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**Article:**
Renshall, L.J., Arnold, N.D. orcid.org/0000-0002-4317-5738, West, L. et al. (10 more authors) (2017) EXPRESS: Selective Improvement of Pulmonary Arterial Hypertension with a Dual ETA/ETB Receptors Antagonist in the Apolipoprotein E-/- Model of PAH and Atherosclerosis. Pulmonary Circulation. ISSN 2045-8932

https://doi.org/10.1177/2045893217752328
Selective Improvement of Pulmonary Arterial Hypertension with a Dual ET<sub>A</sub>/ET<sub>B</sub> Receptors Antagonist in the Apolipoprotein E<sup>−/−</sup> Model of PAH and Atherosclerosis

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Total number of manuscript pages: 30

Total number of figures: 5

Word count: 3073

Original article

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Keywords: endothelin, macitentan, ApoE, pulmonary hypertension, atherosclerosis.
Abstract

Idiopathic pulmonary arterial hypertension (IPAH) is increasingly diagnosed in elderly patients who also have an increased risk of comorbid atherosclerosis. Apolipoprotein E deficient (ApoE<sup>-/-</sup>) mice develop atherosclerosis with severe PAH when fed a high-fat diet (HFD), and have increased levels of endothelin (ET)-1. ET-1 receptor antagonists (ERAs) are used for the treatment of PAH but less is known about whether ERAs are beneficial in atherosclerosis. We therefore examined whether treatment of HFD-ApoE<sup>-/-</sup> mice with macitentan, a dual ET<sub>A</sub>/ET<sub>B</sub> receptor antagonist, would have any effect on both atherosclerosis and PAH. ApoE<sup>-/-</sup> mice were fed chow or HFD for 8 weeks. After 4 weeks of HFD, mice were randomised to a 4-week treatment of macitentan by food (30mg/kg/day dual ET<sub>A</sub>/ET<sub>B</sub> antagonist), or placebo groups. Echocardiography and closed-chest right heart catheterisation were used to determine PAH phenotype and serum samples were collected for cytokine analysis. Thoracic aortas were harvested to assess vascular reactivity using wire myography, and histological analyses were performed on the brachiocephalic artery and aortic root to assess atherosclerotic burden. Macitentan treatment of HFD-fed ApoE<sup>-/-</sup> mice was associated with a beneficial effect on the PAH phenotype and led to an increase in endothelial-dependent relaxation in thoracic aortae. Macitentan treatment was also associated with a significant reduction in interleukin 6 (IL-6) concentration but there was no significant effect on atherosclerotic burden. Dual blockade of ET<sub>A</sub>/ET<sub>B</sub> receptors improves endothelial function and improves experimental PAH but had no significant effect on atherosclerosis.
Introduction

Idiopathic pulmonary arterial hypertension (IPAH) is a devastating life shortening condition. Although often thought to predominantly affect young women, recent data from the European COMPERA study highlights the increasing number of elderly patients over 65 years of age diagnosed with IPAH. Atherosclerosis and atherosclerotic-associated diseases are the leading cause of cardiovascular disease and death worldwide. There are multiple risk factors for atherosclerosis including genetics, high levels of C-Reactive Protein and diet but the impact of more traditional risk factors such as high levels of triglyceride and LDL cholesterol, smoking and age still remains significant. Pro-inflammatory cytokines, such as those from the interleukin family, are upregulated in both atherosclerosis and PAH. Specifically, overexpression of IL-6 in mice can lead to spontaneous PAH and exacerbates chronic hypoxia-induced PAH and high IL-6 concentrations are also associated with increased mortality in patients with coronary artery disease and PAH. Interestingly, IL-1 (sitting upstream of IL-6) is also elevated in both humans and animal models of PAH and atherosclerosis, and inhibition of IL-1 has shown therapeutic potential. Indeed data from our own lab demonstrated therapeutic efficacy of IL-1 receptor antagonist treatment on the HFD-fed ApoE-/- model of PAH.

