Prolactin receptor antagonism uncouples lipids from atherosclerosis susceptibility

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Abstract

The pituitary-derived hormone prolactin has been suggested to stimulate the development of atherosclerosis and cardiovascular disease through its effects on metabolism and inflammation. In this study, we aimed to challenge the hypothesis that inhibition of prolactin function may beneficially affect atherosclerosis burden. Hereto, atherosclerosis-susceptible LDL receptor (*Ldlr*) knockout mice were transplanted with bone marrow from transgenic mice expressing the pure prolactin receptor antagonist Del1-9-G129R-hPRL or their non-transgenic littermates as control. Recipient mice expressing Del1-9-G129R-hPRL exhibited a decrease in plasma cholesterol levels (–29%; $P < 0.05$) upon feeding a Western-type diet (WTD), which could be attributed to a marked decrease (–47%; $P < 0.01$) in the amount of cholesterol esters associated with pro-atherogenic lipoproteins VLDL/LDL. By contrast, Del1-9-G129R-hPRL-expressing mice did not display any change in the susceptibility for atherosclerosis after 12 weeks of WTD feeding. Both the absolute atherosclerotic lesion size ($223 \pm 33 \times 10^3 \mu\text{m}^2$ for Del1-9-G129R-hPRL vs $259 \pm 32 \times 10^3 \mu\text{m}^2$ for controls) and the lesional macrophage and collagen contents were not different between the two groups of bone marrow recipients. Importantly, Del1-9-G129R-hPRL exposure increased levels of circulating neutrophils (+91%; $P < 0.05$), lymphocytes (+55%; $P < 0.05$), and monocytes (+43%; $P < 0.05$), resulting in a 49% higher ($P < 0.01$) total blood leukocyte count. In conclusion, we have shown that prolactin receptor signaling inhibition uncouples the plasma atherogenic index from atherosclerosis susceptibility in *Ldlr* knockout mice. Despite an associated decrease in VLDL/LDL cholesterol levels, application of the prolactin receptor antagonist Del1-9-G129R-hPRL does not alter the susceptibility for initial development of atherosclerotic lesions probably due to the parallel increase in circulating leukocyte concentrations.

Key Words

- ▶ prolactin receptor
- ▶ Del1-9-G129R-hPRL
- ▶ lipoprotein
- ▶ atherosclerosis
- ▶ mouse model

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Introduction

Cardiovascular disease represents a major source of mortality in the Western world. Atherosclerosis, i.e., narrowing of the arteries as a result of lipid deposition, is the primary underlying biological cause of cardiovascular disease. High plasma cholesterol levels are an established risk factor for the development of atherosclerosis. Drug-induced lowering and normalization of cholesterol levels are therefore the primary therapy for patients at risk of cardiovascular disease. However, traditional cholesterol-lowering treatments only reduce the incidence of cardiovascular disease complications by 20–30% (Cholesterol Treatment Trialists' (CTT) Collaborators *et al.* 2012). Thus, there remains a clear need for the development of alternative anti-atherogenic therapies.

Recent clinical evidence has identified the pituitary-derived hormone prolactin as a potential novel target to treat patients at risk of cardiovascular disease. Hyperprolactinemic subjects harboring a prolactin-secreting pituitary adenoma (prolactinoma) also display an increase in carotid intima media thickness (IMT), a sub-clinical marker for atherosclerosis (Arslan *et al.* 2014, Jiang *et al.* 2014). In these patients, prolactin levels independently predict the extent of IMT (Jiang *et al.* 2014). Furthermore, prolactin levels in post-menopausal women correlate with the HeartScore, a composite index that predicts 10-year cardiovascular mortality by taking into account age, sex, systolic blood pressure, total cholesterol, and smoking status (Georgiopoulos *et al.* 2009). Importantly, relatively high prolactin levels are also associated with a sex-independent increase in all the cause and cardiovascular disease-specific mortality rate in the general population (Haring *et al.* 2014).

