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# Visualizing TGF- $\beta$ and BMP signaling in human atherosclerosis: a histological evaluation based on Smad activation

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**Summary.** Background: The TGF- $\beta$  superfamily members Transforming Growth Factor- $\beta$  (TGF- $\beta$ /Activin) and Bone Morphogenetic Proteins (BMP) have been implicated in the pathogenesis of atherosclerosis. However, their role in human disease remains controversial. In this study we used Smad phosphorylation as a read out for TGF- $\beta$  and BMP signaling during the initiation, progression and (de)stabilization of human atherosclerotic disease.

Material and methods: A systematic analysis was performed in 114 peri-renal aortic patches (stained with Movat Pentachrome, H&E, pSmad2, pSmad1,5,8 and PAI-1) covering the entire atherosclerotic spectrum (van Dijk, 2010). Immunostaining against T-cells (CD3) and monocytes and macrophages (CD68) was used to explore a putative association between TGF- $\beta$  and BMP signaling and vascular inflammation.

Results: Smad phosphorylation was present within the normal arterial wall in approximately 10% of the endothelial cells and intimal smooth muscle cells. A significant increase in pSmad2 and pSmad1,5,8 positivity was found in non-progressive lesions (>50% positivity). No further increase or decrease was found in the progressive atherosclerotic lesions, vulnerable and stabilized lesions. No association was found between TGF- $\beta$  and BMP signaling and CD3 and CD68 expression, nor cap thickness.

Conclusion: Activation of the TGF- $\beta$  and BMP pathways is an early event in atherosclerotic lesion formation. No significant relationships were found

between Smad phosphorylation and vessel wall inflammation or plaque vulnerability.

**Key words:** Atherosclerosis, Aorta, TGF- $\beta$ -signaling, BMP-signaling, Inflammation

## Introduction

Atherosclerotic disease is a metabolic disorder with a strong inflammatory component (Hansson, 2001). Early phases of the disease are characterized by progressive lipid accumulation in the intima, followed by formation of an atherosclerotic lesion that is identified by a necrotic core and an overlying collagenous cap. This cap shields the highly prothrombotic contents of the core from the blood stream. Increased inflammation, protease activity and impaired matrix turn-over in the cap area results in destabilization of the cap (Redondo et al., 2006; Mehta and Attramadal, 2007), which may ultimately result in exposure of the necrotic core followed by acute thrombosis and (partial) occlusion of the vessel (Virmani et al., 2000).

The Transforming Growth Factor-Beta (TGF- $\beta$ ) super family of ligands (TGF- $\beta$ , Activin and Bone Morphogenetic Proteins (BMP)) is a group of widely expressed growth factors/cytokines that have broad activities on matrix homeostasis and inflammatory systems (Engelse et al., 1999; Singh and Ramji, 2006; Redondo et al., 2006; Mehta and Attramadal, 2007). TGF- $\beta$  family members exhibit comprehensive anti-inflammatory effects on a wide range of cells, including endothelial cells, macrophages and T-cells, as well as fibroblasts and smooth muscle cells (Grainger, 2004;

Frutkin et al., 2009).

Because of their comprehensive effects on inflammation and matrix homeostasis, members of the TGF- $\beta$  superfamily have been proposed as critical regulators of the atherosclerotic process (Mallat et al., 2001; Lutgens et al., 2002). However, the exact relationship between TGF- $\beta$ /BMP signaling and atherosclerosis is far from clear. Animal studies consistently show a negative association between TGF- $\beta$  concentration and the extent of atherosclerosis. Human studies on the other hand indicate a positive correlation between circulating plasma TGF- $\beta$  and atherosclerosis progression (Wang et al., 1997; Redondo et al., 2006; Grainger, 2007; Volger et al., 2007; Frutkin et al., 2009). The basis for this apparent contrast is unclear. Possible explanations are that it reflects differences between the animal models of atherosclerosis and the actual disease and/or the fact that most human studies have been performed in patients with advanced atherosclerotic disease. A further caveat is that most human studies rely on circulating TGF- $\beta$  levels, which may not necessarily reflect TGF- $\beta$  local levels or activities (Wang et al., 1997). Regulation of the TGF- $\beta$  and BMP signalling pathways is complex, involving extensive regulation of bioavailability of ligands and receptors, as well as tightly controlled intracellular signalling pathways (Heldin et al., 1997; Kalinina et al., 2004; Bobik, 2006; Grainger, 2004; Grainger, 2007).

The canonical TGF- $\beta$  family signalling is via type I and type II cell surface receptors that exhibit serine/threonine kinase activity. Upon induction of heteromeric complex by the ligand, the type I receptor is phosphorylated by type II receptor. This in turn results in phosphorylation of the C-terminus of receptor regulated Smads (R-Smads) that are responsible for the intracellular signaling. Whereas Smad2 is phosphorylated by TGF- $\beta$  type I receptor, Smad1, -5 and -8 are phosphorylated by BMP type I receptors (Heldin et al., 1997). As a consequence the involvement of TGF- $\beta$  activity and BMP signaling pathways in human atherosclerosis is still unclear (Singh and Ramji, 2006; Grainger, 2007).

We performed a systematic histological evaluation of receptor phosphorylated Smad2 (pSMAD2) and -1,5,8 (pSmad1,5,8) as read out for the canonical TGF- $\beta$  and BMP signaling pathways throughout the complete spectrum of human atherosclerotic disease. As well as phosphorylated Smad expression we evaluated the expression of Human Plasminogen Activator Inhibitor type-1 (PAI-1) (Dennler et al., 1998; Lindeman et al., 2007). Expression of PAI-1 is under strict control of Smads (Smad3) and considered one of the classical targets of the TGF- $\beta$  receptor pathway (Mallat and Tedgui, 2002; Vaughan, 2006).

