Gut Microbes

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/kgmi20

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To cite this article: Klytaimnistra Kiouptsi , Giulia Pontarollo , Hristo Todorov , Johannes Braun , Sven Jäckel , Thomas Koeck , Franziska Bayer , Cornelia Karwot , Angelica Karpi , Susanne Gerber , Yvonne Jansen , Philipp Wild , Wolfram Ruf , Andreas Daiber , Emiel Van Der Vorst , Christian Weber , Yvonne Döring & Christoph Reinhardt (2020): Germ-free housing conditions do not affect aortic root and aortic arch lesion size of late atherosclerotic low-density lipoprotein receptor-deficient mice, Gut Microbes, DOI: 10.1080/19490976.2020.1767463

To link to this article: https://doi.org/10.1080/19490976.2020.1767463

GUT MICROBES
https://doi.org/10.1080/19490976.2020.1767463

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ADDENDUM

Germ-free housing conditions do not affect aortic root and aortic arch lesion size of late atherosclerotic low-density lipoprotein receptor-deficient mice

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ABSTRACT

The microbiota has been linked to the development of atherosclerosis, but the functional impact of these resident bacteria on the lesion size and cellular composition of atherosclerotic plaques in the aorta has never been experimentally addressed with the germ-free low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mouse atherosclerosis model. Here, we report that 16 weeks of high-fat diet (HFD) feeding of hypercholesterolemic *Ldlr*$^{-/-}$ mice at germ-free (GF) housing conditions did not impact relative aortic root plaque size, macrophage content, and necrotic core area. Likewise, we did not find changes in the relative aortic arch lesion size. However, late atherosclerotic GF *Ldlr*$^{-/-}$ mice had altered inflammatory plasma protein markers and reduced smooth muscle cell content in their atherosclerotic root plaques relative to CONV-R *Ldlr*$^{-/-}$ mice. Neither absolute nor relative aortic root or aortic arch plaque size correlated with age. Our analyses on GF *Ldlr*$^{-/-}$ mice did not reveal a significant contribution of the microbiota in late aortic atherosclerosis.

ARTICLE HISTORY
Received 4 December 2019
Revised 4 April 2020
Accepted 4 May 2020

KEYWORDS
Microbiota; germ-free; low-density lipoprotein receptor-deficient mouse; atherosclerosis; aortic root; aortic arch; macrophages; smooth muscle cells; inflammatory markers; age; lesion size

Introduction

During the last decade, an increasing number of studies has provided a wealth of association-based[1-3] and causal evidence,[1,4-7] linking the microbiota and specific bacterial community members[8-10] to the development of atherosclerotic lesions and cardiovascular disease (CVD) (for overview see Table 1).[11] The gut microbiota has been recognized as an environmental factor that influences endothelial cell functions and contributes to vascular inflammatory phenotypes,[12–14] fostering arterial thrombosis through various prothrombotic mechanisms.[15–18] While experiments with germ-free (GF) apolipoprotein E (Apoe)-deficient mouse models at chow diet conditions have repeatedly shown a protective role of the microbiota in atherogenesis,[4,6] it remains controversial how HFD and different feeding regimens affect atherosclerotic lesion development in mouse atherosclerosis models under GF housing conditions.

In a study that addressed late carotid artery atherosclerosis in the germ-free *Ldlr*$^{-/-}$ atherosclerotic mouse model,[18] we have recently reported that 16 weeks of feeding with an adjusted calories diet (42% kcal from fat, 17.3% protein, 48.5% carbohydrates, 21.2% [wt/wt] fat, 0.2% cholesterol, 34% [wt/wt] sucrose) that had been vacuum packaged and irradiated, abolishes differences between GF and conventionally raised (CONV-R) mice in the lipoprotein profile and total plasma cholesterol levels, which are apparent characteristics observed under

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Table 1. Studies with antibiotics and germ-free mouse models addressing the functional implication of the microbiota in atherogenesis.

Antibiotic (Abx) microbiota depletion

Study design	Mouse model	Start	End	Diets (producer)	Abx Treatment	Results	Ref
Chow diet, choline/ TMAO-rich diet, HFD: SPF *vs* Abx	*Apoe*$^{-/-}$ males+females C57BL/6 J	4–8 weeks	20–24 weeks	• **Chow diet**: normal chow, 0.08–0.09% choline *(2018)* • **Choline-rich diet**: chow+0.5% or 1.0% *(TD.07863, TD.078634)* • **TMAO-rich**: chow +0.12%*(TD.07865)* • **HFD**: 42% fat, 0.15% cholesterol *(88137). All diets were from Teklad.*	**Abx** in the drinking water for 3 weeks. (1.0 g/L ampicillin, 1 g/L neomycin sulfate, 0.5 g/L vancomycin, 1 g/L metronidazole)	• **Choline/TMAO-rich diet effects**: increased aortic root atherosclerotic plaque area. Similar cholesterol, triglycerides and glucose levels. Enhanced athero-sclerosis in female mice (even on normal chow diet). • **Abx effects**: reduced atherosclerosis; lower macrophage cholesterol accumulation; gut microbiota and dietary choline increase TMAO production.	**Wang Z, Nature., 2011**[1]
HFD: SPF *vs* Abx	*Ldlr*$^{-/-}$ males+females - *background*	10 weeks	26 weeks	• **HFD**: 0.15% cholesterol, 21% fat, 19.5% casein *(WD, TD88137, Harlan Teklad)*	**Abx** in the drinking water. (100 mg/L neomycin, 10 mg/L polymyxin B).	• **Abx effects**: decreased atherosclerotic lesion size in aortic arch and entire aorta; smaller necrotic core; no effects on plasma cholesterol and triglyceride levels; attenuation of glucose intolerance and LPS uptake.	**Ghosh SS, PLoS One., 2014**[26]
HFD without gluten ± gladin: SPF vs Abx	*Apoe*^{tm1Unc}**/N11** females B6.129P2	at birth	16 weeks	• **HFD without gluten** *(D12079B, Research Diet Inc.)* • **HFD without gluten + 1% gliadin** *(D11061501, Research Diet Inc.)*	**Abx** in the drinking water. (1 g/L ampicillin)	• **Abx effects**: decreased aortic atherosclerotic lesion size; improvement of glucose tolerance and reduced insulin levels; reduced LDL, VLDL cholesterol levels. • **Gluten-free diet effects**: no significant effects.	**Rune I, PloS One., 2016**[21]
Chow diet: SPF *vs* Abx *vs* CONV-D	*Apoe*$^{-/-}$, *Ldlr*$^{-/-}$ females C57BL/6 J	4 weeks	8/16 weeks	• **Chow diet**: regular *(RM1, Dietex)*	**Abx** 4 weeks daily gavage. (200 mg/kg neomycin, 200 mg/kg metronidazole, 200 mg/kg ampicillin, 100 mg/kg vancomycin).	• **Abx effects**: increased plasma cholesterol (+55%), VLDL (+53%) and LDL (+36%) levels in microbiota-depleted mice (due to increased cholesterol absorption, hepatic synthesis and clearance).	**Le Roy T, BMC Biol., 2019**[27]
Chow diet, HFD: SPF *vs* Abx	*Apoe*^{tm1Unc}**/J** males B6.129P2	6 weeks	22 weeks	• **Chow diet**: 10% calories from fat *(GLP Mucedola Srl)* • **HFD**: 45% calories from fat *(D12079B, Research Diets)*	**Abx** in the drinking water after 6 weeks of feeding. (1 g/L ampicillin, 1 g/L metronidazol, 1 g/L neomycin, 0.5 g/L vancomycin).	• **Abx effects**: increased aortic root atherosclerotic lesion size (via tryptophan and lipid metabolism and reduction of Bacteroidetes and Clostridia). • **HFD effects**: no significant effects.	**Kappel BA, Mol Metabol. 2020**[7]

