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# Transglutaminase activity regulates atherosclerotic plaque composition at locations exposed to oscillatory shear stress

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ABSTRACT

*Objective:* Atherosclerosis preferentially develops at sites of disturbed blood flow. We tested the hypothesis that transglutaminase activity plays a role in plaque development at these locations.
*Methods and results:* Exposure of endothelial cells to steady flow (7 dynes/cm$^2$) was associated with relatively low transglutaminase activity, whereas under low oscillatory flow (1.3 ± 2.6 dynes/cm$^2$) endothelial cells showed a >4-fold higher level of transglutaminase activity. Under oscillatory flow, transglutaminase activity increased the expression of the chemokine MCP-1 (CCL2). In vivo, oscillatory flow was induced by placement of a tapered perivascular cast around the carotid artery of type 2 transglutaminase (TGM2) knockout mice and WT counterparts. After 2 days, significantly less monocytes adhered to the endothelium in TGM2 knockout mice as compared to WT. In a more chronic setting, ApoE knockout mice that were equipped with the flow-modifying cast developed lesions proximal to the cast (low shear stress), and distal to the cast (oscillatory shear stress). Inhibition of transglutaminase induced a marked reduction in macrophage and fat content in distal lesions only. In addition, lesion size was increased in this area, which was attributed to an increase in smooth muscle content.
*Conclusion:* Oscillatory shear stress increases endothelial transglutaminase activity. In turn, transglutaminase activity affects the expression of MCP-1 in vitro and monocyte recruitment in vivo. In a mouse model of atherosclerosis, transglutaminase activity has a major effect on plaque composition under oscillatory shear stress.



## 1. Introduction

Atherosclerosis is a multi-factorial disease involving genetic factors, life-style, and local hemodynamic conditions. Lesions develop preferably in large vessels at bifurcations, curvatures and side branches [1]. Here, endothelial cells are exposed to oscillatory and low shear stress. This hemodynamic profile affects the local coagulation state, increases the expression of leukocyte adhesion factors, and promotes the formation of reactive oxygen species [2–4]. These conditions may promote the infiltration of leukocytes, a key step in the initiation of atherosclerosis.

Transglutaminases are enzymes with a broad spectrum of actions, including cell signaling and protein cross-linking, by formation of a γ-glutamyl-ε-lysine cross-link [5]. Transglutaminase type 2 (TGM2 or tTG) is highly expressed in endothelial cells, and accumulating evidence suggests a role for TGM2 in processes thought to be relevant for initiation and progression of atherosclerosis. Thus, endothelial expression of TGM2 is increased by turbulent shear stress [6], downregulated by high shear stress [7], and increased after exposure to inflammatory cytokines [8,9]. TGM2 is present at endothelial cell–cell and cell-matrix contact points [10], and inhibition of TGM2 leads to reduced cell spreading and attachment [11]. Activation of TGM2 in endothelial cells by auto-antibodies against TGM2 from patients with celiac disease was found to increase permeability and leukocyte transmigration [12]. We previously found that inhibition of transglutaminase activity reduces the infiltration of macrophages in a model based on a non-flow limiting cuff placed around the femoral artery in ApoE3 Leiden mice on high fat diet [13]. Based on these findings, we hypothesized that TGM2 activity is critically involved in plaque development under disturbed flow. Indeed, in the present study we

