

# The prolactin receptor is expressed in macrophages within human carotid atherosclerotic plaques: a role for prolactin in atherogenesis?

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## Abstract

Atherosclerotic vascular disease is the consequence of a chronic inflammatory process, and prolactin has been shown to be a component of the inflammatory response. Additionally, recent studies indicate that prolactin contributes to an atherogenic phenotype. We hypothesized that this may be the result of a direct effect of prolactin on atherogenesis through activation of the prolactin receptor. Human carotid atherosclerotic plaques were obtained from patients by endarteriectomies. The mRNA of prolactin receptor, but not of prolactin, was detected in these atherosclerotic plaques by quantitative real-time PCR. *In situ* hybridization confirmed the expression of

the prolactin receptor in mononuclear cells. Analysis at the protein level using immunohistochemistry and immunoelectron microscopy revealed that the prolactin receptor was abundantly present in macrophages near the lipid core and shoulder regions of the plaques. Our findings demonstrate that the prolactin receptor is present in macrophages of the atherosclerotic plaque at sites of most prominent inflammation. We therefore propose that prolactin receptor signaling contributes to the local inflammatory response within the atherosclerotic plaque and thus to atherogenesis.

*Journal of Endocrinology* (2011) **208**, 107–117

## Introduction

For decades, lipid accumulation in the arterial wall has been considered to form the core of the pathophysiology of atherosclerosis (Kottke 1986). Currently, however, it is recognized that low-grade chronic inflammation, characterized by inflammatory cell infiltration, accumulation of heterogeneous macrophage populations, T-cell activation, cytokine production, and cell death, is also an integral part of the atherosclerotic process (Packard & Libby 2008). In fact, all stages of atherogenesis are currently thought to be mediated through inflammatory pathways (Rocha & Libby 2009).

Besides this low-grade chronic inflammation, various hormonal disturbances have been shown to be associated with cardiovascular disease (van Zaane *et al.* 2009). Whether prolactin is involved in the pathogenesis of human atherosclerosis and its atherothrombotic complications is currently the focus of our research (Reuwer *et al.* 2009a,b). Recent observations suggest that prolactin, either in excess or at physiological levels, may contribute to an atherogenic phenotype. This is based on the finding that hyperprolactinemia is associated with impaired endothelial function, decreased insulin sensitivity as well as with low-grade

inflammation (Yavuz *et al.* 2003, Serri *et al.* 2006, Dos Santos Silva *et al.* 2010). In addition, high prolactin levels, but still within the physiological range, were found to be correlated to arterial blood pressure, which is a recognized risk factor for cardiovascular disease (Stumpe *et al.* 1977, Georgiopoulos *et al.* 2009, Zhang *et al.* 2010). Last and most importantly, prolactin has been shown to be correlated to a risk score that predicts 10-year cardiovascular mortality ( $P=0.002$ ; Georgiopoulos *et al.* 2009).

The principal function of prolactin is to ensure lactation (Horseman 1999). Its physiological function outside pregnancy and the lactation period in humans is yet to be clarified. Animal and cell studies have shown that prolactin is a pleiotropic hormone that affects various reproductive and metabolic, but also inflammatory pathways (Chikanza & Grossman 1996, Ben-Jonathan *et al.* 2008). In fact, prolactin is widely recognized as an important physiological modulator of the immune response (Dorshkind & Horseman 2000). For instance, prolactin stimulates T-cell proliferation (Clevenger *et al.* 1990), and it supports interferon- $\gamma$  production (Schwarz *et al.* 1992). Furthermore, prolactin has been shown to be involved in regulating monocyte/macrophage function *in vitro* (Aziz *et al.* 2008, Carvalho-Freitas *et al.* 2008).

Prolactin is a 23-kDa hormone that binds to the prolactin receptor, which is a member of the cytokine receptor superfamily, and exerts its action via several interacting signaling pathways (Ben-Jonathan *et al.* 2008). Various isoforms of the prolactin receptor have been identified in several species as a result of alternative splicing. In humans, they mainly differ by their cytoplasmic domains, and with the exception of  $\delta$  S1 isoform, they share an identical extracellular domain (Kline *et al.* 2002, Ben-Jonathan *et al.* 2008).

In addition to being a classical pituitary-secreted endocrine hormone, prolactin is also produced by many extra-pituitary tissues (Ben-Jonathan *et al.* 2008). For instance, human lymphocytes synthesize and secrete prolactin (Pellegrini *et al.* 1992, Sabharwal *et al.* 1992), and express cell surface prolactin receptors (Bresson *et al.* 1999).

The clinical observations which indicate that prolactin may contribute to an atherogenic phenotype led us to hypothesize that prolactin may have a direct effect on atherogenesis through activation of the prolactin receptor. Therefore, we decided to evaluate whether carotid atherosclerotic plaques express the prolactin receptor, and whether this could be validated using *in situ* hybridization (ISH) and immunohistochemistry (IHC). Secondly, we aimed to elucidate whether the ligand prolactin is expressed within the atherosclerotic plaque itself. The expression of prolactin and of its receptor in carotid atherosclerotic plaques, as obtained by endarterectomies, was investigated using quantitative real-time PCR. In addition, ISH, IHC, and immunoelectron microscopy were used to investigate the localization of the prolactin receptor within the plaque.

## Materials and Methods

### *Isolation of carotid atherosclerotic arteries and placenta*

Carotid atherosclerotic plaques from male and female patients were obtained by endarterectomies. The aim of carotid endarterectomy is to prevent the adverse sequelae of carotid artery stenosis secondary to atherosclerotic disease (i.e. stroke). In this procedure, the internal, common, and external carotid arteries are clamped, the lumen of the internal carotid artery is opened, and the atheromatous plaque substance (waste material) is removed. Fresh atheromatous plaque substances were used for quantitative real-time PCR (ultimately  $n=5$  donors) and ISH ( $n=2$  donors). The plaques from which RNA was isolated were freshly collected at the operation room within 30 min after endarterectomy. Part of the tissue was snap frozen in liquid  $N_2$  and was subsequently stored at  $-80^\circ C$ . The other part of the tissue was fixed in formalin. Carotid artery fragments used for IHC were obtained between 1995 and 1998 after endarterectomy, and were routinely fixed in buffered formalin and were paraffin embedded. These atherosclerotic plaques were obtained from 25 patients (9 female and 16 male patients).