The results of this study demonstrate that activation of the TGF- $\beta$  and BMP pathways occurs in the earliest phases of human atherosclerotic disease. This activation level remains stable during further progression of the disease. No significant relationships were found between

Smad2 or Smad1,5,8 phosphorylation, and vessel wall inflammation or plaque vulnerability.

## Materials and methods

### *Patients and tissue sampling*

All sections in the study were selected from a tissue bank of aortic wall patches that were obtained during kidney transplantation with donor kidneys from cadaveric donors. Details of this bank have been described previously by van Dijk et al. (2010). All patches were derived from grafts that were eligible for transplantation (i.e. all donors met the criteria set by The Eurotransplant Foundation). Due to national regulations, only transplantation relevant data from donation is available. Sample collection and handling was performed in accordance with the guidelines of the Medical and Ethical Committee in Leiden, Netherlands and the code of conduct of the Dutch Federation of Biomedical Scientific Societies (<http://www.federa.org/?s=1&m=82&p=0&v=4#827>).

All sections in the bank are Movat stained and classified by two independent observers with no knowledge of the characteristics of the aortic patch (Virmani et al., 2000; van Dijk et al., 2010).

### *Characterization of the lesions and histological definitions*

Allocation to the different groups was based on the most advanced lesion in the section. Classification was done according to the modified AHA classification as proposed by Virmani et al. (2000): viz. adaptive intimal thickening (AIT), intimal xanthoma (IX) (i.e. non-progressive lesions); pathological intimal thickening (PIT), early fibroatheroma (EFA), late fibroatheroma (LFA) (i.e. progressive atherosclerotic lesions); thin cap fibroatheroma (TCFA), acute plaque rupture (PR) (i.e. vulnerable plaques); healed plaque rupture (PR) and fibrotic calcified plaque (FCP) (i.e. stable atherosclerotic lesions).

Normal lesions were defined as aortic wall samples consisting merely of subendothelial proteoglycan rich layers that were devoid of infiltrating smooth muscle cells and/or macrophages (Stary et al., 1992). In order to obtain balanced and representative study groups we randomly selected 10-12 samples from each stage. A total of 114 cases were selected.

### *Immunohistochemistry*

Immunohistochemical single stains were performed on 4  $\mu$ m thick sections prepared from formalin fixed, paraffin embedded tissue, as described previously (van Dijk et al., 2010). The following antibodies were used as read-out for TGF- $\beta$  and BMP signaling: rabbit polyclonal phosphorylated Smad2 (cat#3108, 1:100, Cellsignaling; Leiden, the Netherlands), rabbit

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polyclonal phosphorylated Smad1,5,8 (AB-246-NA, 1:50, R&D Systems, Abingdon), and PAI-1 staining was performed with an in-house rabbit polyclonal antibody that recognizes both the free and complexed form (Lindeman et al., 2007). Conjugated biotinylated anti-rabbit anti-IgG (1:1000, Amersham Biosciences, Buckinghamshire, UK) was used as secondary antibody. Sections were counterstained with Mayer's Haematoxylin.

Quantification of inflammation was performed by staining for macrophages with mouse monoclonal CD68 (KP-1, isotype IgG1-kappa, Dako, Denmark: diluted at 1:400 overnight) and T-lymphocytes with rabbit polyclonal CD3 (AB828, Abcam, Cambridge, UK; diluted at 1:200 overnight).

### Assessment of immunolocalization of the intermediate TGF- $\beta$ pathway, macrophage and T-cell markers

The lesion was defined as the area between the endothelium and internal elastic lamina over a distance of 1mm. In the presence of a necrotic core the distance was expanded another 500  $\mu$ m on both sides of the necrotic core to include the adjacent shoulder region. The regions of interest (ROI) are the intima (in the presence of a (early) necrotic core the intima was divided into a cap and both shoulder regions), media and adventitia. Particular interest was taken in the various cell types present in the atherosclerotic lesion (i.e. monocytes/macrophages, lymphocytes and smooth muscle cells).

The degree of pSmad2, pSmad1,5,8, PAI-1, CD3 and CD68 intensity was assessed semi-qualitatively on a scale of 0-4 by two observers: 0 (absent/no positive staining in ROI); 1 (<10% of cells in ROI stained

positively); 2 (10-50% of cells in ROI stained positively); 3 (50-75% of cells in ROI stained positively) and 4 (>75% of cells in ROI stained positively).

### Statistical analysis

Data in figures are presented as mean  $\pm$  SEM. Mean variables between the various lesions were compared with the one-way analysis of variance (ANOVA; SPSS 17.0; Chicago, IL), followed by post-hoc Fisher's LSD test. A value of  $p < 0.05$  was considered statistically significant.

## Results

### Studied population

Data were obtained from 114 consecutive peri-renal aortic samples from organ donors aged from 5 to 76 years (Table 1). The male/female ratio was evenly distributed between the clusters (Table 2). There was a strong relationship between donor age and the stage of atherosclerosis (van Dijk et al., 2010). Normal aortic wall samples had a mean age of ~18-years, whereas the mean age for aortas with fibrotic calcified plaques was ~58-years.