In the current study, we aimed to challenge the hypothesis that inhibition of prolactin function may beneficially affect atherosclerosis burden. Dopamine receptor agonists such as bromocriptine effectively lower prolactin secretion by the pituitary and are the primary treatment option for prolactinoma patients (Wu *et al.* 2006, Yarman *et al.* 2012). However, these agents may also theoretically affect atherosclerosis susceptibility by altering dopamine action locally in the atherosclerotic plaque, as dopamine receptors have been detected in both smooth muscle cells (Zeng *et al.* 2004, Li *et al.* 2008) and different types of leukocytes (McKenna *et al.* 2002). The prolactin receptor antagonist Del1-9-G129R-hPRL is an inactive prolactin variant that efficiently competes with endogenous prolactin for binding to the prolactin receptor (Bernichtein *et al.* 2003), leading to a disruption of normal

prolactin action *in vivo* (Rouet *et al.* 2010). To specifically show the impact of prolactin function inhibition on atherogenesis, we induced the expression of the prolactin receptor antagonist Del1-9-G129R-hPRL in atherosclerosis-susceptible LDL receptor (*Ldlr*) knockout mice.

Materials and methods

Mice and bone marrow transplantation

Animal experiments were approved by the Ethics Committee for Animal Experiments of Leiden University and carried out at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws and the Directive 2010/63/EU of the European Parliament.

Transgenic mice expressing the *Del1-9-G129R-hPRL* gene under control of the ubiquitous metallothionein I promoter (Rouet *et al.* 2010) and non-transgenic littermate controls were bred at Inserm (Paris, France) and subsequently used as donors for bone marrow transplantation. Genetically hyperlipidemic homozygous C57BL/6J *Ldlr* knockout mice (Ishibashi *et al.* 1993) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) as mating pairs and bred at the Gorlaeus Laboratories, Leiden, The Netherlands. Age-matched *Ldlr* knockout mice served as bone marrow recipients. As prolactin can influence the secretion of female-specific hormones (i.e., estrogens) and *vice versa*, male mice were chosen as recipients to minimize the covariable effects of other hormones on atherosclerosis. Lethally irradiated recipients (2×4.5 Gy; 11–12 mice per group) received 5×10^6 bone marrow cells by i.v. injection into the tail vein 1 day after irradiation. After a recovery period of 8 weeks on a regular chow diet containing 4.3% fat and no cholesterol, the animals were challenged with a Western-type diet (WTD; 0.25% cholesterol and 15% cocoa butter) for 12 weeks to induce atherosclerotic lesion development. At 20 weeks after transplantation, mice were anaesthetized by s.c. injection with a mixture of 70 mg/kg body weight of xylazine, 1.8 mg/kg body weight of atropine, and 350 mg/kg body weight of ketamine. Animals were subsequently killed by cervical dislocation.

Genotyping

DNA was extracted from the bone marrow and spleen of different recipient mice. Genotyping was performed essentially as described previously (Rouet *et al.* 2010).

Analysis of gene expression by real-time quantitative PCR

Quantitative gene expression analysis on snap-frozen liver and spleen was performed as described previously (Hoekstra *et al.* 2003). Total RNA was isolated according to Chomczynski & Sacchi (1987) and reverse transcribed using RevertAid reverse transcriptase. Gene expression analysis was performed using real-time SYBR Green Technology (Eurogentec, Seraing, Belgium). Primers were validated for identical efficiencies and the primer sequences are given in Table 1. Beta-actin (*Actb*), ATP synthase, H⁺ transporting, mitochondrial F0 complex, subunit F6 (*Atp5j*), beta-glucuronidase (*Gusb*), and acidic ribosomal phosphoprotein P0 (*36B4* (*Rplp0*)) were used as the standard housekeeping genes.

Plasma measurements

Plasma prolactin levels were measured using a mouse prolactin-specific ELISA kit (Calbiotech, Inc., Spring Valley, CA, USA). Corticosterone levels were determined using the CORTICOSTERONE Double Antibody ¹²⁵I RIA Kit obtained from MP Biomedicals (Irvine, CA, USA). Murine monocyte chemoattractant protein 1 (MCP1 (CCL2)) levels were assayed in plasma using a MCP-1 instant ELISA kit (eBioscience, Hatfield, UK) according to the manufacturer's instructions. Concentrations of free and total cholesterol, cholesterol esters, and triglycerides were determined using enzymatic colorimetric assays (Roche Diagnostics). The distribution over the different lipoproteins was analyzed by fractionation of 30 µl of plasma of each mouse using a Superose 6 column (3.2 × 300 mm, Smart-system, Pharmacia).