Human placental tissue was used as positive control for prolactin receptor expression in quantitative real-time PCR, ISH, and IHC experiments. This material was freshly obtained from the obstetric unit (waste material) and either kept deep frozen ( $-80^\circ C$ ) or embedded in paraffin following routine formalin fixation, depending on the experiment. Both placental and human pituitary tissues served as positive controls for prolactin expression in quantitative real-time PCR experiments. Pituitary tissue was obtained from The Netherlands Brain Bank (NBB), Netherlands Institute for Neuroscience, Amsterdam, The Netherlands. All materials have been collected from donors from whom a written informed consent for brain autopsy and for the use of the material as deep-frozen ( $-80^\circ C$ ) samples and clinical information for research purposes had been obtained by the NBB.

### *RNA extraction and quantitative real-time PCR*

The plaques that were used for this study were collected within a time frame of 10 months and were stored at  $-80^\circ C$  for not more than 10 months before RNA was extracted. Total RNA was extracted from 10- $\mu m$  thick cryosections of placenta, pituitary, and carotid plaques using TRIzol reagent (Invitrogen), followed by further extraction using the NucleoSpin RNA II kit according to the manufacturer's recommendations (Macherey-Nagel GmbH, Duren, Germany). This protocol included incubation with RNase-free DNase. RNA concentrations were measured using the Nanodrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Atherosclerotic plaques contain variable amounts of necrotic tissue, and endogenous RNA disintegration is expected to occur beyond the control of the investigator. For 11 atherosclerotic plaques, RNA yield was measured by optical density (OD) at 260 nm, RNA purity was assessed by determining the OD 260/280 nm ratio (NanoDrop Spectrophotometer), and RNA integrity was investigated by assessing the RIN score, using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). **Supplementary Table 1**, see section on **supplementary data** given at the end of this article, shows the RNA yield, RNA purity, and RIN score for each plaque. For some plaques, RIN scores were not computed by the bioanalyzer because of anomalies of baseline and in the 5S-region, indicating degradation of the RNA. We decided to select the best samples available: the five included plaques had a 260/280 ratio around 2, and the RNA yield ranged from 30 to 115 ng/ $\mu l$ . In addition, RIN scores for these samples varied between 5.5 and 7.0. Equal amounts of RNA were used to synthesize cDNA, using oligo-(dT)<sub>12-18</sub> and random hexamers as primers, and Superscript II reverse transcriptase, according to the manufacturer's method (Invitrogen). cDNA derived from atherosclerotic plaques was diluted 10 $\times$ , while cDNA from the placenta and pituitary was diluted 20 $\times$ , prior to gene-specific analysis performed on an iCycler MyiQ single-color real-time PCR detection system using the iQ SYBR Green Supermix

(Bio-Rad Laboratories). Expression levels were normalized to those of TATA box binding protein (TBP), porphobilinogen deaminase (PBGD), and hypoxanthine-guanine phosphoribosyltransferase (HPRT). The following primer sequences were used to detect these reference genes: TBP forward primer 5'-GTTCCAGCGCAAGGGTTTCTGGT-3', reverse primer 5'-GAGTCATGGCACCTGAGGGGAG-3' (NM\_003194.4; 234–324); PBGD forward primer 5'-CGGCACCCACACACAGCCTA-3', reverse primer 5'-CCGTCTGTATGCCAGCAAGCTGG-3' (NM\_000190.3; 122–241); HPRT forward primer 5'-TGC-TGGTGAAAAGGACCCACAG-3', reverse primer 5'-TG-GCGATGTCAATAGGACTCCAGA-3' (NM\_000194.2; 655–877). Alternative splicing within the coding region of the prolactin receptor gene yields mRNA isoforms that, for the majority, differ in length of the cytoplasmic domain, i.e. long (NM\_000949), intermediate (AF166329), short 1A (AF416619), short 1b (AF416618), short 1c (GU133399),  $\delta$  S1 (AF349939),  $\delta$  4-SF1b (AF493069),  $\delta$  7/11 (AF4924470), and  $\delta$  4–7/11 (AF493068). Four primer sets were designed to enable detection of all these splice variants, set A forward primer 5'-TCATGATGGTCAATGCCACT-3', reverse primer 5'-GCGTGAACCAACCAGTTTTT-3' (NM\_000949; 829–1007); set B forward primer 5'-CAGCACAACCCCAgATCCTC-3', reverse primer 5'-GGCGTATCCTGGT-CAGTCTC-3' (NM\_000949; 1797–1983); set C forward primer 5'-TGCAAACCAGACCATGGATA-3, reverse primer 5'-GGAAAGATGCAGGTACCAT-3' (NM\_000949; 1152–1317); and set D forward primer 5'-TTCTCAACA-CCTGCCTTCTGAATGTT-3', reverse primer 5'-CCCA-CTCAGCTGCTTTCTCGGGT-3' (AF349939; 47–238). Primer sets A–D are able to detect the various prolactin receptor mRNA splice variants as detailed in Table 1. In addition, the following primers were used: CD68 forward primer 5'-GCTGGCTGTGCTTTTCTCG-3', reverse primer 5'-GTCACCGTGAAGGATGGCA-3' (NM\_001251; 197–307); CCL18 forward primer 5'-CCCAGCTCACT-CTGACCACT-3', reverse primer 5'-GTGGAATCTG-CCAGGAGGTA-3' (NM\_002988; 22–182); TNF $\alpha$  forward primer 5'-GGCGTGGAGCTGAGAGATA-3', reverse primer 5'-CAGCCTTGGCCCTTGAAGA-3' (NM\_000594; 515–603); IL10 forward primer 5'-TGCCTCAGC-AGAGTGAAGACTT-3', reverse primer 5'-TCCTCC-AGCAAGGACTCCTTTA-3' (NM\_000572; 197–277).

Primers were purchased from Biogio (Nijmegen, The Netherlands). Data were analyzed using LinRegPCR software, version 11.6.0.0 (<http://LinRegPCR.nl>) to correct for baseline fluorescence, to estimate the PCR efficiency per amplicon (observed PCR efficiencies for each primer set were between 1.7 and 1.9), and to calculate starting concentrations per sample (data not shown) (Ruijter *et al.* 2009). We specifically controlled for possible contamination with genomic DNA by applying PCR using primers that were designed to detect a region in chromosome 11, located in the 5' upstream sequence adjacent to the first exon of the *PBGD* gene and not being part of the transcribed sequence of any known gene (forward primer 5'-ACAGCTGGGGAATGGGGTGGT-3', reverse primer 5'-GAGGTCCCTCCCTCTGGGCG-3' (NC\_000011.9; 118955492–118955569)). PCR for this region of chromosome 11 yielded the expected 78-bp amplicon on genomic DNA extracted from a colon carcinoma tissue sample. On the other hand, PCR with this primer set on any of the cDNA samples that were prepared from RNA isolated from placenta, pituitary, or plaque tissue did not yield a significant signal (data not shown). In addition, during cDNA synthesis negative control samples were generated in the absence of the Superscript II enzyme (–RT samples). These samples did not show a signal in our PCR analysis with the use of three different primer sets (PRLR A, PRL, and HPRT). We therefore conclude that the RNA samples were not contaminated by genomic DNA.