### TGF- $\beta$ signaling: Smad2 phosphorylation

About 10% of the smooth muscle cells (SMCs) and endothelial cells in normal aortas stain positive for phosphorylated Smad2 (Figs. 1a, 2a). There is an immediate increase in pSMAD2 positivity (>50% positivity ( $p < 0.029$ ) Fig. 2a) in the earliest phases of

**Table 1.** Demographic data of the 114 studied patients.

	Male	Female
N	60	54
Mean age (years)	49.4 [44-58]	43.8 [38-47]
Mean length (cm)	179.9 [175.0-185.8]	166.6 [160.0-175.0]
Mean weight (kg)	80.1 [75.3-85.0]	64.9 [57.0-75.0]
Mean BMI (kg/m <sup>2</sup> )	24.8 [23.0-26.0]	22.9 [21.0-26.0]
Number of patients with known history of nicotine abuse	21	17
Number of patients with known history of hypertension	13	10
Number of patients with known diabetes	0	1
Cause of Death:		
Severe head trauma	11	7
Cerebral vascular accident (CVA)	8	10
Subarachnoidal bleeding (SAB)	15	14
Cardiac arrest	7	0
Trauma	1	1
Other	6	3
Unknown	12	19
Medication:		
Anti-hypertensives	11	6
Statins	1	1
Anti-coagulants	1	2

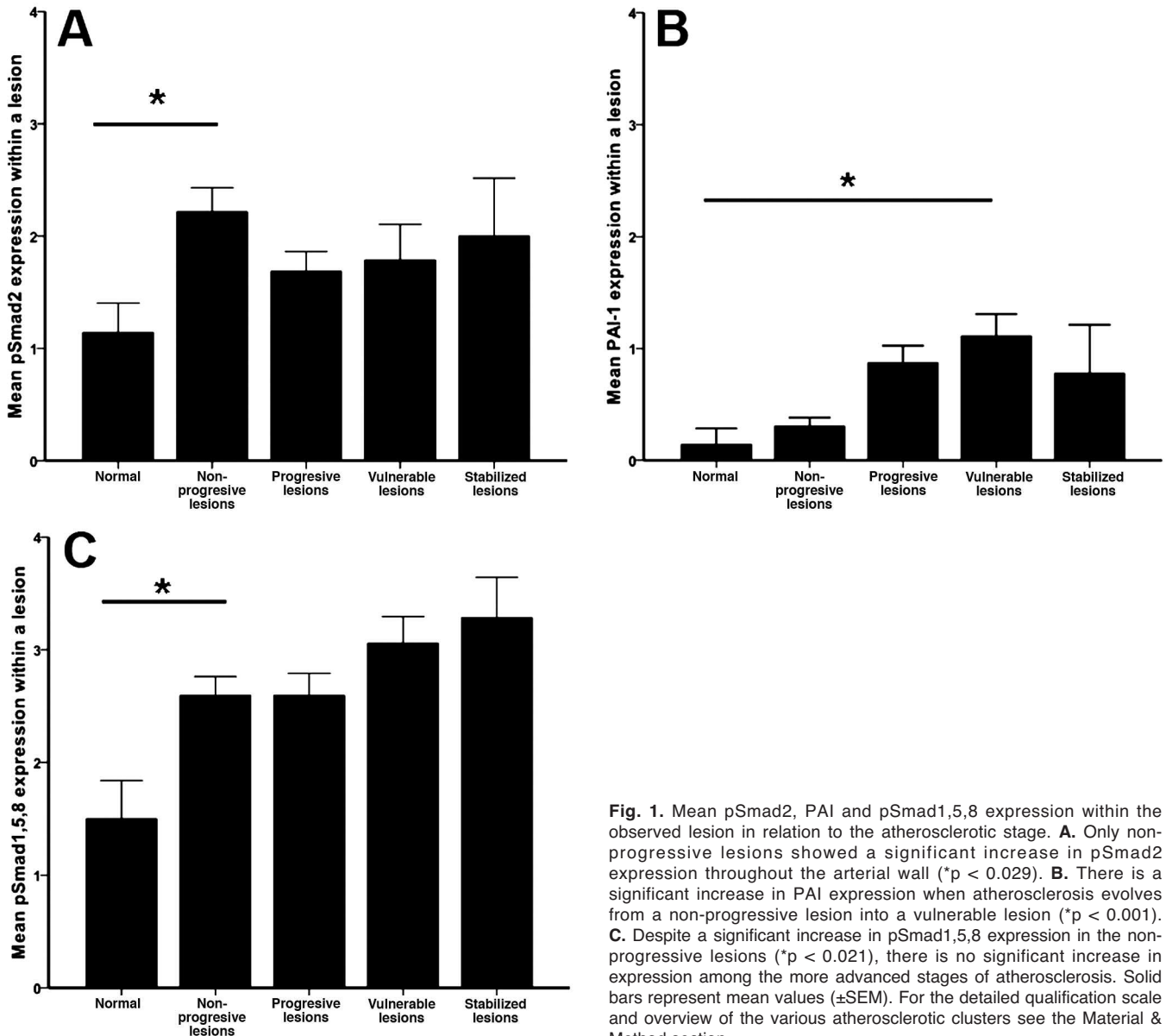
atherosclerotic disease (non-progressive lesions). No further increase in pSMAD2 positivity is observed in the more advanced stages of the disease (i.e. progressive atherosclerotic lesions, vulnerable and stabilized lesions).

Smad2 activation is similar in the different layers of the vessel wall (intima, media or adventitia). We did not observe a shift in pSmad2 positivity over the individual cell types (monocytes/lymphocytes and SMCs) throughout all stages of atherosclerosis (data not shown). No relationship was found between pSmad2 positivity

and cap thickness.