Mechanistically, endothelial dysfunction is evident in both PAH and atherosclerosis in the form of impaired endothelium-dependent and –independent vasorelaxation. ET-1 is a potent vasoconstrictor formed by the conversion of Big ET-1 to ET-1 by endothelin converting enzymes. ET-1 production and secretion can be regulated by inflammatory cytokines, hypoxia and glucose and is increased in the vessel wall of experimental models and human atherosclerotic lesions and PAH. Reviewed in...
The biological function of ET-1 can be reduced through the administration of endothelin receptor antagonists (ERAs). ERAs bind to either one or both ET_A/ET_B receptors on smooth muscle cells (SMC) or endothelial cells and reduce the vasoconstriction associated with ET-1.

ERAs improve the prognosis of WHO functional class II and III PAH patients and ETA-selective or dual ETA/ETB antagonism were shown to increase endothelial function and improve forearm and coronary artery blood flow in patients with atherosclerosis.

Since atherosclerosis and PAH have these shared pathophysiological processes, and that ET-1 stimulates production and secretion of IL-6 in human vascular SMCs via nuclear factor-κB (NF-κB) and with evidence of an aging population of IPAH patients, we sought to further investigate the potential impact of macitentan treatment on the Paigen HFD-fed ApoE⁻/⁻ model of concomitant atherosclerosis and PAH. We hypothesised that treatment with a dual ERA would improve endothelial function, reduce inflammatory cytokines, improve cardiac hemodynamics and thus alleviate atherosclerotic lesion burden.

Methods

Animals

ApoE⁻/⁻ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were on a C57BL/6J background. Male mice weighing between 22 - 30 grams at 12 weeks of age were fed a high-fat, high-cholate Paigen diet (HFD; 18.5% fat, 0.9% cholesterol, 0.5% cholate, and 0.2959% sodium) or standard chow (4.3% fat, 0.02% cholesterol and 0.28% sodium). Diets were supplied by Special Diet Services, Braintree, UK. At 16 weeks of age mice fed a Paigen diet (HFD) were randomised to either intervention (ApoE⁻/⁻ HFD macitentan; 30 mg/kg/day) or treatment-naïve group (ApoE⁻/⁻ HFD) for a further 4 weeks (n= 10/group). Mice fed a standard diet were treatment-naïve (ApoE⁻/⁻ chow). All procedures conformed to UK Home
Office Regulations (Animals Scientific Procedures Act 1986; Project license 40/3517) and were approved by the University of Sheffield Project Review Committee.

**Echocardiography**

At week 20, transthoracic echocardiography was performed (n= 8/group) using the RMV707B scan head on the Vevo 770 system (VisualSonics, Toronto, Canada). There was a single operator blinded to the intervention status of the mice. Mice were placed on a heated platform with rectal temperature, heart rate and respiration rate monitored continuously. Anaesthesia was induced and maintained using isoflurane through oxygen. Right ventricular free wall and left ventricular parameters were obtained as previously described. Cardiac output was derived by measurement of the flow and diameter at the junction between aortic outflow tract and aortic valve. Cardiac index was normalised by body weight and all measurements were taken at non-inspiratory phase of the cardiac cycle. Analysis was performed using the Vevo 770 V3.0 VisualSonics software.

**Cardiac catheterisation**

Immediately after echocardiography, animals were maintained under 1-2% isoflurane whilst performing right and left ventricular catheterisation via either the right internal carotid artery or the right external jugular vein. Pressure-volume (PV) data were collected by Millar ultra-miniature catheters (PVR-1045; mouse LV, PVR-1030; mouse RV: Millar Instruments, Inc., Houston, TX) coupled to a Millar MPVS 300. Data was acquired using Powerlab 8/30 (AD instruments, Oxfordshire, UK) and recorded using LabChart 7 Pro software (AD instruments). PVAN v2.3 (Millar Instruments, Inc.) was used to analyse PV data as previously described.
Assessment of right ventricular hypertrophy.

