and maintenance treatment (1 phlebotomy every 3 months for at least 2,6 years; HH T2). HH patients from all groups were diagnosed at around 45 years of age with serum ferritin levels of 1600-2000 mg/l (Table S2-3). As expected, HH T0 patients presented the highest levels of serum iron and ferritin, transferrin saturation and NTBI, and inappropriately low hepcidin compared to the other groups (Fig.8A,S17, Table S2-3). Iron-loaded, untreated HH T0 patients showed the highest levels of circulating soluble adhesion molecules, lipid and protein oxidation, as well as reduced NO (measured as nitrotyrosine) and

increased circulating inflammatory chemokines/cytokines, (Fig.8B,C,F,G,H) compared to iron-depleted HH T1 and T2 patients. Serum LDL and oxLDL were equally increased in all HH groups, which suggests that even relatively small alterations in iron homeostasis may alter lipid metabolism and oxidation (Fig.8D-E). Serum iron parameters and most of the other markers analyzed progressively normalized following iron depletion by phlebotomy (T0>T1>T2). HH T2 patients, who showed the most effective iron depletion, displayed an almost complete normalization of most markers that we analyzed. These parameters directly correlate with Tf saturation and NTBI in the HH cohort (Fig.8B-G). Stepwise backward selection for multiple linear regression for iron parameters (serum iron, Tf saturation, serum ferritin, NTBI) revealed a highly significant positive association for P-Selectin, and a significant negative association for nitrotyrosine in all cases. A positive trend was observed for LDL in serum iron, and for VCAM1 when modeling Tf saturation, NTBI and serum ferritin. (Table S4-7). These results uncover a clear correlation between the degree of iron accumulation, phlebotomy and markers of endothelial dysfunction, inflammation and circulating lipids. Taken together, our data in mice and HH patients demonstrate that iron exacerbates atherosclerosis, an effect which is preventable by iron restriction strategies.

Discussion

Iron overload disorders are frequent in Western European populations. Whether these disorders promote or prevent atherosclerosis progression is controversial to date[1, 23, 24]. In this study, we utilized a mouse model of type IV HH, as a genetic model of iron overload, to study the effect of iron on atherosclerosis[2]. We show that adult iron-loaded $FPN^{wt/C326S}$ mice on an ApoE-null background develop severe atherosclerosis, associated with vascular damage and inflammation. Consistently, a cohort of HH patients exhibits a clear correlation between the severity of systemic iron overload and hallmarks of endothelial dysfunction and inflammation. These data indicate that high iron promotes the progression and aggravates the severity of atherosclerosis. We further demonstrate that the role of iron is causal, since iron overload promotes well-established pro-atherogenic mechanisms, whereas its depletion prevents disease aggravation. Our data call for the development of therapeutic strategies and effective treatment plans based on iron depletion and NTBI removal.

In our mouse model, the presence of NTBI and vessel iron overload accompanies the development of an advanced atherosclerotic phenotype[1]. Our findings are in line with observations in iron-loaded animal models [7] and a number of studies in humans, where high iron levels, monitored by serum ferritin, labile iron or Tf saturation, positively correlate with an increased risk of CVD[8, 11, 25-34].

Our results identify NTBI as predisposing factor for atherosclerosis, leading to CVD. Elevated NTBI plays a multifactorial role in atherosclerosis by: (1) altering serum composition in a pro-atherogenic and pro-inflammatory manner; (2) activating the vascular endothelium; (3) loading ECs and VSMCs, which generates a highly oxidized and pro-apoptotic vascular

microenvironment; and (4) promoting inflammation, foam cell formation and plaque instability (Figure7F).

Our murine model of iron overload features enhanced circulating LDL as well as lipid and protein oxidation, vascular leakiness, endothelial activation, reduced nitric oxide bioavailability, increased production of pro-atherogenic inflammatory mediators and intra-plaque foam cells. These events all co-occur and accompany the presence of circulating NTBI, which support a framework utilizing multiple actions of free unbound iron in atherosclerosis[35].