Histological analysis of the aortic root

To analyze the development of atherosclerosis at the aortic root, the arterial tree was perfused *in situ* with PBS (100 mmHg) for 10 min via a cannula in the left ventricular apex. The heart along with the aortic root were excised and stored in 3.7% neutral buffered formalin (Formal-fixx; Shandon Scientific Ltd, Runcorn, UK). Serial sections (10 µm) of the aortic root were cut using a Leica CM3050S cryostat. The atherosclerotic lesion areas in Oil red O-stained cryostat sections of the aortic root were quantified using the Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and the Leica Qwin Imaging software (Leica Ltd, Cambridge, UK). Mean lesion area (in µm²) was calculated from ≥5 consecutive Oil red O-stained sections of the aortic root, starting at the appearance of the tricuspid valves. Collagen content of the lesions was determined after Masson's Trichrome staining (Sigma Diagnostics). Sections were stained immunohistochemically for the presence of macrophages using a MOMA-2 antibody (dilution 1:50; Serotec Ltd, Oxford, UK) respectively. A goat anti-rat antibody coupled to HRP (1:100) (Dako, Glostrup, Denmark) was used as a secondary antibody and Nova red substrate (Vector Laboratories, Burlingame, CA, USA) was used for visualization of HRP.

Hematological analysis

Total leukocyte numbers as well as the absolute number of neutrophils, monocytes, and lymphocytes in whole blood samples with EDTA anticoagulant obtained at the time of killing were routinely measured using an automated

Table 1 Primers used for quantitative real-time PCR

Genes	Accession	Forward primer	Reverse primer
<i>36B4</i>	NM007475	CTGAGTACACCTTCCCACTTACTGA	CGACTCTTCCTTTGCTTCAGCTTT
<i>Actb</i>	NM007393	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
<i>Atp5j</i>	NM016755	CAGGGCCGGAAGTAGAACGG	AAGGACAGAGGAGAGCCTGAAGA
<i>Dgat1</i>	NM010046	GGTGCCCTGACAGAGCAGAT	CAGTAAGGCCACAGCTGCTG
<i>Fasn</i>	NM007988	GGCGGCACCTATGGCGAGG	CTCCAGCAGTGTGCGGTGGTC
<i>Gusb</i>	NM010368	TATGGAGCAGACGCAATCCAG	AGCTCTCCGACCACGTATTCTTTAC
<i>Hl</i>	NM008280	AGAGCCTACTTTTACCATGTCGCT	CGGGATGTCTTTGTCCAGTGTG
<i>Ifnγ</i>	NM008337	ATAACTATTTTAACTCAAGTGGCATAGATGT	TCT GGC TCT GCA GGA TTT TCA
<i>Lpl</i>	NM008509	CCCCTAGACAACGTCCACCTC	TGGGGGCTTCTGCATACTCAAA
<i>Lrp1</i>	NM008512	CTTCTGGTGGCTGGCGTGGTG	CATCCGCTGGTGTGGAAGCC
<i>Mcp1</i>	NM011333	CTGAAGCCAGCTCTCTTCTCTC	CCTCAATGAGTAGCAGCAGGTGA
<i>Scd1</i>	NM009127	TACTACAAGCCCGGCTCC	CAGCAGTACCAGGGCACCA
<i>SRBI</i>	NM016741	AAACAGGGAAGATCGAGCCAGTAG	CGTAGTGAAGAACCTGGGGCAT
<i>Tnfα</i>	X02611	GCCTCTTCTCATTCTGCTTGTG	ATGATCTGAGTGTGAGGGTCTGG

Figure 1

Successful introduction of the prolactin antagonist Del1-9-G129R-hPRL by bone marrow transplantation. PCR on genomic DNA of bone marrow and spleens (A) and gene expression analysis on livers and spleens (B) isolated from LDL receptor knockout mice transplanted with bone marrow from Del1-9-G129R-hPRL transgenic (TG/Del1-9-G129R-hPRL) mice and their non-transgenic controls (WT/Control) at 20 weeks after bone marrow transplantation. Data in (B) are expressed as means $\pm$ s.e.m. of 5–6 mice per group. ND, not detected.