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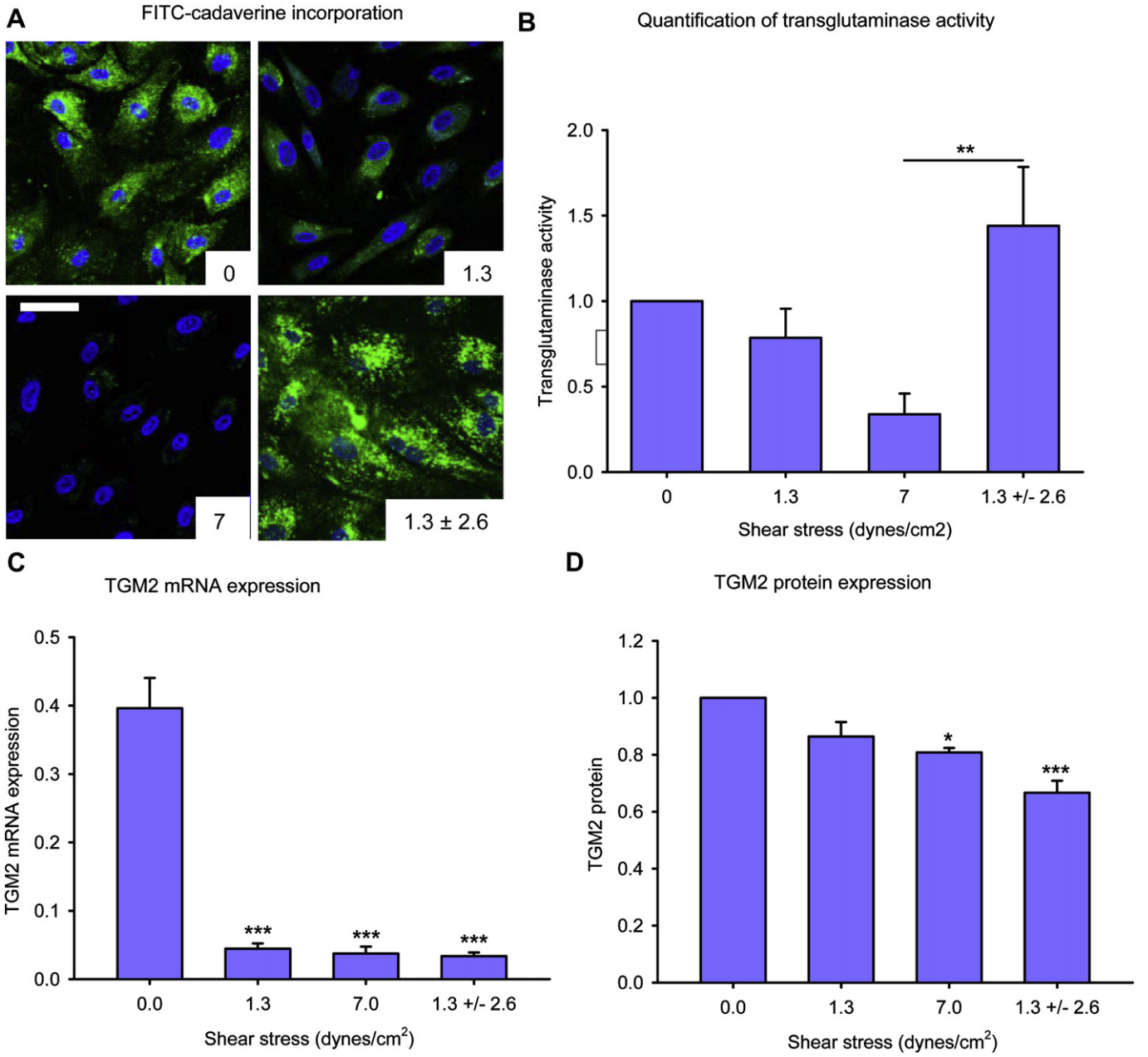


**Fig. 1.** Transglutaminase activity is regulated by shear stress. A) Confocal microscopic images to detect FITC-cadaverine incorporation in HUVECs as a measure of transglutaminase activity. Cells were cultured under static conditions or exposed for 24 h to either laminar shear stress at 1.3 dyne/cm$^2$, 7 dyne/cm$^2$ or oscillatory shear stress at 1.3 ± 2.6 dyne/cm$^2$. Scale bar = 50 μm. B) Quantification of FITC-cadaverine incorporation in endothelial cells exposed to 0, 1.3, 7 and 1.3 ± 2.6 dyne/cm$^2$ respectively ($n = 5$ each). Data are normalized to the number of cells and to the respective static counterparts. C) TGM2 mRNA levels in HUVECs cultured under shear stress levels of 0, 1.3, 7 and 1.3 ± 2.6 dyne/cm$^2$ respectively ($n = 4$ each). Data are normalized to the housekeeping gene, ribosomal protein P0. D) TGM2 protein levels in HUVECs cultured under shear stress levels of 0, 1.3, 7 and 1.3 ± 2.6 dyne/cm$^2$ respectively ($n = 2$–4). Data are normalized to tubulin expression as reference protein. *Indicates $p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

show that endothelial TGM2 is an important regulator of leukocyte infiltration, plaque size and plaque composition in areas exposed to oscillatory shear stress.

## 2. Methods

An expanded description is available in the supplemental data.

### 2.1. In vitro flow setup and endothelial cell culture

HUVECs were cultured in Ibidi flow chambers and exposed to different flow conditions for 24 h: static, steady low flow (1 ml/min), normal flow (5.4 ml/min) or oscillatory low flow (1 ml/min ± 2 ml/min) leading to shear stress levels of 0, 1.3, 7 and 1.3 ± 2.6 dyne/cm$^2$ respectively. Transglutaminase activity was determined using the fluorescent substrate FITC-cadaverine.