Following cardiac catheterisation animals were euthanised by cervical dislocation under 2% isoflurane. Cardiac tissue was collected and fixed in 10% neutral buffered formalin for 24 hours and washed with PBS. Using a modified Fulton index weight of the right ventricle free wall relative to the left ventricle and septum was used to determine an estimate of right ventricular hypertrophy (n= 7/group).

Endothelial function of thoracic aortae

Mounting. Thoracic aortas from HFD-fed mice were harvested to assess vascular reactivity using wire myography (ApoE^−/− HFD macitentan n= 4; ApoE^−/− HFD n= 4 mice). Aortae were mounted onto a Danish Myo Technologies 610M wire myograph (DMT, Denmark), warmed to 37°C, and gassed with 95% air/5% CO$_2$/balance N$_2$ for 30 minutes in physiological salt solution (PSS in mM; 119 NaCl, 25 NaHCO$_3$, 4.7 KCl, 1.17 MgSO$_4$, 1.6 CaCl$_2$, 1.17 KH$_2$PO$_4$, 5.5 glucose, 0.03 EDTA (free acid) at pH 7.4).

Normalisation. Incremental stretch was applied to each vessel using the micrometer attached to the myograph chamber and passive tension was recorded for 30 minutes until a passive steady resting tension of 5 mN was achieved.

Experimental procedure. Post-equilibration, aortas were exposed to two separate washes with depolarizing solution (KPSS in mM: 63.7 NaCl, 25 NaHCO$_3$, 60 KCl, 1.17 MgSO$_4$, 1.6 CaCl$_2$, 1.17 KH$_2$PO$_4$, 5.5 glucose, 0.03 EDTA (free acid) at pH 7.4). After washing with PSS, aortas were pre-contracted with 10 µM phenylephrine and exposed to ACh (1x10^{-9} – 3x10^{-5} M). After washing with PSS, vessels were pre-contracted with 10 µM phenylephrine and exposed to the nitric oxide donor sodium nitroprusside (SNP; 1x10^{-9} – 3x10^{-5} M).
Immunohistochemistry

Left lung was perfusion fixed via the trachea as previously described. Briefly, left lung was inflated with 10% neutral buffered formalin at 20 cm of H2O. Lungs were processed in paraffin wax and sectioned (5 µm). Sections were histologically stained with Alcian Blue Elastin van Gieson (ABEVG). For immunohistochemistry sections were stained with α-smooth muscle actin (SMA; M0851; Dako, Cambridgeshire, UK) for smooth muscle cell identification and von Willebrand factor (vWF; A0082; Dako) to identify endothelial cells. Standard immunohistochemical techniques were applied, and both IgG and no primary antibody negative controls were used. The Axiocam 506 Color System (Zeiss) was used for microscopy and analysis was performed in Zen 2 Blue Edition (Zeiss).

Quantification of pulmonary vascular remodelling

The degree of pulmonary muscularisation was assessed and analysed as a percentage of total non-muscularised vessels. Vessels were categorised into two groups based on their size; small arterioles (<50 µm in diameter) and medium pulmonary arteries (>51 - <100 µm).

Assessment of atherosclerotic lesion burden

Aortic sinus paraffin-embedded sections were stained with ABEVG to assess mean lesion to cross sectional area ratio and martius scarlet blue to assess collagen content. Sections were also stained immunohistochemically with α-SMA, as described above. Whole mount aortae, which included aortic arch and descending aorta were stained with Oil Red O (Sigma, UK) to assess lesion area as a percentage of total aortic area. Scoring was performed in a blinded manner.
Enzyme linked immunosorbent assays

Prior to sacrifice a cardiac puncture was performed and serum samples were collected for cytokine analysis (n=4–9/group). To assess concentration of soluble cytokines the Cytometric Bead Assay Flex sets (BD Biosciences, Oxford, UK) for Insulin, IL-1β, IL-6, IL-10, HGF, TNF-α, MCP-1, Leptin, IP-10, SDF-1a and CRP was used.