Sysmex XT-2000iV Veterinary Hematology analyzer (Sysmex Corporation, Etten-Leur, The Netherlands). Glucose levels were measured in blood drops using ACCU-CHEK compact strips.

Statistical analysis

Statistical analysis was performed using the Graphpad Instat software (San Diego, CA, USA, <http://www.graphpad.com>). Normality of the experimental groups was confirmed using the method of Kolmogorov and Smirnov. The significance of differences was calculated using a two-tailed unpaired *t*-test. Probability values <0.05 were considered significant.

Results

Bone marrow transplantation induces detectable expression of Del1-9-G129R-hPRL

Our previous studies in which we transplanted mice with bone marrow from transgenic/knockout mice have shown the value of bone marrow transplantation to uncover the effect of secreted peptides/proteins on atherosclerosis susceptibility (Van Eck *et al.* 1997, 2007, Vikstedt *et al.* 2007). We therefore transplanted lethally irradiated *Ldlr* knockout mice with bone marrow from either Del1-9-G129R-hPRL transgenic mice or their non-transgenic littermate controls to induce long-term (constitutive) expression of the prolactin receptor antagonist and show the potential of prolactin receptor antagonism to reduce the atherosclerosis risk. At the time

of killing, genotyping was performed on bone marrow and spleen isolated to assess that the Del1-9-G129R-hPRL sequence was present only in the DNA of mice transplanted with the transgenic donor bone marrow (Fig. 1A). WT bone marrow recipients showed undetectable gene expression of Del1-9-G129R-hPRL ($C_t \geq 40$) in their liver and spleen, two tissues rich in bone marrow-derived cells. By contrast, mRNA expression of *Del1-9-G129R-hPRL* could be readily detected in spleens ($C_t = 30.1 \pm 0.5$; $n = 6$) and, to a lesser extent, in livers ($C_t = 35.2 \pm 2.0$; $n = 5$) of transgenic bone marrow recipients (Fig. 1B). Endogenous (mouse) prolactin levels in plasma were not different between the two experimental groups (Table 1). Owing to the mutations harbored by *Del1-9-G129R-hPRL* to convert prolactin into a functional antagonist, the latter is not recognized by human prolactin immunoassays that have been tested thus far (data not shown). As a sensitive assay to detect this antagonist remains yet to be developed, we were unable to determine the actual levels of Del1-9-G129R-hPRL protein in the plasma of the bone marrow recipient mice.

Del1-9-G129R-hPRL expression in bone marrow is associated with a decrease in the plasma atherogenic index

Prolactinoma patients suffer from dyslipidemia. Lowering their circulating prolactin levels using dopamine agonists was shown to decrease plasma LDL cholesterol levels (Fahy *et al.* 1999, Berinder *et al.* 2011). To determine whether such a relation between prolactin signaling and cholesterol levels also existed in our experimental mouse

Figure 2

Body weight profile and blood glucose levels in Del1-9-G129R-hPRL transgenic bone marrow-transplanted LDL receptor knockout mice and their non-transgenic littermate bone marrow-transplanted controls. Body weight was recorded at the indicated points after the bone marrow transplantation (BMT; A). Glucose levels were determined in blood collected from 12-week Western-type-diet-fed mice after 4 h of fasting (B). Data are expressed as means $\pm$ s.e.m. of 11–12 mice per group.

Table 2 Effect of Del1-9-G129R-hPRL expression on plasma parameters. Data are expressed as means $\pm$ S.E.M.