Atherosclerotic lesions from cholesterol-fed rabbits, ApoE-deficient mice and humans exhibit increased ferritin expression and iron deposits[36-38]. We show here that iron massively deposits in the tunica media of arteries as a function of NTBI formation. Our findings may explain iron accumulation in human arterial tissue, which is significantly higher in patients with high plasma ferritin[39]. We propose that the association between elevated systemic/body iron stores and CVD at least partially ensues from the detrimental effect of NTBI-triggered arterial iron deposition, which induces further vascular dysfunction and plaque formation[40]. This concept is supported by our molecular studies of the pro-atherogenic action of NTBI in triggering EC and VSMC iron overload, ROS production, altered cell functionality and apoptosis. By promoting oxidation and mediating VEGF release and enhanced adhesion molecule expression, NTBI increases the permeability and adhesive properties of the vascular endothelium. Increased VEGF levels have been implicated in atherosclerosis due to their pro-inflammatory and permeabilization activity[41, 42]. Finally, iron-loaded apoptotic ECs and VSMCs attract monocytes/macrophages through MCP-1 release, thus accelerating foam cell formation and plaque evolution[43-45]. Consistent with previous findings, elevated NTBI levels stimulate

MCP1 expression [46, 47]. Importantly, Tf and DFO prevent NTBI-triggered apoptosis and dysfunction of ECs and VSMCs, by scavenging iron and counteracting cellular iron overload.

A more recent, refined version of the iron hypothesis proposes that HH-associated iron depletion of macrophages may act as a protective mechanism with an anti-atherogenic effect[48, 49]. In a mouse model of decreased hepcidin levels, iron-depleted macrophages show improved cholesterol handling, which reduces foam cell formation and atherosclerotic plaque generation in mice[49]. However, a recent study failed to show aggravated atherosclerosis in flatiron mice bearing iron-overloaded macrophages[50]. Although iron depletion of macrophages is a hallmark of our mouse model[2], we also failed to observe a protective effect of iron-spared macrophages on atherosclerosis progression in adult mice. This finding seems to exclude a dominant protective effect of low macrophage iron content and suggests that chronically elevated systemic iron levels prevalent in our model, play a dominant role. We do not exclude that the detrimental effects of high NTBI may mask the beneficial action of macrophage-iron depletion during plaque development.

The analysis of serum samples from a cohort of HFE^{C282Y} patients with elevated transferrin saturation and ferritin substantiates the clinical relevance of our findings. HFE^{C282Y} is the most frequent mutation causing HH in the Caucasian population. HH patients display most parameters of vascular activation and oxidative stress according to systemic iron levels and NTBI[1, 35, 51]. The discrepancy of epidemiological studies on HH cohorts may result from variations in the reliability of iron status and treatment regimen indicators. Serum ferritin levels are in fact eventually augmented by inflammation. Both Tf saturation and serum ferritin do not reflect the levels of catalytically active free iron. NTBI together with LPI, are more reliable measures of the circulating iron fraction capable of pro-atherogenic action.

Our results demonstrate that iron restriction by limited dietary iron intake or iron chelation therapy can suppress iron-aggravated atherosclerosis in ApoE-/- FPN$^{wt/C326S}$ mice. Iron depletion strategies can attenuate atherogenesis in animal models[1, 52-54]. Accordingly, in humans, frequent phlebotomy and iron chelation correlate with improved vascular function, artery wall thickness, blood pressure and markers of CVD risk[1, 34] and reduced incidence of death[1, 12, 55]. Here we describe the benefit of phlebotomy in HH patients by showing a reversal of the induction of vascular and inflammatory parameters proportional to the degree of iron depletion. Interestingly, reduced plaque burden of mice receiving anti-inflammatory therapy suggests that inflammation contributes in part to iron-exacerbated atherosclerosis, whereas other non-inflammatory but iron-dependent pro-atherogenic mechanisms (e.g.lipid metabolism alteration, ROS formation, apoptosis) are maintained.

This manuscript addresses a longstanding, controversial problem in a rigorous way based on mouse and cell models as well as human clinical data. Overall, we demonstrate that iron, and specifically its NTBI fraction, plays a crucial role in the exacerbation of atherosclerotic disease, and show that different iron depletion and anti-inflammatory strategies can effectively reduce the development of vascular degeneration and inflammation in mice and humans. While this work does not address the role of iron within physiological limits, it documents the relevance of iron overload in atherogenesis. The identification of NTBI as a risk factor for atherosclerosis may help to clarify some controversies observed in previous studies, where the distinction between body iron stores and NTBI was not examined. Consistently, in absence of NTBI, a reduction of body iron stores below the physiologic level may not provide any benefit in terms of overall CVD survival, as observed in the FeAST trial[12, 13]. Nevertheless additional work needs to explore whether small variations in iron levels under physiologic conditions play a role in

atherosclerosis. We suggest iron restriction as a key intervention to prevent NTBI formation and its pro-atherogenic consequences. Our findings highlight the close correlation between NTBI formation and plaque evolution and the importance of maintaining a transferrin saturation within a range that prevents NTBI to minimize CVD risks. Finally, our results emphasize the need for critical consideration of the treatment regimen for iron-loaded patients, as well as the extent and duration of iron depletion with regard to the possible atherogenic risks induced by iron. Our observations may have implications for other pathological conditions associated with elevated systemic iron levels (e.g. transfusion-dependent or iv iron-treated anemic patients, patients with diabetes and metabolic syndrome who show pronounced iron overload).