(*Continued*)

Table 1. (Continued).

Antibiotic (Abx) microbiota depletion

Study design	Mouse model	Start	End	Diets (producer)	Abx Treatment	Results	Ref
Chow diet, HFD: *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) *vs* *Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$)	female *Casp1*$^{-/-}$ B6 N.129S2 female *Ldlr*$^{-/-}$ B6.129S7	12 weeks	20/25 weeks	• **Chow diet**: *(RMH-BAB diets)* • **HFD**: *(HFC, Research Diets)*	**Abx** 10 d oral gavage. (0.26 mg/g ampicillin, 0.1 mg/g metronidazol, 0.26 mg/g neomycin, 0.13 mg/g vancomycin).	• **Effect of *Casp1*$^{-/-}$ fecal translplantation into *Ldlr*$^{-/-}$ mice**: similar aortic root atherosclerotic lesion size after 8 weeks, but 29% increase after 13 weeks HFD. • **Abx effects**: increase of proinflammatory phenotype.	**Brandsma E, Circ Res., 2019**[28]

Germ-free mice (GF)

Study design	Mouse model	Start	End	Diets *(producer)*	Results	Ref
HFD: SPF *vs* GF	*Apoe*$^{-/-}$/*Ips*^{d} males+females C57BL	4 weeks	22/32 weeks	• **HFD**: 0.15% cholesterol, 21.22% fat, 17.01% protein, 48.48% carbohydrate *(TD88137, Harlan Teklad)*	• **GF housing effects**: no differences in atherosclerotic lesion sizes in the aortic root between GF and CONV-R mice; similar plasma lipid profiles and cholesterol. No effect of gnotobiosis (and infectious agents) on the progress of atherosclerosis: HFD is sufficient for atherogenesis. No sex effects.	**Wright SD, J Exp Med, 2000**[22]
Chow diet, HFD: SPF *vs* GF *vs* CONV-D.	*Apoe*^{tm1Unc}/J males+females C57BL/6	8 weeks	24 weeks	• **Chow diet**: ST-1 *(Bergman, Kocanda)* • **HFD**: 2% cholesterol, 5% tallow fat, 3% fat fish meal	• **GF housing effects**: bigger atherosclerotic lesion sizes in the aorta (lipids deposition with foam cells and macrophages) and higher cholesterol levels. • **Diet effects**: under chow diet, only GF mice developed atherosclerotic lesions in the aorta. Fed HFD, atherosclerosis was similar in GF and CONV-R.	**Stepankova R, J Ather Thromb 2010**[4]
Chow diet: SPF *vs* GF	*Apoe*$^{-/-}$ all females C57BL/6	*not specified*	20 weeks	• **Chow diet**: 20% calories from fat, 50% from carbo-hydrate, 30% from protein *(CMF, Oriental Yeast Co)*.	• **GF housing effects**: reduction in atherosclerotic lesion formation (due to reduced LPS-mediated inflammatory response) and lipid area in the aortic root, with a reduction of intraplaque macrophages. Higher plasma and hepatic cholesterol levels; higher VLDL and LDL-cholesterol; lower triglycerides.	**Kasahara K, J Lipid Res., 2017**[5]
Chow diet, HFD: SPF *vs* GF	*Apoe*$^{-/-}$ males+females C57BL/6 J	8 weeks	20 weeks	• **Chow diet**: chow *(Envigo TD.130104)* or chow +1,2% choline *(Envigo TD.09041)*. • **HFD**: HFD *(D11042101; Research Diets)* + or HFD + 1% choline *(D11042102; Research Diets)*	• **GF housing effects**: bigger aortic atherosclerotic lesion sizes in the aorta and higher cholesterol levels. • **Diet effects**: differences are abolished in HFD conditions. No effect of choline supplementation on aortic lesion sites.	**Lindskog Jonsson A, ATVB, 2018**[6]

chow diet conditions.[19,20] Although GF *Ldlr*$^{-/-}$ mice had reduced counts of adherent leukocytes to the uninjured common carotid artery lesion, absolute and relative carotid artery plaque size was unchanged by GF housing conditions. This was in contrast to plaque rupture-induced atherothrombosis and adhesion-induced platelet activation on type III collagen coatings, which were both diminished in germ-free *Ldlr*$^{-/-}$ mice relative to their CONV-R *Ldlr*$^{-/-}$ counterparts. Thus, our study on GF *Ldlr*$^{-/-}$ mice revealed a prothrombotic role of the gut microbiota in atherothrombosis, but unchanged carotid artery plaque size during late atherosclerosis.[18]

Since several reports demonstrated that the results on GF mouse atherosclerosis models vary dependent on the genetic model, the diet, and the feeding regimen (Table 1),[4–6,18,22] we have comparatively analyzed the aortic lesions of GF *Ldlr*$^{-/-}$ and CONV-R *Ldlr*$^{-/-}$ mice to pinpoint whether the absence of the gut microbiota affects lesion size and cellular plaque composition.