#### Preparation of digoxigenin-labeled cDNA probes and ISH

A human prolactin receptor-specific digoxigenin (DIG)-labeled riboprobe for ISH was prepared. Briefly, cDNA was prepared on total RNA extracted from human placenta using random hexamer priming. A 461-bp cDNA fragment (NM\_000949; 750–1210) was amplified by PCR using 5'-ATTTAGGTGACACTATAGAATGTCCAGACTACA-TAACC-3' and 5'-TAATACGACTCACTATAGGGTATC-TGAATGAAGGTCGCT-3' as forward and reverse primers, of which the underlined regions include SP6 and T7 RNA polymerase binding sequences respectively. To enable detection of the majority of splicing variants, the primers that we used to develop probes for ISH are specific for exon 5 (forward primer) and exon 7 (reverse primer) of the prolactin receptor gene (Ben-Jonathan *et al.* 2008). PCR products were cloned and verified for sequence fidelity. *In vitro* transcription

**Table 1** Four primer sets were designed to enable detection of all nine known human prolactin receptor splicing variants

| Primer sets | Prolactin receptor isoforms |              |          |          |          |             |                 |               |                 |
|-------------|-----------------------------|--------------|----------|----------|----------|-------------|-----------------|---------------|-----------------|
|             | Long                        | Intermediate | Short 1a | Short 1b | Short 1c | $\delta$ S1 | $\delta$ 4-SF1b | $\delta$ 7/11 | $\delta$ 4–7/11 |
| A           | +                           | +            | +        | +        | +        | –           | +               | +             | +               |
| B           | +                           | –            | –        | –        | –        | +           | –               | –             | –               |
| C           | +                           | +            | +        | +        | +        | +           | +               | –             | –               |
| D           | –                           | –            | –        | –        | –        | +           | –               | –             | –               |

of the purified insert as template was performed using SP6 or T7 RNA polymerases and DIG-conjugated UTP (Roche) to produce DIG-labeled sense and antisense riboprobes respectively. Using these probes, ISH was performed as follows: 8- $\mu$ m thick sections from formalin-fixed and paraffin-embedded (FFPE) atherosclerotic carotid sections were deparaffinized, digested with 20  $\mu$ g/ml proteinase K (Roche) for 15 min at 37 °C, treated with 0.2% glycine (Merck) for 5 min, and postfixed with 2% paraformaldehyde (Merck) and 0.1% glutaraldehyde (Merck) in PBS for 10 min. After prehybridization for 1 h at 70 °C, hybridization with 400 ng/ml DIG-labeled riboprobe in hybridization buffer (50% formamide (Merck), 5 $\times$  standard sodium citrate (SSC) (Invitrogen), 1% blocking reagent (Roche), 5 mmol/l EDTA (Sigma), 0.1% Tween-20 (Sigma), 0.1% CHAPS (Sigma), 0.1 mg/ml heparin (BD Biosciences, Alphen aan den Rijn, The Netherlands), and 1 mg/ml yeast tRNA (Invitrogen) was performed at 70 °C O/N. After hybridization, the slides were washed with 2 $\times$  SSC (pH=7), containing 50% formamide at 65 °C, followed by washing with 0.1% Tween-20 in Tris-buffered saline (TBS/T). After blocking non-specific binding sites by 2% blocking reagent (Roche) in TBS/T, sections were incubated with alkaline phosphatase (AP)-conjugated F(ab) fragments of sheep anti-DIG antibody (Roche) at 4 °C for

16 h. The slides were washed with TBS/T and with 0.1 M Tris, pH 9.5, 0.1 M NaCl, 0.05 M MgCl<sub>2</sub>, and 0.1% Tween-20 (NTM/T). Bound AP activity was visualized with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) (Roche), diluted in NTM/T. Subsequently, the slides were rinsed in tap water and mounted with glycerol (Dako, Glostrup, Denmark). All slides were coded and judged by an independent biologist.

#### Immunohistochemistry

Paraffin tissue sections (4  $\mu$ m thick) were cut from placenta and atherosclerotic carotid tissue blocks. Tissue sections were dewaxed in xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked with methanol plus 0.3% peroxide for 20 min at room temperature (Streefkerk *et al.* 1972). Incubation with 10 mM EDTA (pH 9.0) was performed in the PreTreatment Module (PTModule) (Thermo Fisher Scientific/Labvision, Fremont, CA, USA) for 20 min at 98 °C followed by a cooldown to 75 °C. Ultra V Block (Thermo Fisher Scientific/Labvision) was applied for 10 min at RT. After blotting, the monoclonal primary antibody for the prolactin receptor (Galsgaard *et al.* 2009)

**Table 2** Relative mRNA expression of prolactin receptor primer sets A–D and of prolactin in atherosclerotic plaques and in placenta. Expression levels are correlated for those of reference genes PBGD, TBP and HPRT. Data for the first and third columns are presented as median with interquartile range (IQR).