*Phosphorylated Smad2 and vascular inflammation*

The mean expression of lesional monocytes/ macrophages and T cells (CD3) increased with advancing atherosclerosis in all layers of the aortic wall (Fig. 3). No relation was found between Smad2 phosphorylation and the amount of CD3 positive cells (Fig. 4a). Healing of a ruptured lesion (i.e. healed ruptures and fibrotic calcified plaques) was associated



**Fig. 1.** Mean pSmad2, PAI and pSmad1,5,8 expression within the observed lesion in relation to the atherosclerotic stage. **A.** Only non-progressive lesions showed a significant increase in pSmad2 expression throughout the arterial wall (\* $p < 0.029$ ). **B.** There is a significant increase in PAI expression when atherosclerosis evolves from a non-progressive lesion into a vulnerable lesion (\* $p < 0.001$ ). **C.** Despite a significant increase in pSmad1,5,8 expression in the non-progressive lesions (\* $p < 0.021$ ), there is no significant increase in expression among the more advanced stages of atherosclerosis. Solid bars represent mean values ( $\pm$ SEM). For the detailed qualification scale and overview of the various atherosclerotic clusters see the Material & Method section.

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with a significant reduction in CD3 positive cells ( $p < 0.0001$ ).

Most Smad2 phosphorylation was seen in the lesions containing minimal CD68 expressing cells ( $p < 0.038$ ; Fig. 5a). The degree of Smad2 phosphorylation did not significantly change when the amount of CD68 expressing cells in the lesions increased.

*PAI-1 expression*

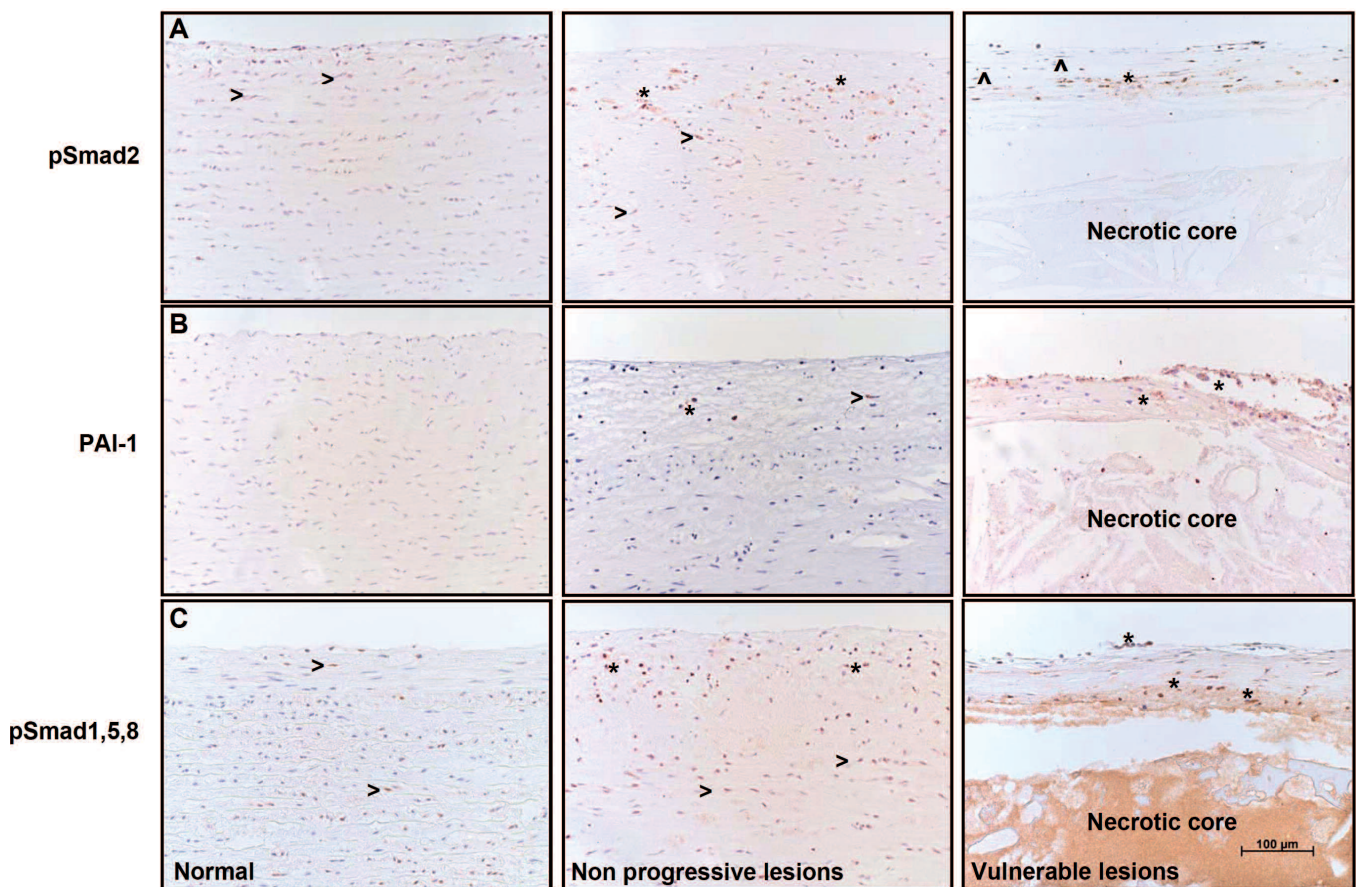
Minimal PAI-1 expression was observed in the intimal layer of normal aortic tissue (Figs. 1b, 2b). PAI-1 positivity was found in 10% of the SMCs (predominantly in the infiltrating SMCs) in pathological intimal thickening (i.e. progressive lesions). A further increase in PAI-1 expressing SMCs was found with advancing atherosclerosis (non-progressive vs. vulnerable lesions  $p < 0.001$ ; Fig. 1b, 2b). In the vulnerable lesions the thin fibrous cap was devoid of

PAI-1 positive SMCs, whereas almost all macrophages stained positive for PAI-1.