Results

To study whether the lack of a gut microbiota impacts atherosclerotic lesion size and cellular composition, we rederived *Ldlr*$^{-/-}$ mice as germ-free (GF), kept those mice for 16 weeks on an irradiated high-fat diet (HFD) and compared their lesions in the aortic root and arch with those of conventionally raised (CONV-R) *Ldlr*$^{-/-}$ counterparts (Figure 1(a)). Histological analyses of fixed-frozen sections revealed no differences in the atherosclerotic plaque areas in the oil-red-O stained aortic roots of male and female, GF *Ldlr*$^{-/-}$ mice on HFD relative to CONV-R *Ldlr*$^{-/-}$ controls (Figure 1(b)). As expected, females in both HFD-fed CONV-R *Ldlr*$^{-/-}$ and HFD-fed GF *Ldlr*$^{-/-}$ groups had increased relative aortic root plaque areas compared to males, irrespectively of the presence of microbiota (Figure 1(c)).[23,24] Thus, our results confirm that sex is a determinant of the atherosclerotic lesion size in 'zero-level' aortic roots.[24] Interestingly, under these circumstances, germ-free housing conditions had no influence on aortic root lesion size.

Next, we comparatively analyzed the cellular composition of aortic root lesions in GF and CONV-R *Ldlr*$^{-/-}$ mice on HFD. Immunostaining of aortic root plaques for the macrophage marker MAC-2 excluded differences in the macrophage content or the necrotic core area in these late atherosclerotic lesions, which was comparable between the two groups of mice (Figure 1(d)). Interestingly, smooth muscle actin staining revealed reduced quantities of smooth muscle cells in the aortic root plaques of GF *Ldlr*$^{-/-}$ mice relative to CONV-R *Ldlr*$^{-/-}$ mice (Figure 1(e)), in agreement with the fibroproliferative response in aortic root lesions that we observed in HFD-fed GF *Ldlr*$^{-/-}$ mice compared with CONV-R *Ldlr*$^{-/-}$ mice. In line with unchanged macrophage content, reactive nitrogen species (RNS) levels in aortic root plaques were comparable between the HFD-fed GF *Ldlr*$^{-/-}$ mice and CONV-R *Ldlr*$^{-/-}$ mice (Figure 1(f)), as indicated by unchanged areas of 3-nitrotyrosine (3-NT) immunostaining (Figure 1(g)). In conclusion, germ-free housing conditions influenced smooth muscle cell content in aortic root lesions, but did not result in altered macrophage content or changed RNS levels.

In addition, we analyzed the aortic arch plaque areas in hematoxylin-and-eosin (HE)-stained cryosections, but did not find differences between HFD-fed GF *Ldlr*$^{-/-}$ mice relative to HFD-fed CONV-R *Ldlr*$^{-/-}$ mice (Figure 2(a)). Furthermore, analyzing the aortic arch plaque areas, we did not find differences in relative plaque size between female and male *Ldlr*$^{-/-}$ mice (Figure 2(b)). In contrast to aortic root plaque size,[24] sex did not influence lesion size in the aortic arch of late atherosclerotic plaques in *Ldlr*$^{-/-}$ mice.

In order to examine the influence of age on aortic plaque size, we calculated the Pearson correlation between age and plaque size. The age of the *Ldlr*$^{-/-}$ mice at the time of sacrifice following the 16 weeks HFD-feeding regimen was not significantly correlated with absolute or relative plaque size in the aortic root overall and when animals were stratified by sex (Figure 3(a)). Aortic arch plaque area was also not dependent on the age of the mice (Figure 3(b)). It is well established that age has a strong influence on aortic root atherosclerosis. To further investigate if age impacted aortic root and aortic arch lesion size, we performed a 2-way analysis of covariance (ANCOVA) with GF or CONV-R housing conditions and sex as factors and age as covariate. The effect of age was not statistically significant ($F_{1,33} = 1.31$, $p = .259$ for aortic root lesion size and $F_{1,35} = 0.716$, $p = .403$ for aortic arch lesion size). Furthermore, the difference