### 2.2. In vivo mouse model of disturbed blood flow

Female TGM2 knockout mice and wild type (WT) counterparts ($n = 5$ each) were equipped with a tapered polymethylpentene cast (diameter 200–400 μm, 1.5 mm length) around the carotid artery [14]. This tapered cast creates different shear stress regions, i.e. lowered shear stress proximal of the cast, an increased shear stress

region inside the cast and an oscillatory shear stress region distal from the cast [15].

### 2.3. Mouse model of atherosclerosis

A mouse model of atherosclerosis based on the perivascular cast was used to investigate the effect of transglutaminase inhibition on plaque development [14]. Female ApoE$^{-/-}$ ($n = 16$) mice were fed a Western type diet containing 0.25% cholesterol. In eight mice, in addition to the cast, an osmotic minipump (Alzet, model 1004) was placed in the intraperitoneal cavity containing the transglutaminase inhibitor L682777. Velocity and diameter measurements were performed using a Vevo 770 system (Visualsonics) and a 40 MHz probe (RMV-704). After sacrifice, sections of carotid arteries were stained with Oil Red O to determine fat-deposits, and antibodies against CD68 and 1A4 to identify macrophages and smooth muscle cells respectively.

## 3. Results

### 3.1. Oscillatory shear stress increases transglutaminase activity in endothelial cells

To investigate the effect of shear stress profiles on transglutaminase activity in endothelial cells, HUVECs were cultured under static, steady, or oscillatory flow profiles for 24 h in the presence of the fluorescent substrate FITC-cadaverine. A clear difference in transglutaminase activity was found between steady shear stress at a level of 7 dynes/cm$^2$ as compared to oscillatory shear stress, which appeared to induce the highest level of transglutaminase activity (Fig. 1A and B). Usually, a granular staining pattern of transglutaminase activity was found, with sometimes an increased density around the nucleus. RT-PCR results show that the levels of TGM2 mRNA were substantially decreased in all groups exposed to flow as compared to static, with no apparent differences between shear stress patterns (Fig. 1C). The effect of flow on TGM2 mRNA levels was reflected in protein levels, albeit to a lesser extent. Thus, western blot analysis showed that protein levels decreased in all groups exposed to flow, which reached statistical significance for the highest steady flow group and the oscillatory flow.

### 3.2. Inhibition of TGM2 decreases the expression of MCP-1 under oscillatory shear stress

Oscillatory shear stress is often reported to promote a pro-inflammatory state of the endothelium. To study the relevance of the relatively high transglutaminase activity in this context, we studied the expression of MCP-1, ICAM-1, VCAM-1 and E-selectin under oscillatory shear. We found that when HUVECs exposed to oscillatory shear were incubated with the transglutaminase inhibitor L682777, the expression of MCP-1 was significantly decreased, to less than 25% of the control level. E-selectin, ICAM-1, and VCAM-1 expression was not significantly changed (Fig. 2). In comparison to laminar shear stress (7 dynes/cm$^2$), oscillatory shear did not significantly affect the expression of MCP-1, ICAM-1, or E-selectin (data not shown).

### 3.3. TGM2 knockout mice show reduced monocyte/macrophage recruitment

Since we found that transglutaminase activity is increased by oscillatory shear stress and affects the expression of MCP-1, we reasoned that this could affect monocyte recruitment. Therefore, in vivo experiments were done on the carotid artery using a tapered perivascular cast. This flow-modifying cast induces oscillatory shear stress in the area distal to the cast, and reduces shear stress