|                   | Relative expression to PBGD in atherosclerotic plaques median (IQR) | Relative expression to PBGD in placenta | Atherosclerotic plaque/placenta ratio for PBGD-corrected expression median (IQR) |
|-------------------|---|---|--|
| <b>Primer set</b> |   |   |  |
| PRLR A            | 0.22 (0.04–0.71)  | 25.44                                   | 0.02 (0.01–0.04)   |
| PRLR B            | 0.56 (0.20–1.10)  | 16.91                                   | 0.01 (0.00–0.04)   |
| PRLR C            | 1.33 (0.56–3.15)  | 43.45                                   | 0.03 (0.01–0.07)   |
| PRLR D            | 0.02 (0.01–0.03)  | 1.22                                    | 0.01 (0.00–0.03)   |
| Prolactin         | 0   | 0.03                                    | 0  |
|                   |   |   |  |
|                   | Relative expression to TBP in atherosclerotic plaques median (IQR)  | Relative expression to TBP in placenta  | Atherosclerotic plaque/placenta ratio for TBP-corrected expression median (IQR)  |
| PRLR A            | 0.06 (0.02–0.12)  | 4.15                                    | 0.05 (0.02–0.06)   |
| PRLR B            | 0.20 (0.07–0.23)  | 2.76                                    | 0.02 (0.01–0.04)   |
| PRLR C            | 0.38 (0.20–0.65)  | 7.09                                    | 0.05 (0.03–0.09)   |
| PRLR D            | 0.01 (0.00–0.01)  | 0.20                                    | 0.03 (0.01–0.04)   |
| Prolactin         | 0   | 0.00                                    | 0  |
|                   |   |   |  |
|                   | Relative expression to HPRT in Atherosclerotic plaques median (IQR) | Relative expression to HPRT in placenta | Atherosclerotic plaque/placenta ratio for HPRT-corrected expression median (IQR) |
| PRLR A            | 0.03 (0.00–0.05)  | 8.13                                    | 0.01 (0.00–0.11)   |
| PRLR B            | 0.04 (0.01–0.93)  | 5.40                                    | 0.00 (0.00–0.01)   |
| PRLR C            | 0.14 (0.02–2.21)  | 13.9                                    | 0.01 (0.00–0.16)   |
| PRLR D            | 0.00 (0.00–0.01)  | 0.39                                    | 0.00 (0.01–0.02)   |
| Prolactin         | 0   | 0.01                                    | 0  |



(mouse IgG2b, clone 1A2B1, Zymed/Invitrogen) was diluted (1:500 v/v) with TBS + 1% BSA (TBS/BSA) and incubated overnight at 4 °C. This antibody reacts with the long form of the human prolactin receptor and may also identify the human prolactin receptor intermediate and  $\delta$  S1 isoforms (product sheet Zymed/Invitrogen).

Both placenta and atherosclerotic plaques were also tested with a negative isotype control antibody (mouse IgG2b, Dako) applied in a concentration that matched with the prolactin receptor antibody. TBS was used for washing (3 × 2 min) throughout. A three-step HRP polymer technique was applied: post-antibody blocking, 1:1 diluted in TBS/BSA (15 min, room temperature) followed by anti-mouse/rabbit/rat Powervision polymer/HRP (both ImmunoVision, ImmunoLogic, Duiven, The Netherlands), 1:1 diluted in TBS/BSA (30 min, room temp); HRP activity was visualized with diaminobenzidine (DAB+, Dako) in 8 min. Sections were counterstained with hematoxylin (Dako) and mounted with VectaMount (Vector Labs, Burlingame, CA, USA).

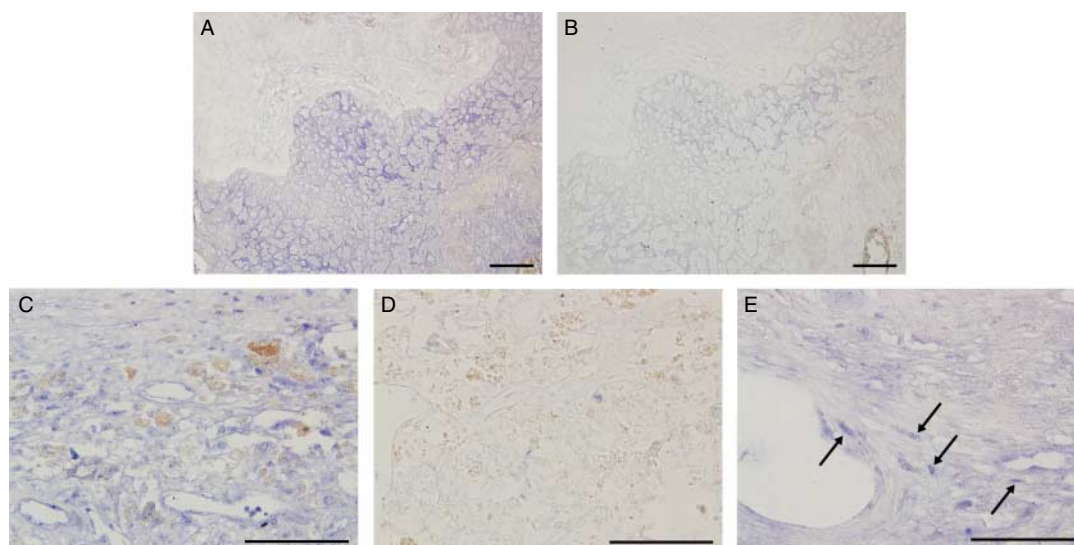
To reveal the cellular origin of prolactin receptor expression, a sequential double AP staining was performed (van der Loos & Teeling 2008) with prolactin receptor antibody and antibodies to CD3 (rabbit monoclonal, clone SP7, Thermo Fisher Scientific/Labvision) or CD68 (mouse monoclonal, clone PG-MI, Dako) to detect co-localization with T-cells or macrophages respectively. CD3 or CD68 antibody binding was detected with an appropriate anti-mouse or anti-rabbit AP-conjugated polymer (Immuno-Logic), and AP activity was visualized using Vector Blue

(Vector Labs). After a short 10-min heat step using one of the Tris-EDTA (pH 9.0) HIER buffer to remove the first set of immunoreagents, but leaving the blue AP reaction product unchanged, the prolactin receptor antibody was detected by a second AP staining using Vector Red (Vector Labs). Sections were mounted with VectaMount, without a nuclear counterstain. All slides were coded and judged by an independent pathologist.

### Spectral imaging

Datasets were acquired from 420 to 720 nm at 20 nm intervals. Spectral library of single Vector Red and single Vector Blue was used to unmix the double staining into the individual components. Using the Nuance software version 2.9, an exclusive image of prolactin receptor and CD3 or CD68 co-localization as well as fluorescent-like pseudo-colored images was created (van der Loos 2008).

IHC and ISH slides were analyzed by light microscopy (Olympus BX51 microscope, Olympus UPlanFl 20 × / 0.5 ∞ / 0.017 objective, Olympus DP70 digital camera; Olympus, Zoeterwoude, The Netherlands). In addition, double-stained IHC slides were analyzed using light microscopy (Leica DM5000B microscope, Leica HC PLAN APO 20 × / 0.70 ∞ / 0.017 / C objective; Leica Microsystems, Rijswijk, The Netherlands) and a multispectral imaging system and software (Nuance MSI camera, Nuance software vs 2.8.0; Cambridge Research and Instrumentation, Inc., Woburn, MA, USA).