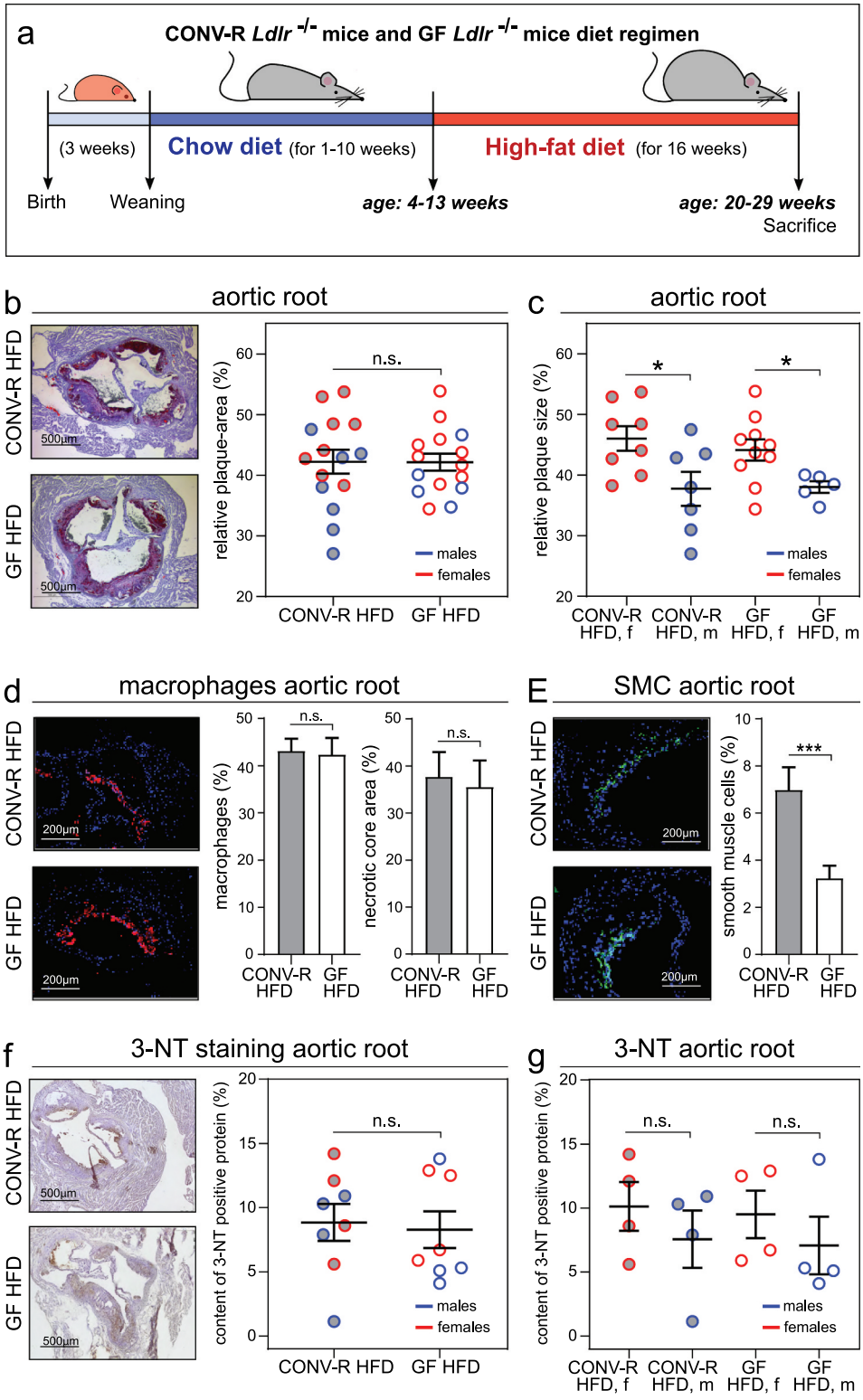

Figure 1. (a) Applied diet regimen to study late atherosclerosis in GF and CONV-R *Ldlr*$^{-/-}$ mice. (b, c) Atherosclerotic plaque area in cross-sections at the zero-level of the aortic root of CONV-R (15 mice/group, 8 females, and 7 males) and GF (15 mice/group, 10 females, and 5 males) *Ldlr*$^{-/-}$ mice on HFD, (b) overall values or (c) sex-split. Mean ± SEM. Representative histology images showing Oil-Red O-stained sections. Scale bar 500 µm. (d) Quantification (% of total plaque nuclei) of MAC-2 positive cells in cross-sections of the aortic root (3–7 mice/group). Scale bar 200 µm. Mean ± SEM. Based on the % of plaque area, the necrotic core area was calculated for seven CONV-R and six GF mice, all males. (e) Quantification (% of total nuclei) of smooth muscle cells (SMC) by SMC-actin immunostaining in cross-sections of the aortic root (3–7 mice/group). Scale bar 200 µm. Mean ± SEM. (f, g) Immunostained atherosclerotic plaque area stained for 3-nitrotyrosine (3-NT) in cross-sections at the zero-level of the aortic root of CONV-R (8 mice/group, four females and four males) and GF (8 mice/group, four females, and four males) *Ldlr*$^{-/-}$ mice on HFD, (f) overall values, or (g) sex-split. Mean ± SEM. Representative histology images showing 3-NT-stained sections. Scale bar 500 µm. Independent samples Student's t-tests, * $p < .05$, ** $p < .01$, *** $p < .001$. For all panels, CONV-R mice are shown in gray and GF mice in white. For panels (b, c, and f, g), the sex of the mice is color-coded: females: red; males: blue.

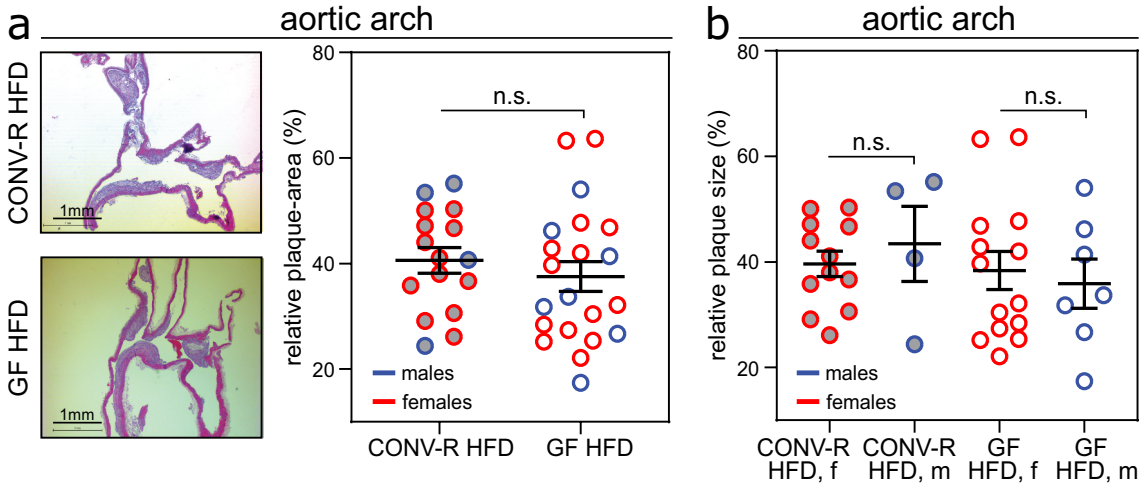

Figure 2. Atherosclerotic plaque area in longitudinal-sections at the aortic arch of CONV-R (16 mice/group, 12 females, and 4 males) and GF (21 mice/group, 14 females, and 7 males) *Ldlr*$^{-/-}$ mice on HFD, (a) overall values, or (b) sex-split. Mean ± SEM. Representative histology of hematoxylin and eosin-stained sections. Scale bar 1 mm. CONV-R mice are shown as gray dots, and GF animals as white dots. The sex of the mice is color-coded: females: red; males: blue.

between GF *Ldlr*$^{-/-}$ and CONV R *Ldlr*$^{-/-}$ in aortic root plaque size ($F_{1,33} = 1.141$, $p = .293$) or in aortic arch plaque size ($F_{1,35} = 0.21$, $p = .649$) was not significant even after adjusting for age differences.

Since our previous study identified altered plasma cytokine levels between HFD-fed GF *Ldlr*$^{-/-}$ and CONV R *Ldlr*$^{-/-}$ mice,[18] we went on to identify additional biomarkers influenced by colonization with microbiota under normal and HFD conditions. We analyzed 92 proteins of the Mouse Exploratory Panel (Olink Proteomics AB, Uppsala, Sweden) in citrated plasma samples by an immuno-PCR-based proximity extension assay with high sensitivity to detect low-level proteins. Principal component analysis showed robust clustering of the individual samples according to experimental conditions (Figure 4(a)). The greatest difference was observed for the profiles of HFD-fed *Ldlr*$^{-/-}$ mice compared to WT mice on a chow diet. However, the microbiome status separately affected the analytes measured by proximity ligations assay. We fit the data of HFD-fed *Ldlr*$^{-/-}$ mice with housing conditions as a factor to model the difference in plasma proteins induced by GF conditions. This allowed us to identify markers that were altered in HFD-fed *Ldlr*$^{-/-}$ mice in dependence of the microbiota (Figure 4 (b)). Analogously, we identified a set of markers that were altered in GF WT mice in comparison to CONV-R WT mice (Figure 4(c)). The altered biomarkers had no apparent enrichment in specific GO terms.