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Autorship

Contribution: F.V. designed the project, performed the experiments, analyzed data, wrote the manuscript; G.P. provided samples and contributed to the design of human study; A.S. and R.S. helped with in vivo experiments; S.A. generated mice; S.T.P. and K.Y. helped with in vitro experiments; M.G. and A.S. performed NTBI/LPI measurement on murine and human samples, respectively; S.S. performed mouse echocardiography; S.A. and S.E.S performed statistical analysis; M.W.H. and M.U.M designed and supervised the project and wrote the manuscript. Authors declare no competing financial interests.

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Figure 1. Iron-overloaded ApoE-/- FPN$^{wt/C326S}$ mice accumulate iron in vessels and develop severe atherosclerosis. Data are shown on 6/12 month-old ApoE-/- and ApoE-/-FPN$^{wt/C326S}$ mice. (**A**) En face staining with Sudan IV on whole aortae, quantification of aortic lesion area and number, and (**B**) aortic iron content (n=8-12). (**C**,**E**) qRT-PCR analysis of aortic L-Ferritin and HO-1 mRNA levels (n=6-12). mRNA levels expressed in relative quantity of averaged ApoE-/- samples (RQ=1). (**D**,**F**) Representative western blot analysis of L-/H-Ferritin, HO-1 and SOD-1 protein levels in aortae (12 month-old). Graphs show densitometric analyses expressed in arbitrary unit to the average of ApoE-/- samples (AU=1, n=8-10). (**G**) Aortic lipid peroxidation (n=4-7).

Figure 2. Iron-overloaded mice show NTBI and LPI formation and pro-atherogenic changes in serum composition. Data are shown on 3/6/12 month-old ApoE-/- and ApoE-/-FPN$^{wt/C326S}$ mice. (**A**,**B**) Measurement of serum iron levels, % Tf saturation, NTBI and LPI (n=4-7). (**C**) Correlation of % aortic lesion area with aortic iron levels/NTBI and aortic iron levels with NTBI. (**D-H**) Measurement of serum LDLs, oxidized LDLs (n=4-21), lipid peroxide levels (TBARS, n=4), advanced oxidized protein products (AOPP, n=20-23), protein carbonylation (n=11-12), serum VEGF and MCP- (n=7-10). (**I**) qRT-PCR analysis of aortic VEGF, MCP-1, IL-6 and TNFα mRNA levels (n=4-13) (**C-I**:12 month-old).

Figure 3. Iron-overloaded mice present marked endothelial activation and dysfunction. Data are shown on 3/12 month-old ApoE-/- and ApoE-/-FPN$^{wt/C326S}$ mice. (**A**) Vascular permeability: quantification of Evans Blue dye accumulation in tissues (n=10). Representative macroscopic pictures of whole aortae showing Evans Blue staining. (**B**) qRT-PCR analysis of

aortic E-selectin, VCAM-1 and ICAM-1 mRNA levels (3 month-old, n=9-10). (**C,F**) Representative western blot and densitometric analysis of aortic P-selectin, VCAM-1 and ICAM-1 protein levels and serum protein nitrosylation (n=4-5). (**D**) ELISA measurement of serum soluble P-selectin, E-selectin, VCAM-1 and ICAM-1 (n=15-16). (**E**) qRT-PCR analysis of aortic endothelial nitric oxide synthase (eNOS) mRNA levels (n=11-13) (**A,B-E**:12 month-old).