**Figure 1** *In situ* hybridization (ISH) for prolactin receptor mRNA. A shows the results for hybridization with the antisense probe directed against prolactin receptor mRNA on placental tissue (positive control, positive staining blue) (scale bar = 100  $\mu$ m). B shows background staining of the placenta after hybridization with the sense probe (negative control) (scale bar = 100  $\mu$ m). C shows ISH with the antisense probe for the prolactin receptor mRNA within the shoulder region of the atherosclerotic plaque (scale bar = 100  $\mu$ m). D shows the same area of the plaque but hybridized with the sense probe (negative control) (scale bar = 100  $\mu$ m). E represents a higher magnification of the shoulder region after ISH with the antisense probe (scale bar = 50  $\mu$ m).

### Immunoelectron microscopy

Dehydration of the tissue samples was done by means of progressive lowering of temperature in an ascending concentration of ethanol and embedded in LR Gold (Polysciences, Warrington, PA, USA). Polymerization was carried out at  $-20^{\circ}\text{C}$  under u.v. light for 24 h, following which the blocks were kept for an additional 48–72 h at room temperature for polymerization under u.v. light. Sections, 50–70 nm thick, were cut and collected on formvar-coated 50-mesh copper grids. Before staining with antibody, free aldehyde groups were blocked with 50 mM glycine/PBS. After washing the grids with PBS+0.2% BSA-c (Aurion, Wageningen, The Netherlands), incubation with the prolactin receptor antibody was performed at RT for 60 min. After washing of the grids, the secondary antibody, Protein-G gold conjugate (15 nm 1/70; CMC-Utrecht, The Netherlands) was applied for 30 min. Finally, the grids were thoroughly washed with distilled water and lightly counterstained with uranyl acetate (Leica) and lead citrate (Leica). Grids were examined in a Philips CM 10 electron microscope.

### Statistical evaluation

Detection of the prolactin receptor was scored for the plaques, stained using IHC, as follows: 0=no expression, += low expression, ++= medium expression, and +++= high expression. The morphology of the plaque was evaluated as well (stable versus non stable), and with a 2-sided *P* value Fisher's exact test (hereby the expression levels of the prolactin receptor were merged in two groups, 0/+ vs ++/+++),

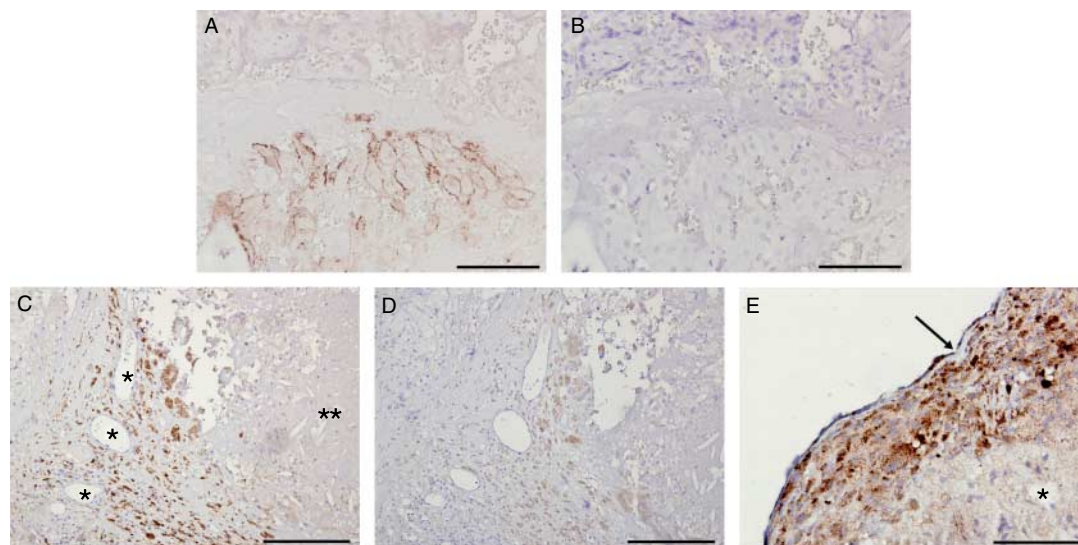
the significance of the association between these two classifications was examined. The same test was used to evaluate whether the extent of prolactin receptor expression was different between genders, and whether plaque morphology was related to gender.

## Results

### Quantitative real-time PCR

RNA was isolated from 11 atherosclerotic plaques. Out of the eleven RNA samples obtained, only five were satisfactory for further analysis with quantitative real-time PCR based on their RNA yield, RNA purity, and RNA integrity (Supplementary Table 1, see section on supplementary data given at the end of this article). Detection of all known prolactin receptor isoforms (i.e. long, intermediate, short 1a, short 1b, short 1c,  $\delta$  S1,  $\delta$  4-SF1b,  $\delta$  7/11, and  $\delta$  4-7/11) (Kline *et al.* 2002, Ben-Jonathan *et al.* 2008) was covered with four different primer sets (A–D) (Table 1). Herein, 'A' refers to the prolactin receptor amplicon obtained when using primer set A; the same holds true for primer sets B, C, and D respectively. Specificity of each primer set was verified by PCR of cDNA from placenta and pituitary as positive controls, followed by evaluation of the amplicons with the use of gel electrophoresis and melting curve analysis (Supplementary Figures 1 and 2, see section on supplementary data given at the end of this article respectively).

Atherosclerotic plaques contain mRNA of the prolactin receptor but not of its ligand prolactin, as was revealed by



**Figure 2** Immunohistochemical staining of the prolactin receptor. A and B show staining of placenta with an antibody directed against the prolactin receptor or an isotype control respectively (brown) (scale bar=100  $\mu\text{m}$ ). C shows positive staining for the prolactin receptor within the shoulder region of the atherosclerotic plaque (scale bar=200  $\mu\text{m}$ ); \* indicates small vessels and \*\* represents the necrotic core. In D, the corresponding area of the atherosclerotic plaque is stained with an isotype control (scale bar=200  $\mu\text{m}$ ). E confirms abundant presence of the prolactin receptor in macrophages (scale bar=100  $\mu\text{m}$ );  $\rightarrow$  refers to the fibrotic cap and \* represents the necrotic core.

quantitative real-time PCR analysis of cDNA from carotid atherosclerotic plaques (Table 2). Prolactin receptor mRNAs of isoforms, which are detected by PCR using primer sets A–C, and to a lesser extent using D, were detected in the plaques.