	Control (n=11)	Del1-9-G129R- hPRL (n=12)	P value
Chow diet			
Triglycerides (mg/dl)	162 $\pm$ 9	155 $\pm$ 7	0.55
Total cholesterol (mg/dl)	250 $\pm$ 10	252 $\pm$ 7	0.89
Western-type diet			
Triglycerides (mg/dl)	498 $\pm$ 67	457 $\pm$ 57	0.64
Total cholesterol (mg/dl)	1788 $\pm$ 173	1262 $\pm$ 164	0.038
Free cholesterol (mg/dl)	796 $\pm$ 67	642 $\pm$ 52	0.082
Cholesterol esters (mg/dl)	1676 $\pm$ 193	891 $\pm$ 133	0.003
Prolactin (OD)	0.091 $\pm$ 0.008	0.101 $\pm$ 0.006	0.35

model, we measured plasma lipid levels in bone marrow-transplanted mice both after 8 weeks recovery on a chow diet and after subsequent WTD feeding for 12 weeks. No apparent changes in food consumption were noted. In accordance with similar food intake, body weights as well as blood glucose levels were identical in the two transplant groups (Fig. 2). No difference in plasma total cholesterol or triglyceride levels was noted with respect to bone marrow genotype while feeding a chow diet (Table 2). By contrast, under WTD feeding conditions, plasma total cholesterol levels were significantly lowered (-29% ; $P<0.05$) in Del1-9-G129R-hPRL bone marrow-transplanted mice when compared with WT bone marrow recipients (Table 2). Triglyceride levels were also not different between the two groups upon feeding the WTD, indicative of a similar intestinal lipid absorption rate. Further specification of the effect on plasma cholesterol levels indicated that the decrease in total cholesterol levels observed on the WTD was mainly due to a marked reduction (47% , $P<0.01$) in the plasma cholesterol ester concentration, as free cholesterol levels only tended to decrease (-19% ; $P=0.08$). Lipoprotein distribution analysis on pooled plasma revealed that Del1-9-G129R-hPRL exposure reduced the atherogenic index of the plasma compartment. The level of cholesterol associated with pro-atherogenic lipoproteins VLDL and LDL was decreased, while anti-atherogenic HDL cholesterol levels were similar between the two groups of mice (Fig. 3). The decrease in VLDL/LDL cholesterol levels could not be attributed to changes in hepatic expression levels of key lipogenic genes (*Fasn*, *Dgat1*, and *Scd1*). In addition, relative mRNA

expression levels of proteins associated with the catabolism of triglyceride-rich VLDL particles (LPL and HL) and the uptake of smaller sized LDL particles by the liver (LRP1 (LIPC) and SRBI (SCARB1)) were not significantly different upon Del1-9-G129R-hPRL exposure (Table 3).

Del1-9-G129R-hPRL expression in bone marrow does not affect the initial development of atherosclerotic lesions

Mice transplanted with WT bone marrow developed macrophage-rich early atherosclerotic lesions in the aortic root, which were almost devoid of collagen ($<2\%$; Fig. 4) in response to 12 weeks of WTD feeding. In accordance with an initial stage of lesion development, no atherosclerotic plaques (white lipid deposits) were microscopically visible in the aortic arch and descending aorta in either transplant groups upon killing. Strikingly, the size of the atherosclerotic lesions in the aortic root was not different in Del1-9-G129R-hPRL recipients ($n=10$, mean lesion size: $223 \pm 33 \times 10^3 \mu\text{m}^2$) when compared with the WT bone marrow-transplanted mice ($n=11$, $259 \pm 32 \times 10^3 \mu\text{m}^2$). Lesional macrophage and collagen contents did not differ

Figure 3

Lipoprotein profiles of Del1-9-G129R-hPRL transgenic bone marrow-transplanted LDL receptor knockout mice and their non-transgenic littermate-transplanted controls. Pooled plasma, collected in the *ad libitum* fed state after 12 weeks of WTD feeding, was subjected to fast performance liquid chromatography. Distribution of free cholesterol, cholesterol esters, and triglycerides over the different lipoprotein fractions was determined enzymatically.

Table 3 Effect of Del1-9-G129R-hPRL expression on hepatic gene expression levels. Given the high sensitivity of the quantitative real-time PCR method, five out of six livers were randomly chosen from the two groups of mice for biochemical analysis. Data are expressed as fold change compared with control values and represent means $\pm$ S.E.M.