Figure 4. ApoE-/- FPN$^{wt/C326S}$ mice show iron deposition in the VSMC media layer and vulnerable plaque formation. Data are shown on 12 month-old ApoE-/- and ApoE-/- FPN$^{wt/C326S}$ mice. Representative DAB-enhanced Perls' staining for iron (**A**), and immunohistochemistry for α-smooth muscle actin (α-SMA), FPN and HO-1 (**B-D**) on consecutive sections of innominate artery (IA). Arrows indicate iron deposition and FPN/HO-1 expressing cells. Masson trichrome (**E**) and Von Cossa staining for calcification (**H**), immunohistochemistry for MAC2 and α-SMA (**F,G**) on IA sections. (**I**) Quantification of collagen (blue), VSMC fiber (violet), lipid droplets (white) (Masson stain), foam cells (MAC2^{+}) and calcification (Von Cossa). Plaque vulnerability index: (collagen+VSMC % plaque area) / (lipids +foam cells % plaque area). (**J)** Tunel staining on IA sections. Arrows indicate the blu/purple Tunel^{+} areas within the plaque and media layer. (**K**) Ecocardiographic analysis. LV Mass: left ventricular (LV) mass; LVEDV/LVESV:LV end-diastolic/end-systolic volume; LVEDA/LVESA: left ventricular end-diastolic area/end-systolic area (n=11).

Figure 5. NTBI triggers functional impairment and apoptosis of human ECs and VSMCs and stimulates monocyte recruitment. Human HAECs and HAVSMCs remained untreated or were exposed to 100μM FAC, FAC-Tf or FAC-DFO for the time indicated. **(A)** Measurement of

cellular iron content (14h), **(B)** ROS production (6h, total ROS production and %ROS^{+}cells), **(C)** cell viability (7AAD viability/Annexin V staining, 14h) and **(D)** adhesion molecule expression (E-/P-selectin, ICAM-1,24h). **(E)** ELISA quantification of MCP-1 and VEGF in culture medium of HAECs/HAVSMCs treated for 14h. **(F)** Endothelial permeability assay of HAECs treated for 6h: quantification of FITC-dextran leaking during 30' through a confluent HAEC layer into the medium of a 1 μm pore transwell bottom chamber. **(G)** Adhesion assay: number of THP-1 monocytes which adhered in 15' to treated-HAECs (6h). **(H)** Migration assay: number of THP-1 monocytes which migrated in 2h towards the conditioned medium of treated-HAVSMCs (6h). **(I)** In vivo immune cell recruitment: number of macrophages, neutrophils and lymphocytes recruited in 6h into the peritoneum of mice injected with 150.000 treated-HAVSMCs (14h,n=5-6).

Figure 6. Iron restriction strategies prevent iron-aggravated atherosclerosis in ApoE-/-FPN$^{wt/C326S}$ mice. Analysis of 6/10 month-old ApoE-/- and ApoE-/-FPN$^{wt/C326S}$ mice administered a standard diet (200ppm iron) and ApoE-/- FPN$^{wt/C326S}$ mice administered a low-iron diet or a chelator-enriched standard diet. (**A,E**) En face staining with Sudan IV on whole aortae and quantification of aortic lesion area and number (n=3-4). (**B,F**) Measurement of transferrin saturation and aortic iron content. ELISA measurement of serum sVCAM-1, ICAM-1(**C,H**), VEGF and MCP-1(**D,I**), AOPP and nitrotyrosine(**G**) (n=4-6).

Figure 7. Anti-inflammatory therapy partially prevents iron-aggravated atherosclerosis in ApoE-/- FPN$^{wt/C326S}$ mice. Analysis of 6 month-old ApoE-/- and ApoE-/-FPN$^{wt/C326S}$ mice administered a dexamethasone therapy (0.15 mg/kg/day) for 2 months (n=6). (**A**) En face

staining with Sudan IV on whole aortae and quantification of aortic lesion area and number. (**B,C**) Measurement of iron content in serum and aortae. ELISA measurement of serum sVCAM-1, ICAM-1 (**D**), VEGF and MCP-1 (**E**). The partially protective effect of dexamethasone might be explained by its known interference with lipid metabolism. (**F**) Model depicting the multifactorial action of iron in atherosclerosis.

Figure 8. Markers of vascular dysfunction and atherosclerosis in hemochromatosis HFE^{C282Y} patients vary according to iron overload and iron depletion regimen. Analysis of serum samples from control healthy subjects (Ctrls,n=28), untreated HH patients (T0,n=14), HH patients under intensive (T1,n=13) and maintenance phlebotomy (T2,n=42). (**A**) Measurement of serum iron levels, Tf saturation, Ferritin and NTBI levels and correlation Tf saturation versus NTBI. ELISA measurement of serum sP-/E-selectin and sV/ICAM1 (**B**), LDLs (**D**) oxLDLs (**E**), AOPP (**F**) and protein nitrotyrosilation (**G**). (**C**) Serum lipid peroxide measurement. (**H**) Multiplex bead-array measurement of serum VEGF, MCP-1, TNFα and IL-6. Correlations of each marker (including all groups) versus Tf saturation and NTBI are shown.