Evaluation of the relative expression levels of the various isoforms was beyond the scope of this study. As expected, CD68, CCL18, TNF $\alpha$ , and IL10, four markers of inflammatory activity in atherosclerosis, were also expressed in the atherosclerotic plaques, indicating that the tissues used for qRT-PCR analysis were harvested from inflammatory sites containing a large amount of macrophages, as can be expected from atherosclerotic tissue (data not shown).

#### In situ hybridization

The DIG-labeled antisense prolactin receptor probe was tested on placental tissue and stained positively for the decidua (Fig. 1A, blue signal). Negative controls contained ISH with DIG-labeled sense riboprobes (Fig. 1B), and positive controls included ISH with antisense riboprobes against *CTGF* mRNA, a factor well known to be increased in placenta as well as in atherosclerotic lesions (Oemar *et al.* 1997, Ito *et al.* 1998) (data not shown).

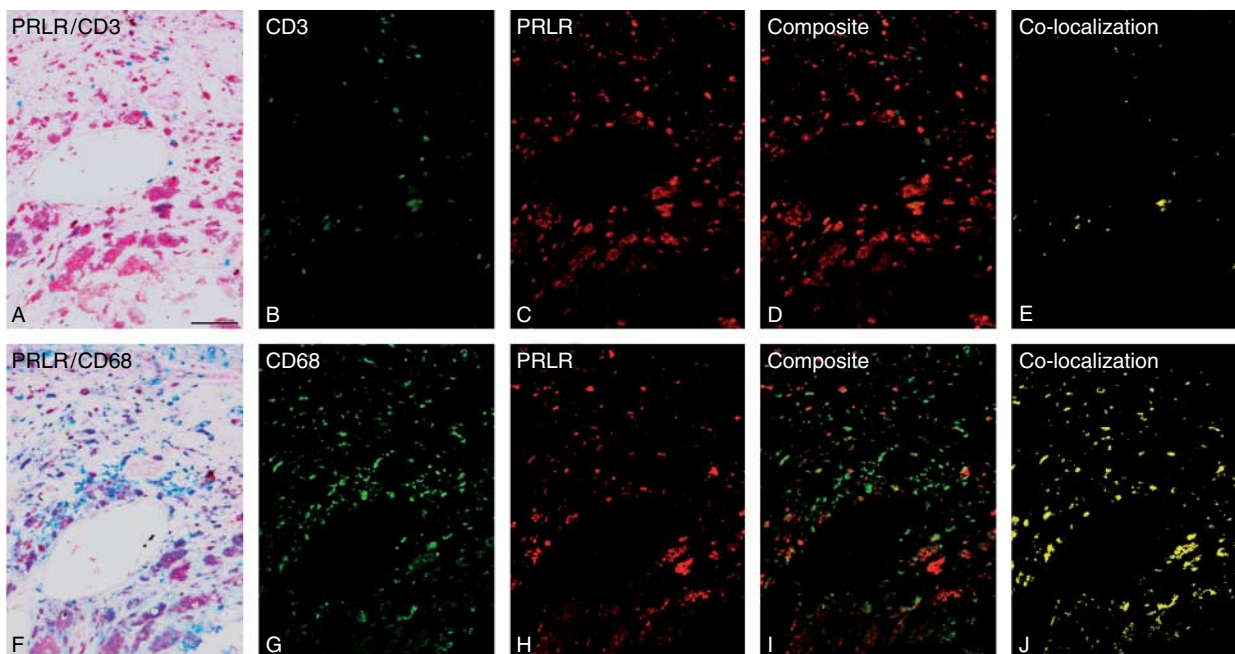
Prolactin receptor mRNA was most abundant in the inflamed shoulder regions of atherosclerotic plaques (Fig. 1C) and appeared to be present mainly within mononuclear cells (Fig. 1E, arrows), which may represent macrophages. ISH with the DIG-labeled sense control probe did not yield staining (Fig. 1D).

#### Immunohistochemistry

To investigate the location of the prolactin receptor protein within atherosclerotic plaques, a series of carotid atherosclerotic arteries were immunohistochemically stained using a monoclonal antibody directed against the human prolactin receptor. This antibody was tested on placental tissue and stained positively for decidual cells, with a cell membrane pattern (Fig. 2A, brown staining signal). This observation was in line with previous findings described elsewhere (Galsgaard *et al.* 2009) and with the presence of prolactin receptor mRNA in decidua as shown when using ISH (Fig. 1A). The placenta tissue was fully negative with the isotype control (Fig. 2B). Figure 2C–E are representative pictures of the series of atherosclerotic plaques stained using the same conditions. Figure 2C shows positive intracellular staining for the prolactin receptor within the shoulder region of the atherosclerotic plaque. The corresponding isotype negative control is depicted in Fig. 2D. Abundant presence of the prolactin receptor is observed in a subpopulation of lipid-laden macrophages as displayed in Fig. 2E.

#### Spectral imaging

To gain information regarding the exact cell types expressing the prolactin receptor in the shoulder region of the atherosclerotic plaque, we used spectral imaging for analysis of the prolactin receptor/T-cell and prolactin receptor/macrophage double staining slides. After unmerging,



**Figure 3** Cell-specific immunohistochemical staining of the prolactin receptor (scale bar=100  $\mu$ m in fig A–J). A shows staining of the shoulder region of an atherosclerotic plaque for the prolactin receptor (pink) and staining for T-cells (blue), analyzed by spectral imaging in B–E, demonstrating weak co-localization (E). F shows staining of the prolactin receptor (pink) and macrophages (blue), analyzed by spectral imaging in G–J, demonstrating extensive co-localization (J).