Statistical analyses

When comparing two groups a Mann-Whitney U Test was used for statistical analyses. When comparing the medians of three groups or more a Kruskal-Wallis test followed by Dunns post-hoc test (95% confidence interval) was undertaken. For myography data in thoracic aortae two-way analysis of variance was performed with Bonferroni post-hoc test. P < 0.05 was deemed to be significant (Prism 6.0; Graphpad Software). Data are presented as mean ± SEM, unless otherwise stated. For technical reasons certain procedures were unable to be completed on each individual animal and thus there is variation in the number of data points. An inability to quantify pulmonary artery flow using echocardiography, inability to catheterise left ventricle and a serum sample out of detection range of standard curve were the most common reasons for missing data.
Results

Macitentan improves cardiac haemodynamics associated with PAH phenotype in HFD-fed ApoE<sup>−/−</sup> mice. HFD-fed ApoE<sup>−/−</sup> developed a PAH phenotype similar to that previously described<sup>[16,36]</sup> as assessed by a decrease in pulmonary artery acceleration time (PA-AT, Figure 1A), increase in right ventricular systolic pressure (RVSP, Figure 1B) and changes in right ventricular function (Figure 1C-D). Treatment with macitentan significantly increased PA-AT (Figure 1A), reduced RVSP (23 mm Hg vs. 38 mm Hg, Figure 1B) and improved RV function (Figure 1C-D). There was no effect of macitentan on left ventricular (LV) cardiac output (Figure 1E), mean aortic blood pressure (Figure 1F) or LV end-diastolic pressure (LVeDP, Figure 1G). As previously reported<sup>[16,36]</sup> there was no significant increase in right ventricular hypertrophy (RVH) in HFD-fed ApoE<sup>−/−</sup> mice, and there were no significant differences in macitentan treated HFD-fed ApoE<sup>−/−</sup> when compared with treatment-naïve ApoE<sup>−/−</sup> mice (data not shown).

Macitentan reverses pulmonary vascular remodelling associated with the PAH phenotype in HFD-fed ApoE<sup>−/−</sup> mice. Histologic and immunohistochemistry analysis of serial lung tissue sections revealed significant pulmonary vasculopathy consistent with a PAH phenotype in HFD-fed ApoE<sup>−/−</sup> mice. Analysis of media/CSA ratio in small resistance pulmonary arterioles <50 µm in diameter (Figure 2A) and the small to medium 51-100 µm pulmonary arteries (Figure 2B) demonstrated significant muscularisation in treatment-naïve HFD-fed ApoE<sup>−/−</sup> animals when compared to standard chow-fed ApoE<sup>−/−</sup> mice. Treatment with macitentan resulted in a significant decrease in the media/CSA for both the small pulmonary arterioles (<50 µm, Figure 2A&E), and the small to medium sized pulmonary arteries (51-100 µm,
Figure 2B). Similarly, there was a significant increase in the percentage of muscularised arterioles (< 50 µm, Figure 2C&E) and arteries (51 – 100 µm, Figure 2D) in HFD-fed ApoE⁻/⁻ mice that was also reduced by treatment with macitentan.

Macitentan improves endothelial-dependent vascular function associated with atherosclerotic phenotype in HFD-fed ApoE⁻/⁻ mice. In thoracic aortae explanted from macitentan treated HFD-fed ApoE⁻/⁻ mice, endothelial-dependent relaxation of pre-contracted thoracic aortas was markedly increased compared to untreated HFD-fed ApoE⁻/⁻ mice (Figure 3A). Conversely, when thoracic aortas were once again pre-contracted with phenylephrine there was no significant effect of sodium nitroprusside-mediated endothelial-independent relaxation in macitentan-treated animals compared with treatment-naïve littermates (Figure 3B). There were no differences in maximal contraction to 60 mM high-potassium depolarising salt-solution or 10 µM phenylephrine between treatment-naïve and macitentan-treated ApoE⁻/⁻ littermates (data not shown).