No association was found between PAI-1 positivity and Smad2 phosphorylation ( $p = 0.407$ ). Similarly, no relationship was observed between PAI-1 positivity and the amount of inflammatory cells (CD3 or CD68 expressing monocytes ( $p < 0.804$  and  $p < 0.557$  resp., Fig. 4b, 5b)).

*BMP signaling: Smad1,5,8 phosphorylation*

Approximately 10% of the endothelial and intimal SMCs within normal aortic tissue were positive for pSmad1,5,8. Early non-progressive lesions were characterized by a significant increase in pSmad1,5,8 positivity ( $p < 0.02$ ; Figs. 1c, 2c). No further increase was found in the progressive phases of the atherosclerotic process (i.e. progressive atherosclerotic lesions, vulnerable and stabilized lesions).



**Fig. 2.** Expression of pSmad2, PAI-1 and pSmad1,5,8 in the human aorta. **A.** Within the normal aortic wall only few SMCs stain positively for pSmad2. Macrophages and SMCs expressing pSmad2 are abundantly present within the intima and media of non-progressive lesions. The SMCs resident in the thinning cap of the vulnerable lesions remain devoid of Smad2 activation. **B.** Minimal PAI-1 expression was found in the intimal layer of normal aortic tissue. In the vulnerable lesions the thin fibrous cap lacks PAI-1 positive SMCs, whereas almost all macrophages stained positive for PAI-1. **C.** Early non-progressive lesions are characterized by a significant increase in Smad1,5,8 positivity. The vulnerable thin cap mainly consists of pSmad1,5,8 expressing monocytes and/or macrophages. All images were taken at 20x magnification and correspond with the indicated 100 $\mu$ m scalebar. (>: Intimal or medial positively stained SMCs; \*: Positively stained monocytes or macrophages; ^: Intimal SMCs devoid of Smad2 activation).

No relationship was found between Smad1,5,8 phosphorylation and plaque stability (i.e. cap thickness; data not shown).

#### *pSmad1,5,8 and vascular inflammation*

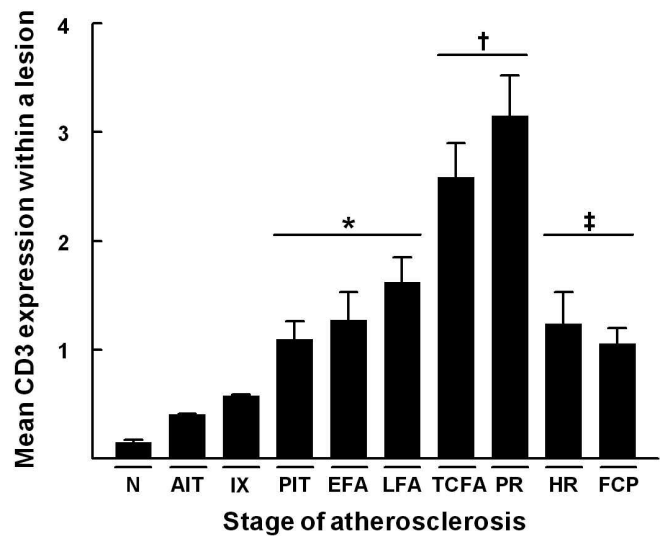
We did not observe a relation between Smad1,5,8 phosphorylation and the CD68 intensity or the amount of CD3 positive cells (Figs. 4c, 5c).

## Discussion

Despite a wealth of animal data, there is limited data available on a putative role of the TGF- $\beta$  superfamily in human atherosclerosis (Bobik, 2006). In the present observational study we focussed on Smad phosphorylation at the C-terminus (the receptor phosphorylated site) to evaluate the activation of the canonical TGF- $\beta$  and BMP signaling pathways during progression and (de)stabilization of human atherosclerotic disease (Heldin et al., 1997). We have established a unique database of vascular tissue containing aortic samples that originate from the perirenal region, a recognized lesion prone location (Stary et al., 1992; Negishi et al., 2001; van Dijk et al., 2010). The results of this study show an immediate activation of TGF- $\beta$  and BMP pathways upon initiation of the atherosclerotic process. No further activation was observed during further progression of the disease. No associations were found between Smad phosphorylation and indices of vascular inflammation or plaque stability.

A large number of animal studies imply members of TGF- $\beta$  family as key regulators of the atherosclerotic process. It was demonstrated that interference with TGF- $\beta$  signaling results in an increased tendency to form lipid lesions, as well as progression towards a more vulnerable plaque phenotype (Mallat and Tedgui, 2001; Grainger, 2007; Frutkin et al., 2009). Based on these animal data it was postulated that the TGF- $\beta$  superfamily members are critically involved in the atherosclerotic process. Data from human studies on the other hand are less consistent and are difficult to interpret, as they are

based on studies on isolated endothelial cells (Volger et al., 2007), surgical material representing the final stages of atherosclerotic disease (Grainger et al., 1995a,b) or TGF- $\beta$  plasma levels (Wang et al., 1997; Bot et al., 2009). Studies on the other superfamily members (Activin and BMP) are currently missing (Heldin et al., 1997; Bobik, 2006). Signaling of these factors occurs



**Fig. 3.** Mean number of CD3 positive cells per lesion plotted against the stage of atherosclerosis. There is no significant increase in CD3 positive cells until the stage of pathological intimal thickening (PIT). As the degree of atherosclerosis advances up to plaque rupture (PR), the amount of CD3 positive cells increases significantly (\*† $p < 0.000$ ). Within stabilized lesions (i.e. HR and FCP) the CD3 positive cells decrease significantly (‡ $p < 0.000$ ). Solid bars represent mean values ( $\pm$ SEM). (Abbreviations; N: Normal, AIT: Adaptive Intimal Thickening, IX: Intimal Xanthoma, PIT: Pathological Intimal Thickening, EFA: Early Fibroatheroma, LFA: Late Fibroatheroma, TCFA: Thin Cap Fibroatheroma, PR: Plaque Rupture, HR: Healed Rupture and FCP: Fibrotic Calcified Plaque).