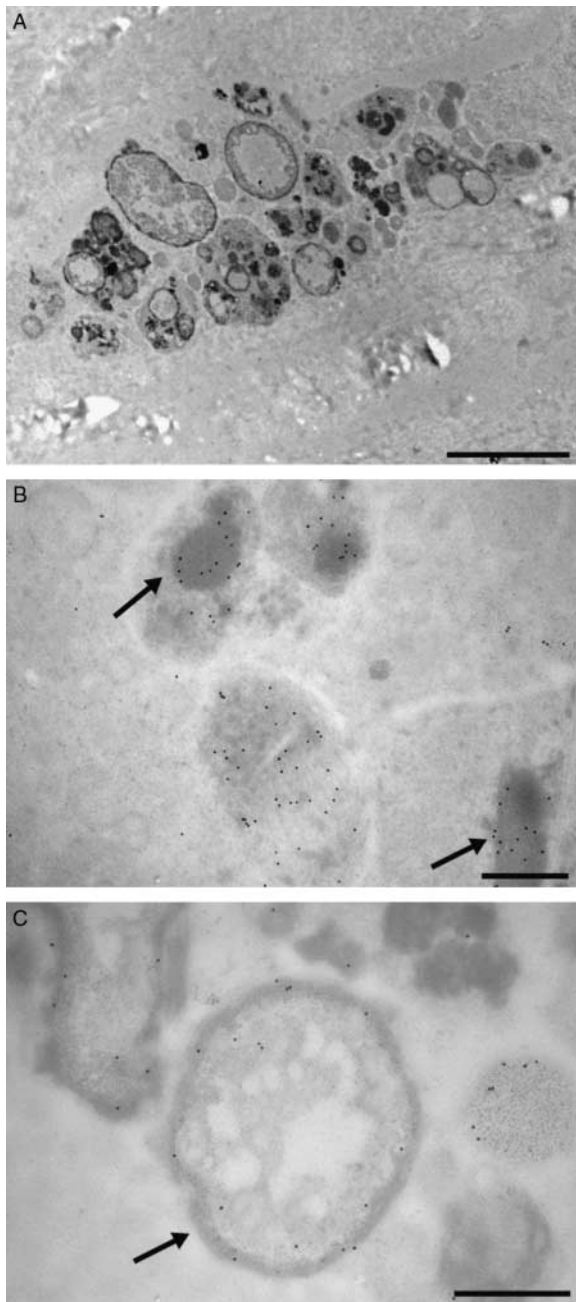


pseudo-colored fluorescent-like images as well as exclusive images of co-localization were obtained. Figure 3A–E show minimal co-localization of prolactin receptor and T-cells (identified by CD3 staining), whereas Fig. 3F–J reveal abundant co-localization with a subset of resident

macrophages (identified by CD68 staining) in the shoulder region of an atherosclerotic plaque.

#### Immunoelectron microscopy

Our observation that prolactin receptors are present within intracellular compartments of macrophages is in line with another recent study, which reports that the prolactin receptor can be internalized and subsequently degraded via the lysosomal pathway (Varghese *et al.* 2008). However, the mechanisms of prolactin receptor proteolysis remain largely to be determined. To investigate whether a lysosomal localization could explain the positive staining for the prolactin receptor within the intracellular compartments of macrophages in atherosclerotic plaques, we used immunoelectron microscopy. Figure 4A provides an overview of the different organelles within the macrophage, and Fig. 4B and C demonstrate localization of the prolactin receptor (black particles) in intracellular organelles, with specific enrichment in lysosomes (arrows), displaying both a central and a membranous localization in these structures.



**Figure 4** Prolactin receptor immunoelectron microscopy in macrophages is shown. A provides an overview of the different organelles that are present within the macrophages (scale bar=5000 nm). B and C indicate that there is specific enrichment of the prolactin receptor in lysosomes (arrows), displaying both a central and a membranous localization in these structures (scale bar=500 nm).

#### Correlation between prolactin receptors and plaque stability

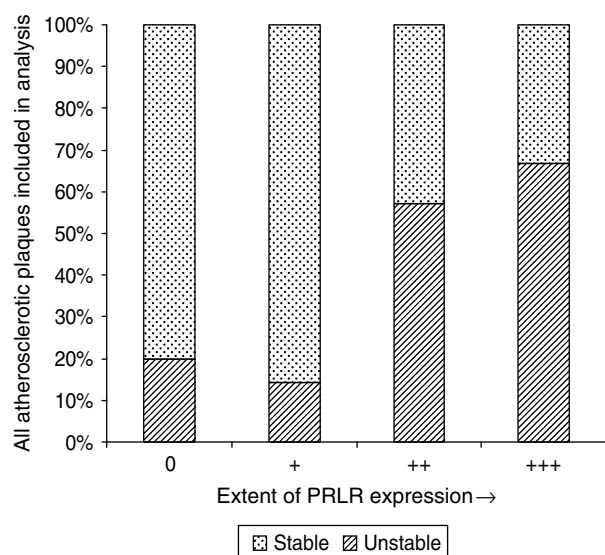
Next, the intensity of prolactin receptor staining was linked to lesion stage of the plaques. Prolactin atherosclerotic plaques were considered unstable when they contained a thin fibrotic cap and a necrotic core, which indicated that the plaques were prone to rupture and thus to subsequently cause an ischemic event (Yuan *et al.* 2002); 10 plaques were found to be stable and 15 were unstable. We observed that plaques with a higher expression of prolactin receptors turned out to be more often unstable (Fig. 5). In fact, the extent of prolactin receptor expression was found to be significantly higher in unstable plaques than in less advanced stable lesions ( $P=0.04$ ; Supplementary Figure 3, see section on supplementary data given at the end of this article).

We also analyzed the expression of prolactin receptors in male versus female patients, and we observed that the expression of prolactin receptors was higher in atherosclerotic plaques obtained from male patients than in plaques obtained from female patients ( $P=0.04$ ; Supplementary Table 2, see section on supplementary data given at the end of this article). The two classifications ‘plaque stability’ and ‘gender’ were not associated ( $P=0.23$ ; Supplementary Table 3, see section on supplementary data given at the end of this article), suggesting there was no confounding effect from plaque morphology on the results in Supplementary Table 2, see section on supplementary data given at the end of this article.

#### Discussion

We seek to define the role of prolactin receptor signaling in atherogenesis. In this study, quantitative real-time PCR indicated that several isoforms of the prolactin receptor, but not of prolactin, are expressed within human carotid





0 = No PRLR expression; + = Low PRLR expression;  
 ++ = Medium PRLR expression; +++ = High PRLR expression

**Figure 5** Immunohistochemical staining of the prolactin receptor in relation to presumed morphological signs of plaque instability (semi-quantitative analyses). Each bar in Fig. 5 represents a specific intensity of prolactin receptor staining: 0 = no expression (five plaques), + = low expression (seven plaques), ++ = considerable expression (seven plaques), and +++ = extensive expression (six plaques). Prolactin receptor expression was associated with lesion stage: high positive staining occurred especially in unstable plaques.

atherosclerotic plaques. ISH analysis and immunohistochemical staining of atherosclerotic carotid artery sections showed that the prolactin receptor is highly expressed in mononuclear cells within advanced atherosclerotic lesions. With spectral imaging, we could show that the prolactin receptor is predominantly detected in macrophages, and to a lesser extent in T-cells. With the use of immunoelectron microscopy, we assessed the presence of the prolactin receptor within lysosomes of macrophages inside the plaques. In addition, a high extent of prolactin receptor staining was especially noticeable in morphologically unstable plaques. The small sample size of our experiments does not allow us to draw firm conclusions about gender-specific expression of the prolactin receptor in atherosclerotic plaques, although our data suggest it could be higher in men.