A set of markers were concordantly altered under HFD and normal diet. Interleukin 23 receptor (IL23r) was decreased whereas epithelial cell markers, such as epithelial cell adhesion molecule (EPCAM), and the incretin glucagon-like peptide-1 (Glp-1, Gcg) with vascular protective functions were increased in the absence of microbiota irrespectively of the diet and the genotype. In contrast, microbiota had also differential effects when comparing WT mice on a normal diet with *Ldlr*$^{-/-}$ mice on an HFD. Specifically, under HFD inflammation markers, e.g. tumor necrosis factor (Tnf), interleukin 1 alpha (IL1a), glial cell derived neurotrophic factor family receptor alpha 1 (Gfra1), and C-X-C motif chemokine ligand 9 (Cxcl9), as well as follistatin (Fst) levels, were reduced in GF *Ldlr*$^{-/-}$ mice relative to their CONV-R counterparts. In addition, proteins known to be expressed in the gastrointestinal track, i.e. integrin subunit beta 6 (Itgb6) and v-set and immunoglobulin domain containing 2 (Vsig2) were increased, possibly related to changes in gut permeability. GF HFD *Ldlr*$^{-/-}$ mice also had higher markers associated with metabolic processes, e.g. carbonic anhydrase 13 (Ca13), quinoid dihydropteridine reductase (Qdpr), mitogen-activated protien kinase 6 (Map2k6), Axin1, which in part may be related to differences in the formulation of the diet independent of lipid content. Interestingly, the downregulation of interleukin 17a/f (IL17a/f) in response to

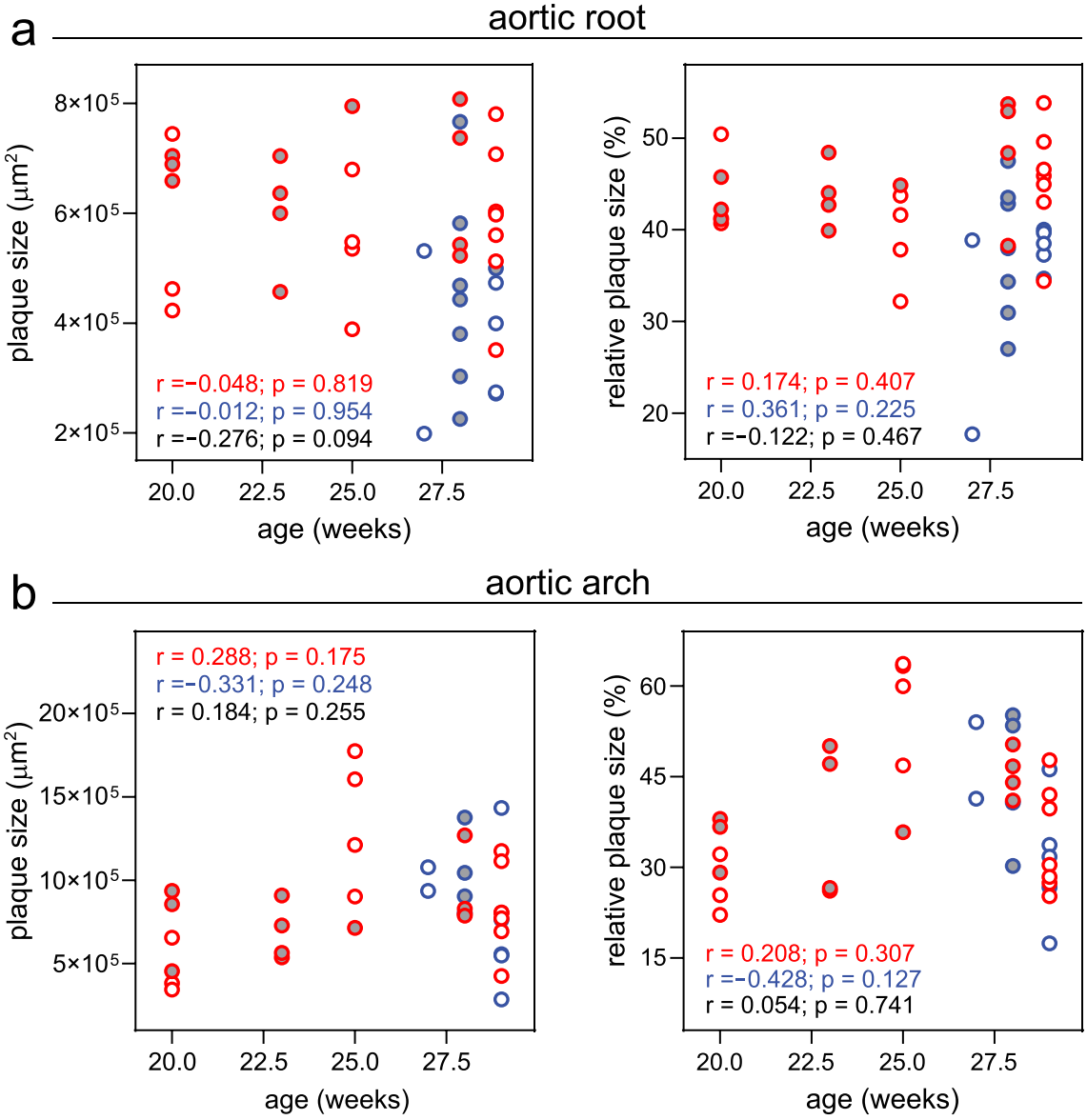

Figure 3. Correlation between the age and the absolute (µm^2) or relative (%) plaque size at the (a) zero-level of the aortic root or (b) at the aortic arch in CONV-R and GF *Ldlr*$^{-/-}$ mice on HFD. The groups of mice are the same as detailed in Figures 1 and 2. For all panels, CONV-R mice are shown as gray dots, and GF animals as white dots. The sex of the mice is color-coded: females: red; males: blue.

loss of the gut microbiota in GF WT mice was no longer seen in GF HFD-fed *Ldlr*$^{-/-}$ mice. While macrophage densities were not changed in late atherosclerotic lesions, these proteome changes indicate that GF conditions nevertheless alter immune cell activation or polarization in atherosclerotic mice.