**Table 2.** Histological classifications of the aortic tissue.

Atherosclerotic cluster	Virmani classification	Male		Female	
		N	Mean age in years [range]	N	Mean age in years [range]
Normal aorta	Normal aorta	4	23.8 [11-40]	3	10.0 [6-13]
Nonprogressive lesions	Adaptive intimal thickening	9	40.5 [31-46]	12	30.4 [21-40]
	Intimal xanthoma	10	37.3 [33-48]	8	34.2 [19-45]
Progressive atherosclerotic lesions	Pathological intimal thickening	5	46.0 [38-55]	7	55.4 [44-67]
	Early fibroatheroma	7	50.9 [44-55]	6	46.0 [42-48]
	Late fibroatheroma	6	55.0 [51-58]	10	51.1 [43-57]
Vulnerable lesions	Thin cap fibroatheroma	6	56.5 [54-59]	3	60.3 [57-67]
	Plaque rupture	7	56.9 [56-59]	2	45.0 [30-54]
Stabilized lesions	Healing rupture	4	58.1 [57-67]	1	57.0 [57-57]
	Fibrotic calcified plaque	2	62.7 [56-67]	2	53.0 [45-63]

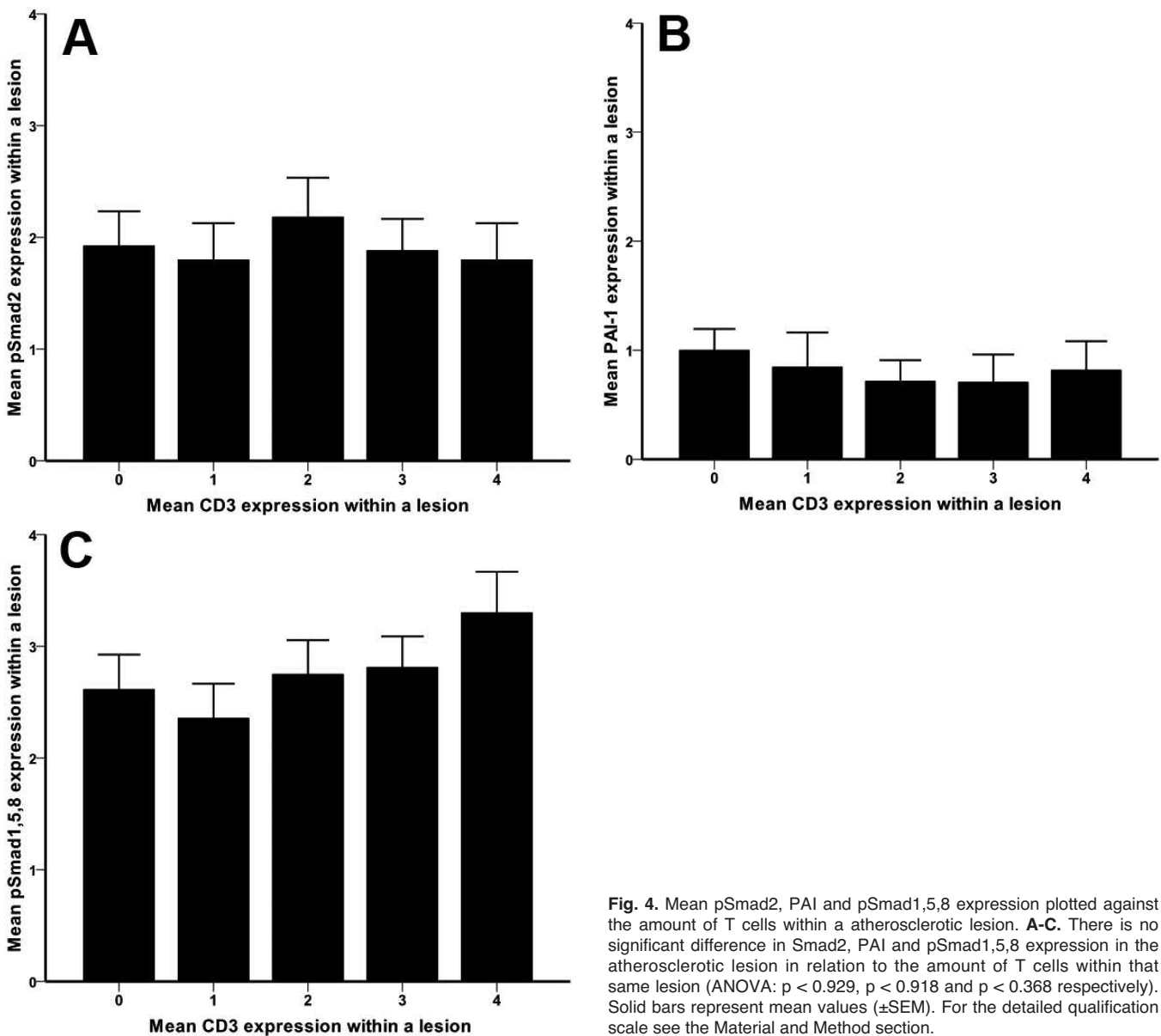
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through both Smad-dependent (Smad2 for TGF- $\beta$  and Activin; Smad1,5,8 for BMP) and Smad-independent pathways (Heldin et al., 1997; Persson et al., 1998). They play a key role in the regulation of cellular proliferation, differentiation, adhesion, apoptosis and production of extracellular matrix components. With respect to inflammation, these factors are generally considered anti-inflammatory (Engelse et al., 1999; Smith et al., 2004; Singh and Ramji, 2006).