Macitentan treatment had no effect on atheroma lesion burden in HFD-fed ApoE⁻/⁻ mice. On examination of explanted whole aortae stained en face with Oil Red O (ORO) there appeared to be a small but significant increase in lipid deposition in macitentan-treated animals (Figure 4A&C). This appeared to be due to greater accumulation of lipid specific to the aortic arch region (Figure 4B) and not the descending aorta (data not shown). However immunohistological analysis of both the brachiocephalic artery (Figure 4D-E) and aortic root sinus (Figure 4F-I) demonstrated that there was no significant effect of macitentan treatment on atherosclerotic lesion burden or composition. Macitentan treatment did not alter the size of
the lesion, as assessed by the ratio of lesion to cross sectional area (Figure 4D), or the content of SMA in the lesion of the brachiocephalic artery (Figure 4E) compared with sections from control mice. Further histological assessment of the aortic root sinus also showed no significant change in the ratio of lesion to cross sectional area (Figure 4F&I), collagen content (Figure 4G&I) or vascular smooth muscle cell content (Figure 4H&I) of macitentan treated mice compared with treatment-naïve controls.

Macitentan decreases IL-6 levels in HFD-fed ApoE−/− mice. Treatment with macitentan resulted in a significant reduction in circulating levels of IL-6 (Figure 5A) and increased the concentration of leptin (Figure 5B) in serum. There were no significant differences in TNF-α, MCP-1, IL-1β, IL-10, IP-10, SDF-1, CRP, Insulin or HGF (Figure 5C-K).

**Discussion**

To our knowledge, this is the first time macitentan has attenuated established PAH in mice. In a novel model of concomitant PAH and atherosclerosis, we found a significant reduction in muscularisation of small vessels and associated improvements in haemodynamics. Our results are consistent with pre-clinical studies using other models of pulmonary hypertension and with clinical studies in PAH patients. Macitentan treatment increased endothelium-dependent dilatation but was not associated with a reduction in atherosclerosis lesion burden at the aortic root or the brachiocephalic artery.

The full molecular mechanisms by which ERAs attenuate PAH are still not fully understood, although there is clear evidence for vasodilatory effects that may result in flow-mediated improvements in remodelling. However, we also found a significant reduction in circulating
IL-6 in macitentan treated Paigen diet-fed ApoE−/+ mice. Preclinical[14] and clinical[10,11] studies identify IL-6 as potentially a key regulator of PAH pathogenesis and therefore a putative drug target. Indeed, the direct effect of targeting IL-6 in PAH will soon become clearer with the conclusion of the TRANSFORM-UK study (NCT02676947). Studies by McMillen have previously demonstrated that ET-1 can stimulate IL-6 production from human monocytes[39]. We propose that the same is occurring in this model, driving increased IL-6 in the Paigen diet-fed ApoE−/− mice, and that levels fall following the addition of Macitentan. Previous studies have shown that administration of an IL-6R antagonist in patients at high risk of coronary artery disease led to a reduction in IL-6, an increase in endothelial function, induction of dyslipidaemia and an increase in total lipid plasma content[40]. There is a suggestion that the dyslipidaemia as a result of reduced IL-6 may in fact be causing the lipid profile to alter to a more anti-inflammatory composition[41]. In our model, quantitative assessment of aortic sinus and brachiocephalic lesion burden suggested there were no differences in lesion size or content between treatment-naïve and macitentan-treated HFD-fed ApoE−/+ mice. Oil red O staining of the uppermost surface of the aortic tree dissected from experimental animals suggested increased lipid rich areas in the treated mice. However, as this technique can be highly influenced by tissue preparation, and since the much more robust and accepted histological assessment of aortic root and the brachiocephalic artery did not show any significant difference, we conclude that Macitentan does not adversely affect experimental atherosclerosis in our joint model of PAH and atherosclerosis. Our data contrast with other animal studies demonstrating that either dual ET_{A}/ET_{B}, or selective ET_{A} receptor antagonists reduce the development and progression of atherosclerosis in both Western diet-fed ApoE−/+ and Ldlr−/−-deficient mice[42–44]. We speculate that there may be two explanations for a lack of effect of
Macitentan on atherosclerotic lesion burden in our study; the length of treatment may not have been sufficient and/or atherosclerotic lesion development and progression was more pronounced in our novel model compared with the traditional Western diet. However, consistent with studies assessing the effects of ERAs on clinical atherosclerosis, macitentan was able to increase endothelium-dependent relaxation of thoracic aortas.