Alternative splicing generates multiple prolactin isoforms, as we indicated in Table 1, and these isoforms are classified by the length of their intracellular domain. The prolactin receptor has been shown to be ubiquitously expressed in human tissues, with the ratio of isoforms varying among tissues, each with a potential for unique signaling (Kline *et al.* 2002, Ben-Jonathan *et al.* 2008). The observations in our study are descriptive; therefore we can only speculate on the functional role that the prolactin receptor may have in atherosclerotic plaques. The abundance of prolactin receptors

therein suggests that prolactin might be able to modulate the atherosclerotic process. Prolactin is widely accepted as an important physiological modulator of the immune response (Pellegrini *et al.* 1992, Schwarz *et al.* 1992, Dorshkind & Horseman 2000). In addition, atherosclerosis is now recognized as a chronic low-grade inflammatory condition of the vessel wall, characterized by infiltration of macrophages and T-cells, which interact with one another and with other cells of the arterial wall (Packard & Libby 2008). Whether activation of the prolactin receptor within the atherosclerotic plaque could result in pro- or anti-inflammatory effect is beyond our knowledge.

The immunohistochemical analysis of coronary atherosclerotic sections showed that the prolactin receptor is highly expressed in advanced atherosclerotic coronary arteries, but not in the absence of such lesions, suggesting that this receptor could modulate the atherosclerotic process (Reuwer *et al.* 2009b). However, as we reported earlier that systemic prolactin levels are not predictive of coronary artery disease (Reuwer *et al.* 2009b), this could suggest that the involvement of the prolactin receptor in this process does not require enhanced systemic prolactin levels. In the latter study, we speculated that local expression of prolactin leading to receptor activation by an autocrine/paracrine loop might be one of the mechanisms involved (Reuwer *et al.* 2009b). We here invalidate this hypothesis, as we could not detect any evidence of prolactin expression within atherosclerotic plaques using a highly sensitive quantitative real-time PCR approach. This suggests that expression of the prolactin receptor in a site where it is normally absent is intrinsically sufficient to confer sensitivity to circulating prolactin. Alternatively, prolactin receptors within the atherosclerotic plaque could be stimulated by GH, another natural ligand of the prolactin receptor in humans (Nicoll *et al.* 1986). Subtle disturbances in the GH-IGF1 axis are indeed correlated with increased prevalence of ischemic heart disease (Colao 2008).

IHC for the prolactin receptor revealed predominantly cytoplasmic staining of macrophages in atherosclerotic plaques, whereas decidual cells in the placenta were stained at the cell membrane. The prolactin receptor, being a member of the cytokine receptor superfamily, is known as a single-pass transmembrane protein (Kelly *et al.* 1991). In addition, the prolactin receptor can reside for a large fraction in the cytoplasm (Clevenger *et al.* 1995). Interestingly, internalization of the receptor is stimulated by prolactin, but basal internalization can also occur in the absence of prolactin (Swaminathan *et al.* 2008). Downregulation of the prolactin receptor is a well-known phenomenon in prolactin-induced signaling, since it leads to less availability of the receptor on the surface of the cell to bind prolactin (Djiane *et al.* 1979). It is known that ubiquitination of cell surface proteins can serve as a pivotal endocytosis signal (Dunn & Hicke 2001). Indeed, it has been shown that ubiquitination of the prolactin receptor plays a role in the negative regulation of cellular responses to prolactin. Once activated, the prolactin receptor is rapidly ubiquitinated, internalized, and degraded primarily

via the lysosomal pathway (Varghese *et al.* 2008). In this study, we indeed found, by using immunoelectron microscopy, that prolactin receptors are particularly enriched within the lysosomal compartment of macrophages of the plaque. This may result from internalization of the receptors, which supports the concept that prolactin receptor activation can occur within the atherosclerotic plaque and that activation of the prolactin receptor pathway may contribute to the inflammatory response.

Based on the available literature and our current results, we propose that prolactin plays the following role in the pathophysiology of atherosclerosis: increased prolactin receptor signaling within macrophages inside the atherosclerotic plaque may aggravate local inflammation, and subsequently, the enhanced inflammatory response may contribute to atherogenesis. Testing this hypothesis requires further investigation.

### Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/JOE-10-0076>.

### Declaration of interest

Dr Th B T is the inventor of a patent application related to the work in this manuscript.

### Funding

Dr Th B T is supported by the Netherlands Organization of Health and Science (NWO-ZonMW; project number 916.66.124). Dr J H von der T is an awardee of a Dutch Heart Foundation Dekker Junior Staff Member Grant (project number 2008T050).

### Author contribution statement

A Q R conceived and designed the research, partly acquired the data, partly analyzed and interpreted the data, and drafted the manuscript. M van E partly conceived and designed the research, and partly analyzed and interpreted the data. F M H-B, C M van der L, N C, and P T partly acquired the data, and analyzed and interpreted the data. J J P K made critical revision of the manuscript for important intellectual content and handled supervision. J H made critical revision of the manuscript for important intellectual content. V G made critical revision of the manuscript for important intellectual content and advised specific tools. J H von der T partly analyzed and interpreted the data, and performed statistical analysis. Th B T partly conceived and designed the research, and handled funding and supervision. J A partly conceived and designed the research, partly analyzed and interpreted the data, and made critical revision of the manuscript for important intellectual content.

### Acknowledgements

We owe many thanks to the vascular surgeons of the Academic Medical Center, Amsterdam, since they provided us the carotid atherosclerotic plaques that we used for qRT-PCR. In addition, we would like to thank Dr Jan

Ruijter (Department of Anatomy, Embryology and Physiology, Academic Medical Center, Amsterdam) for his advice with regard to the analysis of the qRT-PCR data with LinRegPCR.

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Received in final form 4 November 2010

Accepted 10 November 2010

Made available online as an Accepted Preprint  
10 November 2010