Our results for Smad activation show that TGF- $\beta$  family members affect all vascular associated cell types, in all three layers of the arterial wall throughout all stages of human atherosclerosis (de Sousa Lopes et al., 2003). Within the normal aortic wall approximately 10-

15% of the endothelial cells and intimal SMCs express pSmad2 and/or pSmad1,5,8. These findings correspond with previous *in vitro* studies implicating a baseline level of 'naturally' active shear stress induced TGF- $\beta$  (Grainger et al., 1995a,b; Wang et al., 1997; Engelse et al., 1999).

Our study shows an increase in expression of pSmad2 and pSmad1,5,8 in intimal SMCs and macrophages during the transition from normal aortic tissue to early atherosclerotic lesions. These lesions are characterized by adaptive intimal thickening due to infiltrating SMCs, increased extra cellular matrix production and macrophage foamcell formation in response to accumulation of LDL cholesterol (Virmani



**Fig. 4.** Mean pSmad2, PAI and pSmad1,5,8 expression plotted against the amount of T cells within a atherosclerotic lesion. **A-C.** There is no significant difference in Smad2, PAI and pSmad1,5,8 expression in the atherosclerotic lesion in relation to the amount of T cells within that same lesion (ANOVA:  $p < 0.929$ ,  $p < 0.918$  and  $p < 0.368$  respectively). Solid bars represent mean values ( $\pm$ SEM). For the detailed qualification scale see the Material and Method section.

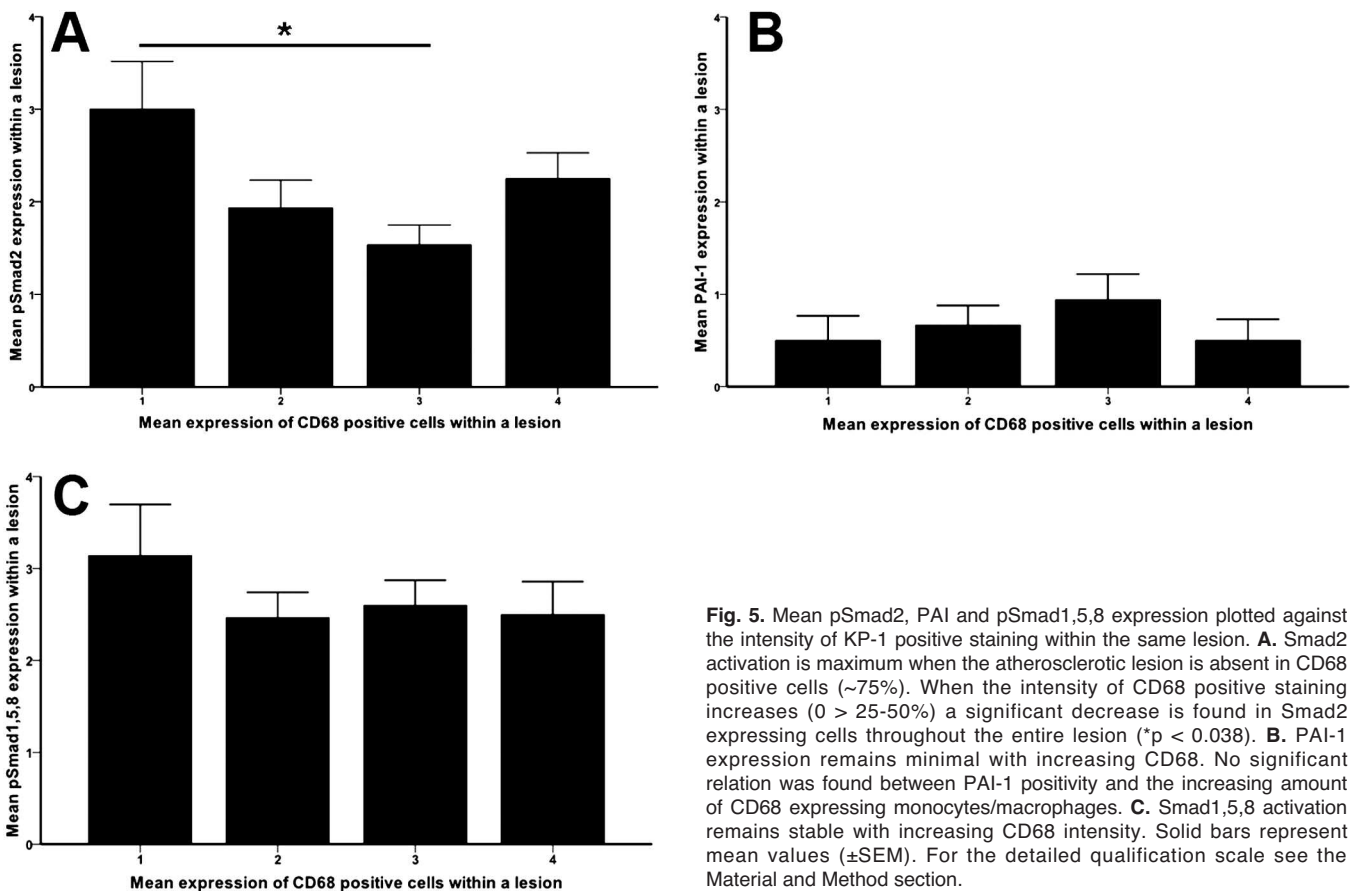
et al., 2000; Hansson, 2001). The immediate increase in Smad phosphorylation in these early stages may reflect induction of TGF- $\beta$  or Activin expression by oxidized LDL (Leonarduzzi et al., 2001). TGF- $\beta$  and Activin are both involved in macrophage biology but their exact role remains unclear. They both enhance the differentiation of monocytic cells into macrophages. TGF- $\beta$  stimulates leukocyte and SMC chemotaxis and as such it may contribute to early macrophage migration and lipid accumulation, whereas Activin-A has been reported to inhibit foamcell formation (Yamada et al., 1992; Engelse et al., 1999; Bobik, 2006).