Discussion

More than one century ago, in 1910, in his book "The Prolongation of Life. Optimistic Studies" Ilja Metchnikoff proposed that "auto-toxication from microbial poisons absorbed and microbes themselves may pass through the walls of the intestine and enter the blood" and he discussed the poisons of microbes as one possible cause for the development of inflammatory artery lesions.[25] Metchnikoff clearly recognized chronic inflammation, triggered by resident microbes, as one of the causes that endangers vascular health and restricts human lifespan. In recent years, this hypothesis from 1910 was refurbished, as it now could be experimentally addressed thanks to genome-wide sequencing approaches and the depletion of gut microbiota in mouse models of atherosclerosis. While the development of next-generation sequencing enabled the detection of abundant bacterial species

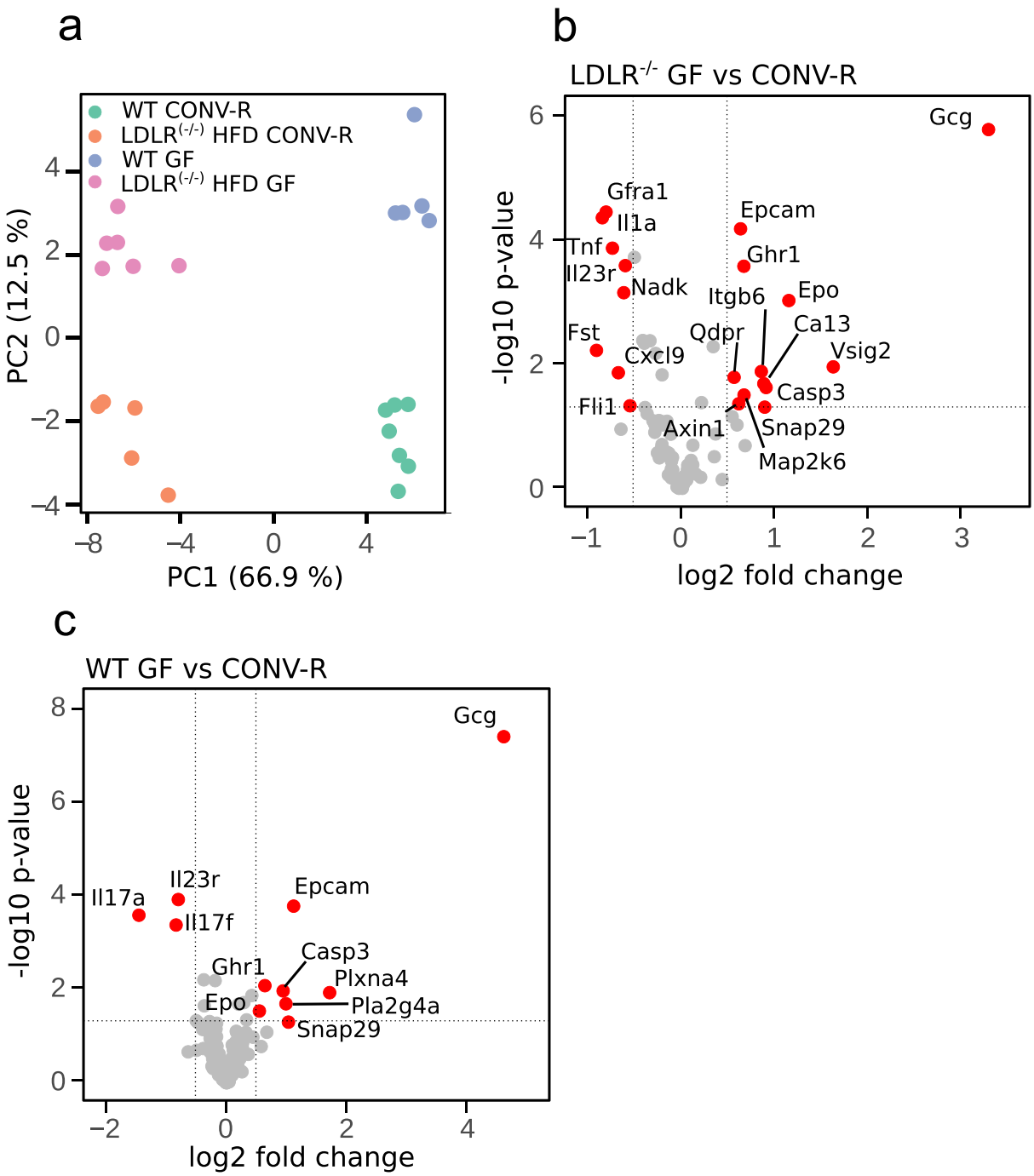

Figure 4. (a) Principal component analysis of plasma proteome changes in GF WT, GF *Ldlr*$^{-/-}$, CONV-R WT, and CONV-R *Ldlr*$^{-/-}$ mice. Scatter plot of the first two principal components for all 92 analytes measured in the indicated mice and feeding as well as colonization conditions. Each point represents a biological replicate from independent animals. (b) Volcano plot of the differential abundance of circulating biomarkers in HFD-fed GF *Ldlr*$^{-/-}$ mice compared to HFD-fed CONV-R *Ldlr*$^{-/-}$ mice and (c) GF WT mice compared to CONV-R WT mice. Positive log2 fold change values correspond to higher protein levels and negative values correspond to reduced protein levels in GF condition mice. The horizontal line reflects the cutoff for statistical significance ($p < 0.05$) while vertical lines represent threshold for minimum effect size (|log2 fold change| >0.5). Red highlights proteins with a significant difference ($p < 0.05$) above the threshold for effect size. Grey dots represent proteins with either no statistical significance or small effect size or both.

in atherosclerotic patients,[2] decimation of the gut microbiota by antibiotic treatments, and/or analysis of germ-free atherosclerotic mouse models unravel the global impact of the resident bacterial community on the host.[6,7,20] In our recent work, we analyzed carotid artery atherosclerosis and atherothrombosis

evoked by ultrasound-induced rupture of carotid artery plaques in the germ-free *Ldlr*$^{-/-}$ mouse model kept for 16 weeks on HFD.[18] We found a phenotype of decreased adhesion-induced platelet activation in the GF *Ldlr*$^{-/-}$ mice. Clearly, with regard to the microbiota's influence on the development of atherosclerotic lesions, controversies persist. In part, experiments may yield divergent results due to different mouse atherosclerosis models studied, inappropriate comparison of GF status with antibiotic decimation of commensals, variations in feeding regimens, time point of diet switch, and the analysis of different endpoints (Table 1). For this reason, detailed reports on atherosclerotic phenotypes with gnotobiotic mouse atherosclerosis models are timely and required to achieve a complete picture on the contribution of the microbiota to atherogenesis. In this addendum article, we provide additional information on atherosclerotic lesion formation at the aortic root and the aortic arch, comparing the same group of GF *Ldlr*$^{-/-}$ mice with their CONV-R *Ldlr*$^{-/-}$ counterparts, kept for 16 weeks on irradiated HFD.[18]

Similar to recent work on germ-free *Apoe*-deficient mice kept on an atherogenic HFD,[6] our study on GF *Ldlr*$^{-/-}$ mice did not find an impact of the gut microbiota on atherosclerotic lesion size, neither in the aortic root, nor in the aortic arch. These results are consistent with unchanged carotid artery lesion areas in 16 weeks HFD-fed GF *Ldlr*$^{-/-}$ mice relative to CONV-R *Ldlr*$^{-/-}$ mice, reported in our previous study, implicating the microbiota in atherothrombosis and adhesion-induced platelet activation.[18] While in this study we found increased total plasma cholesterol levels and increased lipoprotein levels in GF *Ldlr*$^{-/-}$ mice on a chow diet, which is due to the microbiota's role in cholesterol excretion,[20] the lipoprotein profile was unchanged at conditions of excess cholesterol from the diet (0.2% cholesterol). In particular, at conditions of limited cholesterol in the diet, the microbiota has a critical role in the deconjugation, dehydroxylation, and oxidation of primary bile acids, thus promoting their excretion.[29–33] On the other hand, the relatively high cholesterol content of the HFD used in our study and the late endpoints of the analyses could in principle explain why there was no difference in aortic root and aortic arch lesion size between HFD-fed GF *Ldlr*$^{-/-}$ mice and CONV-R *Ldlr*$^{-/-}$ mice.