	Control (n=6)	Del1-9-G129R- hPRL (n=5)	P value
<i>Dgat1</i>	1.00 $\pm$ 0.11	1.27 $\pm$ 0.15	0.22
<i>Fasn</i>	1.00 $\pm$ 0.18	1.13 $\pm$ 0.14	0.49
<i>Hl</i>	1.00 $\pm$ 0.09	1.04 $\pm$ 0.10	0.75
<i>Lpl</i>	1.00 $\pm$ 0.12	1.30 $\pm$ 0.09	0.07
<i>Lrp1</i>	1.00 $\pm$ 0.14	0.96 $\pm$ 0.09	1.00
<i>Scd1</i>	1.00 $\pm$ 0.17	0.97 $\pm$ 0.32	0.66
SCARB1	1.00 $\pm$ 0.25	0.91 $\pm$ 0.19	0.94

between the two experimental groups (Fig. 4), which suggests that the stability of the atherosclerotic lesions was also not affected by prolactin receptor antagonism. It thus appears that the lowered atherogenic index in Del1-9-G129R-hPRL bone marrow-transplanted mice does not translate into an altered susceptibility for the development of initial atherosclerotic lesions.

Del1-9-G129R-hPRL expression in bone marrow is associated with increased blood leukocyte counts

As inflammation is a key feature in the pathogenesis of atherosclerosis, we determined whether introduction of Del1-9-G129R-hPRL in our *Ldlr* knockout mice was associated with a change in immune status. Splenic mRNA expression levels of the pro-inflammatory cytokines *Mcp1*, tumor necrosis factor alpha (*Tnf α* (*Tnf*)), and interferon gamma (*Ifn γ* (*Ifng*)) were not significantly altered by Del1-9-G129R-hPRL treatment (Table 4). In parallel, plasma protein levels of MCP1 were not notably different (Fig. 5A). By contrast, Del1-9-G129R-hPRL application induced a significant change in the concentration of circulating white blood cells. Total leukocyte numbers were 49% higher ($P < 0.01$) in Del1-9-G129R-hPRL bone marrow recipients, which could be attributed to an increase in concentrations of neutrophils (+91%; $P < 0.05$), lymphocytes (+55%; $P < 0.05$), and monocytes (+43%; $P < 0.05$) (Fig. 6). Previous studies have suggested a possible effect of prolactin on the secretion of anti-inflammatory glucocorticoids by the adrenals (Chang *et al.* 1999, Lo & Wang 2003, Dugan *et al.* 2007, Jaroenporn *et al.* 2007). However, plasma levels of corticosterone, the primary glucocorticoid circulating in mice, were not different between the two treatment groups (Fig. 5B).

Discussion

As a clinical association between prolactin levels and the risk of cardiovascular disease exists, we evaluated the impact of treatment with the prolactin receptor antagonist Del1-9-G129R-hPRL on atherosclerosis susceptibility in the commonly used *Ldlr* knockout disease mouse model. Bone marrow-derived Del1-9-G129R-hPRL lowered the atherogenic index of the plasma compartment as measured by a decrease in VLDL/LDL cholesterol levels. Although it is recognized that lowering the prolactin levels by bromocriptine treatment executes a beneficial impact on plasma cholesterol levels in hyperprolactinemic patients (Fahy *et al.* 1999, Berinder *et al.* 2011); to our knowledge this drug has not been tested in the experimental mouse model used in this study. Our findings suggest that lowering prolactin signaling by receptor targeting may constitute an alternative therapeutic approach to lower the plasma levels of pro-atherogenic lipoproteins also in the general normo-prolactinemic population.

Effective retrospective analysis of the relation between plasma lipid parameters and atherosclerosis susceptibility by VanderLaan *et al.* (2009) has indicated that, in *Ldlr* knockout mice, VLDL cholesterol levels and the non-HDL cholesterol to HDL cholesterol ratio served as the two best predictors of the aortic root atherosclerosis extent.