Our study utilised an 8-week fat-feeding regimen commencing at 3 months of age. While this is a similar age used in previous studies investigating the effect of ERAs on atherosclerosis, macitentan was only administered for the final 4 weeks of the model. In the context of atherosclerosis there seems little consensus on the length of treatment of ERAs; with some studies opting for a 6 – 10-week treatment period whilst others opting for a longer 30-week period. We chose a 4-week treatment period as this is known to be beneficial in PAH and as such we wanted to assess whether it could impact on atherosclerosis in our Paigen diet-fed model. Indeed, a recent study from Houde and colleagues showed that a 6-week treatment of macitentan was sufficient to reduce aortic lesion development in ApoE<sup>-/-</sup> mice after 17 weeks of traditional Western diet. However, it must be noted that there is a far greater atherosclerotic lesion burden in ApoE<sup>-/-</sup> mice fed a Paigen diet when compared with ApoE<sup>-/-</sup> mice fed a traditional Western diet. Taken together, these data suggest either increasing the length of treatment or commencing treatment at an earlier stage in atherogenesis may reduce lesion burden or dampen the progression of atherosclerosis in our model. Indeed clinical data suggest that ERAs are only able to reduce plaque progression in coronary arteries at the early stages of atherosclerosis and in the presence of endothelial dysfunction.

Macitentan treatment convincingly led to increased endothelium-dependent relaxation in aortae and a reduction in the pro-inflammatory cytokine IL-6. Serum IL-1β was not altered
but this is unsurprising given the inherent difficulties in measuring this cytokine. In high-risk patients with PAH and concomitant atherosclerosis, worsening endothelial function and raised inflammatory markers such as CRP and IL-6 are quantifiable; thus, our data suggest macitentan may be of most benefit to these patients.

In summary, macitentan significantly reduced the main pathological features associated with PAH such as pulmonary vascular remodelling and cardiac dysfunction. Macitentan treatment was associated with increased endothelium-dependent relaxation in aortae, a surrogate marker of endothelial function. Using our model of advanced atherosclerosis, there was no effect of macitentan on atherosclerotic lesion burden and treatment did not alter the morphology or the stability of plaques within the aortic sinus or brachiocephalic artery. We therefore suggest that macitentan treatment is able to improve endothelial function, reduce inflammatory mediators, such as IL-6 but this did not translate through to reduced atherosclerosis in our study setting. Given the numerous successes of ERAs in experimental and clinical atherosclerosis, combined with our results suggesting improvements in endothelial function and markers of inflammation, we propose future studies should assess the effects of the current generation of ERAs, such as macitentan, in clinical atherosclerosis.

References


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Figure Legends

Figure 1. Macitentan treatment reduces haemodynamic indices of PAH in HFD-fed ApoE\(^{-/-}\) mice. Bar graphs show (A) pulmonary artery acceleration time (PA-AT), (B) right ventricular systolic pressure (RVSP), (C) right ventricular arterial elastane (RVEa), (D) right ventricular dP/dt\(_{\text{max}}\) in mm Hg/s, (E) cardiac output, (F) mean aortic pressure (mAoP), (G) left ventricular end-diastolic pressure (LVeDP). A and F: Measures were made using Vevo 770 echocardiography v3.0. All other measures were made using closed chest cardiac pressure volume catheterisation. Bars represent mean ± SEM, n= 6 – 20, individual animals represented by dots. A - G: Statistical differences between chow-fed ApoE\(^{-/-}\), HFD-fed and HFD-fed + macitentan was assessed by Kruskal-Wallis analysis of variance with Dunn’s multiple comparison post-hoc test; *P< 0.05, **P< 0.01.