A remarkable observation in our study was the dominance of pSmad2 positive macrophages over pSmad2 negative macrophages. The Smad2 positive macrophages are often referred to as M2-subtype macrophages, suggesting dominance of the anti-inflammatory class of macrophages in atherosclerotic disease (Shimada, 2009; Spencer et al., 2010). This notion is supported by dominance of CD163 positive macrophages in human atherosclerosis (unpublished data by van Dijk et al.). Although the M2-type of macrophage is generally considered tissue protective, our findings may indicate that, in the context of

atherosclerosis, M2-type macrophages can promote lesion formation.

The TGF- $\beta$  super family of ligands has also broad activities on endothelial cells and matrix homeostasis (Redondo et al., 2006; Singh et al., 2006; Mehta and Attramadala, 2007). Inhibition of TGF- $\beta$  signaling in atherosclerosis-prone mice significantly accelerates lesion development. This suggests an important protective role of endogenous TGF- $\beta$  activity against the development of atherosclerosis (Mallat et al., 2001). Our findings however show that progression of atherosclerotic lesions into more complicated plaques (viz. progressive atherosclerotic lesions, vulnerable lesions and stabilized lesions) is not paralleled by changes in Smad phosphorylation. These findings are not necessarily in conflict with the animal derived data and may suggest that TGF- $\beta$  is involved in the (prevention of) initiation of atherosclerosis.

Destabilization of the fibrous cap is a key event in the development of vulnerable atherosclerotic lesions (Virmani et al., 2000). Thinning of the fibrous cap is associated with a decreasing SMC/macrophages ratio (van Dijk et al., 2010). We did not observe a change in the overall expression in pSmad 2 and pSmad 1,5,8



**Fig. 5.** Mean pSmad2, PAI and pSmad1,5,8 expression plotted against the intensity of KP-1 positive staining within the same lesion. **A.** Smad2 activation is maximum when the atherosclerotic lesion is absent in CD68 positive cells (~75%). When the intensity of CD68 positive staining increases (0 > 25-50%) a significant decrease is found in Smad2 expressing cells throughout the entire lesion (\* $p < 0.038$ ). **B.** PAI-1 expression remains minimal with increasing CD68. No significant relation was found between PAI-1 positivity and the increasing amount of CD68 expressing monocytes/macrophages. **C.** Smad1,5,8 activation remains stable with increasing CD68 intensity. Solid bars represent mean values ( $\pm$ SEM). For the detailed qualification scale see the Material and Method section.

phosphorylation during the transition from stable to vulnerable lesions. As such our findings do not support animal observations suggesting that impaired TGF- $\beta$  signaling underlies formation of an unstable plaque phenotype (Mallat et al., 2001; Lutgens et al., 2002; Frutkin et al., 2009). However, it should be noted that TGF- $\beta$  is a potent stimulus of connective tissue accumulation, and is implicated in the pathogenesis of fibrotic disorders with Smad3 as a key intracellular signal transducer (Lakos et al., 2004). Human Plasminogen Activator Inhibitor -1 (PAI-1) is a gene that is potently induced by TGF- $\beta$  and specifically binds the TGF- $\beta$ /activin pathway-restricted Smad3 protein (Dennler et al., 1998). Our results show a significant increase in PAI-1 expression with progression towards vulnerable lesions, indicating that TGF- $\beta$  is involved in the more advanced stages of the atherosclerotic disease. Without a valid pSmad3 antibody available for paraffin embedded tissue we cannot firmly conclude whether or not TGF- $\beta$  is highly relevant as regards to cap thickness.

Mallat et al. highlight the specific involvement of TGF- $\beta$  in the regulation of T-cell biology and postulated disruption of TGF- $\beta$  signaling as an explanation for the increased number of T-cells in atherosclerosis (Mallat et al., 2002; Gojova et al., 2003). Our results show a remarkable increase in T-cell content during the evolution of the atherosclerotic lesion towards a vulnerable plaque. Such a mechanism is not supported by observations from this study, in which no association between TGF- $\beta$  and BMP signaling and T-cell content was observed.

Although our findings are highly consistent and show a distinctive pattern throughout the entire atherosclerotic spectrum, a potential limitation of the present study arises from the fact that we were restricted to paraffin embedded aortic tissue. Moreover, although the immunohistochemical staining procedures used for the detection of phosphorylated Smads allow for a spatial evaluation, they are semi-quantitative and do not allow direct conclusions with respect to the TGF- $\beta$  or Activin signaling (Heldin et al., 1997). As such, it should be noted that we cannot exclude that the Activin or TGF- $\beta$  pathway are activated simultaneously during the atherosclerotic process.

### Conclusion

All in all, our findings in this unique database covering the entire spectrum of human atherosclerotic disease do not specifically characterize TGF- $\beta$  and BMP signaling as key regulators in the progression and complications of atherosclerosis. Our material does not rule out a role for TGF- $\beta$  and BMP in the initiation of the earliest atherosclerotic lesions. As this paper focuses on Smad phosphorylation as read-out for TGF- $\beta$  and BMP signaling, it cannot be excluded that the signaling through different pathways also mediate the activity of TGF- $\beta$  and BMP in atherosclerotic disease. No relationship was found between Smad2, or Smad1,5,8

phosphorylation and plaque growth, cap destabilization or plaque stabilization.

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