Figure 4 Analysis of atherosclerosis in the aortic root of bone marrow-transplanted LDL receptor knockout mice after 12 weeks of WTD feeding. Sections of the aortic root area were stained with Oil red O to visualize the total lesion area. MOMA-2 and trichrome stainings were used to show the presence of macrophages (red) and collagen (blue) respectively. Bullets in the graphs on the right represent the values of individual mice, while horizontal lines indicate the group averages $\pm$ S.E.M.

Table 4 Effect of Del1-9-G129R-hPRL expression on splenic gene expression levels. Given the high sensitivity of the quantitative real-time PCR method, six spleens were randomly chosen from the two groups of mice for biochemical analysis. Data are expressed as fold change compared with control values and represent means $\pm$ S.E.M.

	Control (n=6)	Del1-9-G129R-hPRL (n=6)	P value
<i>Mcp1</i>	1.00 $\pm$ 0.12	1.11 $\pm$ 0.13	0.63
<i>Tnfα</i>	1.00 $\pm$ 0.39	0.58 $\pm$ 0.08	0.63
<i>Ifnγ</i>	1.00 $\pm$ 0.20	1.15 $\pm$ 0.24	0.74

Strikingly, the lowered VLDL/LDL cholesterol levels in the context of unchanged HDL cholesterol levels associated with Del1-9-G129R-hPRL treatment did not translate into a lower susceptibility for the development of atherosclerotic lesions. An apparent discrepancy between VLDL cholesterol levels and the extent of atherosclerosis thus exists in the *Ldlr* knockout mice model in our current experimental setting.

Blood leukocyte concentrations were markedly increased in Del1-9-G129R-hPRL bone marrow recipients when compared with *Ldlr* knockout mice transplanted with WT bone marrow. Importantly, multiple clinical studies have indicated that higher blood leukocyte numbers are an independent risk factor for cardiovascular disease (Tong *et al.* 2004, Jee *et al.* 2005, Twig *et al.* 2012, Hou *et al.* 2013). Thus, we hypothesize that the pro-atherogenic increase in leukocyte counts may balance the beneficial effect of Del1-9-G129R-hPRL-induced cholesterol lowering on atherosclerosis outcome.

We attempted to understand the mechanism by which the pure PRLR antagonist could increase leukocyte populations. Given that we used a bone marrow transplantation approach in which recipient mice are provided with the immune system of donor mice, a non-specific inflammatory (host vs graft) response to the antagonist can be excluded, in particular in light of the effective repopulation of leukocyte-rich organs such as the spleen. It is therefore assumed that the effect on leukocyte populations can directly be attributed to the prolactin receptor antagonism.

MCP1, the ligand for the CC-chemokine receptor CCR2, drives the accumulation of monocytes in the arterial sub-endothelial space. In accordance with an essential role for the MCP1/CCR2 axis in the development of atherosclerotic lesions, disruption of either MCP1 or CCR2 function lowers the extent of atherosclerosis in *Ldlr* knockout mice (Gu *et al.* 1998) as well as in

atherosclerosis-susceptible *Apoe* knockout mice (Boring *et al.* 1998, Ni *et al.* 2001). As no changes in the plasma levels of MCP1 were noted, we anticipate that an increased mobilization of cells from bone marrow does not represent the underlying cause of the leukocytosis observed in Del1-9-G129R-hPRL-expressing mice.

Endogenous glucocorticoids, i.e., corticosterone in rodents and cortisol in humans, are some of the most potent immunosuppressive molecules as they inhibit the proliferation of lymphocytes and induce cell death in multiple white blood cell lineages. Accordingly, disruption of the endogenous glucocorticoid function through adrenalectomy was shown to be associated with an increased blood leukocyte concentration both in normolipidemic WT mice and *Ldlr* knockout and *Apoe* knockout hyperlipidemic mice (van der Sluis *et al.* 2012, Hoekstra *et al.* 2013). In our study, we failed to observe a change in plasma corticosterone levels between the two groups of transplanted mice at the time of killing, which does not argue for a major role for the glucocorticoid system.