Figure 2. Macitentan treatment reduces pulmonary vascular remodelling in HFD-fed ApoE\(^{-/-}\) mice. Bar charts (A and B) illustrating the degree of medial wall thickening (identified by smooth muscle actin; SMA) as a ratio of total vessel size (SMA / CSA) in <50 µm arteriole (A) and small to medium sized pulmonary arteries (B). (C and D) represent the percentage muscularised and non-muscularised arterioles (C) and arteries (D). Representative images of ABEVG and SMA stained tissue (E); ABEVG and SMA stained images of pulmonary arteries from ApoE\(^{-/-}\) mice that were fed either chow (top), HFD with no treatment (middle) and HFD with macitentan (bottom). Scale bar represents 100 µm. A and B: Bars represent mean ± SEM, n= 7. Kruskal-Wallis analysis of variance *P<0.05 ***P< 0. C and D: stars represent level of statistical significance when compared with chow-fed ApoE\(^{-/-}\) mice; ****P< 0.0001. Post hoc analysis shows statistical differences between macitentan-treated and treatment-naïve HFD-fed mice (P< 0.0001).
**Figure 3. Macitentan treatment improves vascular function in thoracic aortae.** Graphs show cumulative dose response curves to increasing concentrations of acetylcholine (A) or sodium nitroprusside (B) in thoracic aortae pre-contracted with 10 µM phenylephrine. Data are expressed as mean ± SEM and presented as a % of endothelium-dependent (A) or – independent (B) relaxation, n= 4. *P< 0.05, ***P< 0.001. Statistical differences between treated and untreated HFD-fed mice were assessed by two-way analysis of variance with Bonferroni post hoc analysis.

**Figure 4. Macitentan treatment does not alter the composition or atherosclerotic burden in HFD-fed ApoE^{-/-} mice.** Bar charts show Oil Red O stain as a % of total aortic area (A), and within the aortic arch as a % of total arch area (B). (C) Representative images of Oil Red O in control and macitentan-treated mice. (D) Bar charts show the ratio of atherosclerotic lesion area within the brachiocephalic artery normalised to cross sectional area, and (D) the % α-smooth muscle actin positive tissue within the brachiocephalic sections. (F) shows the ratio of lesion area to cross sectional area within the aortic root sinus, (G) % collagen content, and (H) % α-smooth muscle actin positive tissue in aortic root sections. Panel I shows representative aortic root histological sections from control and macitentan-treated HFD-fed ApoE^{-/-} mice stained with ABEVG, martius scarlet blue (collagen) and α-smooth muscle actin. Bars represent mean ± SEM. Statistical differences between sections from treated and untreated mice were assessed by Mann-Whitney U test, n= 11 – 12. individual mice are represented by dots. *P< 0.05. Statistical differences between groups were measured by Mann-Whitney U test.
Figure 5. Macitentan treatment induces changes in inflammatory plasma cytokines in HFD-fed ApoE<sup>−/−</sup> mice. Bar charts show the plasma concentration of (A) interleukin-6 (IL-6), (B) leptin, (C) interleukin-10 (IL-10), (D) interleukin 1 beta (IL-1β), (E) monocyte chemoattractant protein 1 (MCP-1), (F) tumor necrosis factor alpha (TNFα), (G) hepatocyte growth factor (HGF), (H) insulin, (I) interferon-gamma-inducible protein-10 (IP-10), (J) stromal cell-derived factor 1 (SDF-1) and (K) C-Reactive protein (CRP). Bar graphs represent mean ± SEM, n= 4 – 9 with individual animals represented by dots. *P< 0.05. Statistical differences were assessed by Mann-Whitney U test.