It is well established that the dietary cholesterol content strongly influences aortic root lesion size.[23] Hence, the dietary cholesterol content and the applied feeding regimen may influence the extent of atherosclerosis in terms of plaque size and cellular plaque composition with respect to the analyzed vascular bed. This may at least in part explain the seemingly controversial data of different mouse atherosclerosis studies addressing the role of the microbiota in atherogenesis (Table 1).[1,4-6,18,21,22,34] In late atherosclerosis, at 14 weeks of HFD feeding, female *Ldlr*$^{-/-}$ mice kept on the same HFD we used in our studies show significantly increased total cholesterol levels, low-density lipoprotein cholesterol, and significantly reduced relative aortic root lesions compared with age-matched male *Ldlr*$^{-/-}$ mice.[24] Likewise, larger lesion areas were reported in young female *Apoe*$^{-/-}$ mice on chow diet at the age of 16 weeks compared with age-matched male *Apoe*$^{-/-}$ mice.[35] Increased lesion area was also found by *en face* aorta analyses.[36] Altogether, these studies indicate that in addition to the genetic mouse atherosclerosis model, the dietary cholesterol content, sex, and the chosen endpoint of the feeding regimen are pivotal for the conclusions drawn from functional microbiome studies on atherosclerosis.

Of note, our study did not exclude that specific microbes could impact atherogenesis. Diet has a dominant influence in shaping the diversity and composition of the gut microbial ecosystem.[18] As metagenomics studies have identified specific taxa[2,3,37-39] and as there is ample experimental evidence suggesting that microbiota composition may significantly influence the development of atherosclerotic lesions,[9,40] future functional microbiome studies should aim to understand how specific diets affect the abundance of specific gut microbes linked to atherosclerosis. Similar to the selective inhibition of trimethylamine (TMA)-lyase enzymes of gut microbes associated with atherosclerosis,[39] this may lead to new pharmacologic interventions that may prevent atherogenesis by targeting specific metabolic functions of gut microbes.[40] Furthermore, there certainly is a need for in-depth knowledge on the protective role of some community members and the dietary conditions that increase their relative abundance.[9,10]

In addition to specific bacteria, also certain microbial-associated molecular patterns (MAMPs) were recognized to contribute to chronic inflammation, driving atherogenesis by influencing different leukocyte subsets.[41–43] Studies with germ-free mouse models have revealed that gut microbiota-derived compounds promote steady-state granulopoiesis and regulate the lifespan of neutrophils and inflammatory monocytes.[44–46] In our study on hyperlipidemic *Ldlr*$^{-/-}$ mice, we could confirm the influence of the gut microbiota, as total leukocyte counts and in particular the proportion of monocytes and neutrophils were significantly reduced, whereas we found a slight increase in the proportion of lymphocytes in GF *Ldlr*$^{-/-}$ mice.[18] This was also reflected by reduced counts of rolling and adherent leukocytes at the uninjured carotid artery lesion. Unexpectedly, the observed reduction in blood monocytes in the blood of GF *Ldlr*$^{-/-}$ mice on an HFD was not associated with diminished macrophage content in the aortic root lesions or with a smaller necrotic core area, but this might be due to the high cholesterol content of the HFD and the late time point analyzed (16 weeks). In support of a reduced fibroproliferative response, we found reduced numbers of vascular smooth muscle cells but unchanged staining of the vascular RNS marker 3-NT in the aortic root plaques. This is in accordance with previous work that did not detect changed vascular superoxide formation, comparing dihydroethidium staining in the aorta of unchallenged GF to CONV-R C57BL/6 WT mice.[13] Hence, in future studies, it will be interesting to explore if the absence of a gut microbiota influences the expression of growth factors in the developing atherosclerotic lesions, thus affecting the migration and proliferation of vascular smooth muscle cells.[47] In line with the lower number of vascular smooth muscle cells, plasma follistatin (Fst) levels were reduced in HFD-fed GF *Ldlr*$^{-/-}$ mice compared to their CONV-R counterparts.[48] In contrast to WT mice on a normal diet, inflammation markers Tnf, IL1a, and Cxcl9 were downregulated in HFD-fed GF *Ldlr*$^{-/-}$ mice compared to HFD-fed CONV-R *Ldlr*$^{-/-}$ mice. Thus, despite unaltered macrophage numbers in the atherosclerotic lesions, GF mice display reduced inflammation detected by circulating markers.

In addition, we detected a reduction of Gfra1 in GF *Ldlr*$^{-/-}$ mice. Recently it has been shown that low Gfra1 levels result in enterocolitis with abnormal mucin production and retention causing epithelial damage.[49] Conversely, lack of microbiota increases circulating markers of proteins that are expected to be expressed in the intestine in HFD-fed GF *Ldlr*$^{-/-}$ mice. It will be of interest whether such unexpected markers reflect changes in gut permeability that are influenced not only by commensal microbiota but also HFD.

Therefore, future work should explain if the observed differences in vascular smooth muscle cell content in the aortic root plaques are related to increased collagen synthesis and if this also applies to carotid artery plaques.[50] This aspect is of particular relevance, since vascular smooth muscle cell-derived collagen fibers could promote plaque rupture-induced atherothrombosis and adhesion-dependent platelet activation, as indicated by increased phosphatidylserine exposure in HFD-fed CONV-R *Ldlr*$^{-/-}$ mice relative to HFD-fed GF *Ldlr*$^{-/-}$ mice.[18,20] To grasp the influence of the microbiome on cellular plaque composition during atherogenesis, a time-course analysis of atherosclerotic lesion development with a well-defined feeding regimen on gnotobiotic atherosclerosis mouse models is required.