In mice, the majority of circulating white blood cells as well as all lymphoid tissues express the prolactin receptor (Dardenne *et al.* 1994, Touraine & Kelly 1995). Of note, PRLR protein expression has also been detected on macrophages locally within human atherosclerotic lesions (Reuwer *et al.* 2011). Thus, prolactin can also directly modulate the immune function. Although total

Figure 5 Plasma levels of (A) MCP1 and (B) corticosterone in Del1-9-G129R-hPRL transgenic bone marrow-transplanted LDL receptor knockout mice and their non-transgenic littermate-transplanted controls. Plasma samples were collected in the *ad libitum* fed state after 12 weeks of WTD feeding. Data are expressed as means $\pm$ S.E.M. of six (MCP1) and 11–12 (corticosterone) mice per group.

Figure 6

Leukocyte concentrations in the blood of Del1-9-G129R-hPRL transgenic bone marrow-transplanted LDL receptor knockout mice and their non-transgenic littermate-transplanted controls. Blood samples were collected after 12 weeks of WTD feeding. Data are expressed as means $\pm$ S.E.M. of 11–12 mice per group. * $P < 0.05$, ** $P < 0.01$ (t-test).

abolition of prolactin receptor signaling failed to affect immune cell development or function, which may reflect cytokine receptor signaling redundancy (Bouchard *et al.* 1999), many studies have shown that (over-)stimulation of PRLR signaling impacted on the immune response. For example, hyperprolactinemia exacerbates *Salmonella enterica* infection-related pro-inflammatory cytokine secretion in BALB/c mice (Meli *et al.* 2003). Furthermore, expressions of co-stimulatory molecules and cytokine secretion by splenic dendritic cells of BALB/c mice are induced by prolactin (Yang *et al.* 2006). Moreover, persistently elevated serum prolactin levels interfere with B cell tolerance induction in BALB/c mice (Saha *et al.* 2009). However, it appears that the impact of the prolactin/prolactin receptor axis on immune function is highly dependent on the genetic background. Accordingly, high levels of prolactin underlie a break in B cell tolerance and cause a lupus-like phenotype in R4A- γ 2b BALB/c mice but not in R4A- γ 2b C57BL/6 mice (Peeva *et al.* 2003). In our study, while the *Ldlr* knockout recipients were fully (greater than ten times) backcrossed onto the C57BL/6 background, Del1-9-G129R-hPRL bone marrow donors were on a mixed background (BALB/c/C57BL/6). However, given the fact that we used non-transgenic littermates as control donors, a difference in genetic background can be ruled out as the reason for the unanticipated Del1-9-G129R-hPRL-induced increase in leukocyte numbers.

Most evidences regarding the pro-inflammatory effect of prolactin are derived from experimental settings in which the effect of relatively high levels of prolactin (hyperprolactinemia) on immune function has been evaluated. Importantly, our prolactin receptor antagonist studies were carried out in a normoprolactinemic setting.

As Del1-9-G129R-hPRL was shown to be devoid of any residual agonism toward the mouse prolactin receptor (Bernichtein *et al.* 2003, Rouet *et al.* 2010), any increase in PRL-like activity in transplanted mice is unlikely. It can therefore be anticipated that a u-shaped relationship between prolactin levels/action and inflammation status may exist. However, additional studies in other experimental inflammation-related, i.e., endotoxemia and sepsis, mouse models are warranted to prove such a relation and suggest the potential relevance for the application of prolactin receptor antagonists in the human situation.

In conclusion, our studies show that prolactin receptor signaling inhibition uncouples the plasma atherogenic index from atherosclerosis susceptibility in *Ldlr* knockout mice. Despite an associated decrease in VLDL/LDL cholesterol levels, application of the prolactin receptor antagonist Del1-9-G129R-hPRL does not alter the susceptibility for atherosclerotic lesion development probably due to the parallel increase in circulating leukocyte concentrations.

Declaration of interest

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Author contribution statement

R J van der S, T van den A, and F B executed the experiments and performed data analysis. A Q R, M T T, and M V E supplied essential scientific input into the project. V G and M H initiated and supervised the experimental work and wrote the manuscript.

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