Age is believed to strongly impact the pathogenesis of aortic atherosclerosis. Therefore, previous experimental studies, investigating the effect of microbiota on atherosclerotic lesion development, have used age-matched animals.[1,4,6] In contrast, our study included mice of varying age at the start of HFD. Importantly, age was not significantly correlated with absolute and relative plaque size in the aortic root and aortic arch. Additionally, the effect of GF housing conditions was not significant even after accounting for age differences. Thus, our study provides statistical evidence that conflicting results on the role of microbiota on atherogenesis might stem from factors other than age. However, it is important to mention that our results might be attributable to the late time point analyzed. Additional studies with animals of wider age range or a systematic meta-analysis of existing reports would be instrumental to resolve the complex interaction of age, diet, and gut microbiota on the development of atherosclerotic lesions.

Materials and methods

Animals – B6.129S7Ldlrtm1Her/J mice (1) (*Ldlr*$^{-/-}$ mice) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and were treated as previously described. Briefly, *Ldlr*$^{-/-}$ mice were rederived as germ-free (GF) by aseptic hysterectomy. *Ldlr*$^{-/-}$ and WT mice on a C57BL/6 J background were maintained as a GF mouse colony in sterile flexible film mouse isolator systems checking weekly for the germ-free status of the mice by detection of 16S rDNA by PCR and by bacterial culture. All experimental animals were 4–14 weeks old male or female mice housed in the Translational Animal Research Center (TARC) of the University Medical Center Mainz under specific pathogen-free (SPF, CONV-R) or GF conditions in EU type II cages with 2–5 cage companions with standard autoclaved lab diet and water ad libitum, 22 ± 2°C room temperature and a 12 h light/dark cycle. All groups of mice were free of clinical symptoms. All procedures performed on mice were approved by the local committee on legislation on protection of animals (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany; 23177–07/G12-1-100; 23 177–07/G 16-1-013).

Treatment of mice

Ldlr$^{-/-}$ mice were fed for 16 weeks with an adjusted calories diet (42% from fat, vacuum-packaged, irradiated, and microbial analyzed, TD.88137, Envigo, Venray, the Netherlands).

Analysis of atherosclerotic lesions

For analysis of mouse atherosclerotic lesions, the aortic roots at zero-level were stained for lipid depositions with Oil-Red-O or HE (hematoxylin-and-eosin) staining. In brief, hearts with aortic root were embedded in Tissue-Tek O.C.T. compound (Sakura) for cryo-sectioning. Atherosclerotic lesions were quantified in 5 µm transverse sections and averages were calculated from 3 to 5 sections for each mouse. For analysis of the cellular composition or inflammation of atherosclerotic lesions, sections were stained with an antibody to MAC2 (AbD Serotec), or SMA (Dako), Nuclei were counterstained by 4′,6-Diamidino-2-phenylindol (DAPI). After incubation with a secondary FITC- or Cy3-conjugated antibody (Life Technologies), sections were analyzed using a Leica DMLB fluorescence microscope and charge-coupled device (CCD) camera. Blinded image analysis was performed using Diskus, Leica Qwin Imaging (Leica Lt.) or Image J software. For each mouse and staining, 2–3 root sections were analyzed and data were averaged.

3-Nitrotyrosine immunostaining

The cryosections were dried at 37°C for 1 h, then fixed at −20°C in acetone for 10 minutes. To block the nonspecific bindings, the sections were incubated with 2.5% horse normal serum (Vector laboratories, Burlingame, CA94010) for 60 minutes. Sections were stained for 3-nitrotyrosine made in rabbit (Millipore, Germany) diluted 1:100 in antibody dilution medium (Agilent, Germany) overnight. Following the species of primary antibody, an appropriate biotinylated secondary antibody was used following the manufacturer's instructions. For immunochemical detection ABC reagent (Vector) and then DAB reagent (Peroxidase substrate Kit, Vector) as substrate were used.

Proximity ligation assay

Simultaneous-targeted protein profiling of 92 proteins of the Mouse Exploratory panel (Olink Proteomics AB, Uppsala, Sweden) was performed in 1 µl of once-thawed citrate anticoagulated plasma samples by real-time PCR using the Fluidigm BioMarkTM HD real-time PCR platform based on multiplexing proximity extension assay (PEA) technology (Olink Proteomics AB).[51] For exploratory data analysis, a principal component analysis of the Olink NPX values was performed using the R version 3.6.3 prcomp() function. A linear model was fitted to all data for individual *Ldlr*$^{-/-}$ mice or WT respectively with housing condition as a factor to model the effect of GF conditions using the R version 3.6.3 lm() function. Log2 fold changes and associated *p*-values were calculated by Student's t-test and returned by the summary function of the R lm fit object. *P*-values less than 0.05 were considered statistically significant.

Statistical analysis

Data are presented as mean ± SEM and/or individual data points. Statistical calculations were performed with GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, US) using the independent samples Student's t-test to compare two groups. Pearson correlation coefficients and 2-way ANCOVA were calculated with R version 3.5.3 (R Core Team, Austria, Vienna). *P*-values less than 0.05 were considered statistically significant.

Acknowledgments

We are grateful to Klaus-Peter Derreth for expert technical assistance.

Author Contributions

K.K., S.J., C.K., T.K., Y.J., Y.D., and C.R. performed experiments and analyzed data. K.K., G.P., H.T., J.B., S.J., F.B., Y. J., S.G., P.W., W.R., A.D., E.v.d.V., C.W., and Y. D. analyzed data. K.K., G.P., and H.T. participated in manuscript writing. Y.D. and C.R. designed experiments and wrote the manuscript.

Disclosure of Potential Conflicts of Interest

The authors declare that no conflicts of interest exist.

Funding

The project was funded by the CTH Junior Group Translational Research in Thrombosis and Hemostasis (BMBF 01EO1003 and 01EO1503), by the German Center for Cardiovascular Research (DZHK, Pillar B Project, FKZ 81 × 2210106 to C.R., Y.D., and C.W.), by a project grant from the Boehringer Ingelheim Foundation (Consortium Grant "Novel and neglected cardiovascular risk factors") to C.R. and WR, by a project grant from the Naturwissenschaftlich-Medizinisches Forschungszentrum (NMFZ) to C.R., by the European Research Council (ERC AdG °692511) to C.W. Supported by a grant from the Interdisciplinary Center for Clinical Research within the faculty of Medicine at the RWTH Aachen University, and NWO-ZonMw Veni (91619053) to E.P.C.v.d.V. S.J., Y.D., and C.R. are members of Young DZHK. W.R. is PI of the DZHK. The work of G.P. was supported by an EMBO Short Term Fellowship (No. 7605) and by an intramural Stufe1 project grant (Inneruniversitäre Forschungsförderung).

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