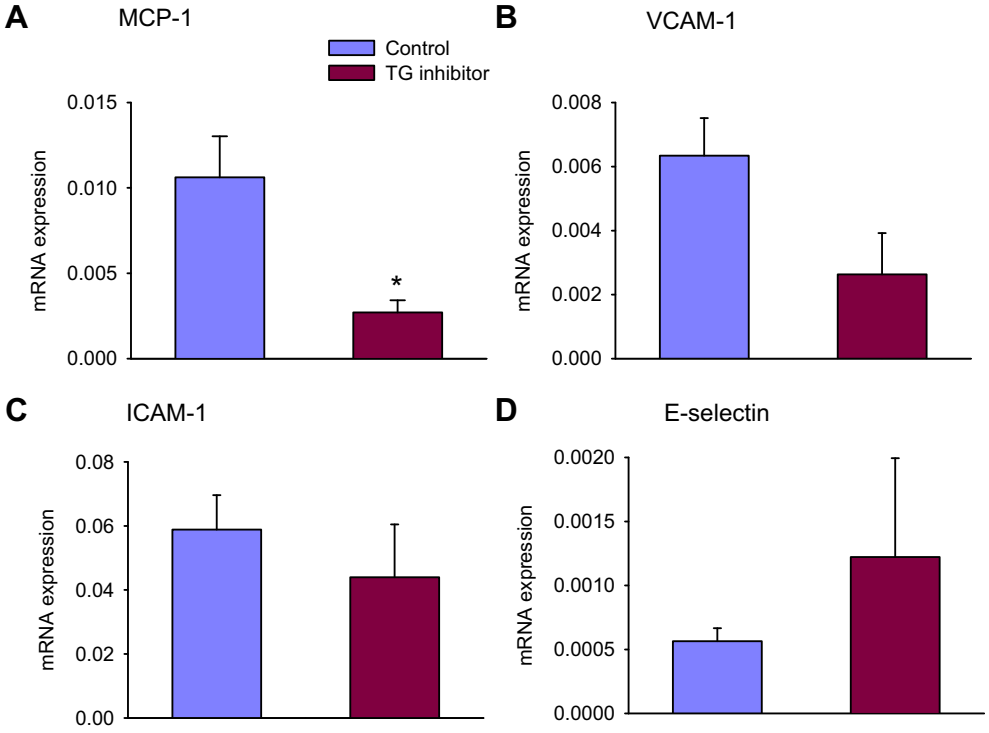


**Fig. 2.** Transglutaminase regulates MCP-1 expression under oscillatory shear stress effect of transglutaminase inhibition (TG inhibitor) on mRNA expression of A) MCP-1, B) VCAM-1, C) ICAM-1 and D) VCAM-1 in HUVECs exposed to oscillatory shear stress. Transglutaminase inhibition induced a significant decrease in the expression of MCP-1 ($P < 0.05$, $n = 5$ each). Data are normalized to the housekeeping gene, ribosomal protein P0. *Indicates $p < 0.05$.

proximal to the cast [14]. The cast was placed in TGM2 knockout mice and WT counterparts. After 2 days, an en face staining of the endothelium for CD68 positive cells was performed. Monocytes/macrophages were counted in the area exposed to oscillatory shear stress. TGM2 knockout mice showed significantly less CD68 positive cells (Fig. 3A and B). The other areas, both proximal to the cast and within the cast, were devoid of CD68 positive cells.

### 3.4. Mouse model of atherosclerosis

Subsequent experiments were aimed to test the relevance of transglutaminase activity in a setting of atherosclerosis induced by altered shear stress. For this purpose, the constrictive cast was placed around the carotid artery of ApoE$^{-/-}$ mice that were fed a high fat diet. In this model, atherosclerotic lesions are induced by low shear stress and oscillatory shear stress patterns before and after the cast respectively. To study the expression of TGM2 in this model, sections of carotid arteries of ApoE$^{-/-}$ mice were stained. Supplemental Fig. 1 shows that TGM2 staining co-localizes with endothelial cells, which both cover the lesion as well as the unaffected part of the vessel wall. Staining was also present in smooth muscle cells, particularly towards the lumen of the vessel. Weak staining was also observed in the lesion, possibly related to infiltrating macrophages. To test the role of transglutaminase activity, eight mice additionally received the transglutaminase inhibitor L682777, delivered continuously by an osmotic minipump. After the experimental period of four weeks, no differences were found in body weight of the control mice and mice receiving the inhibitor (see Supplemental Table 1). Cholesterol levels as well as triglyceride levels in plasma were also not significantly different between control mice and TG inhibitor treated mice.

### 3.5. Transglutaminase inhibition affects lesion size and composition in the oscillatory shear stress region

Atherosclerotic lesion development was analyzed hemodynamically and histologically four weeks after cast placement. The combination of cast placement and lesion growth resulted in a similar flow reduction in control mice and mice that received the transglutaminase inhibitor (control: $-58 \pm 4\%$, inhibitor: $-58 \pm 7\%$ lower than contralateral flow, Supplemental Table 1). Irrespective of the presence of the inhibitor, plaques in the low shear stress region were much larger as compared to lesions in the oscillatory shear stress regions ($p < 0.01$, see Fig. 4). When comparing plaque sizes in the low shear stress region, transglutaminase inhibition did not significantly influence the atherosclerotic lesion size ($88.5 \pm 8.1 \times 10^3$ μm$^2$ in control mice versus $96.9 \pm 12.9 \times 10^3$ μm$^2$ in mice receiving the transglutaminase inhibitor, $p = 0.6$, Fig. 4A and B). In the oscillatory shear stress region however, transglutaminase inhibition resulted in larger lesions ($12.7 \pm 1.3 \times 10^3$ μm$^2$ in control mice and $24.1 \pm 3.7 \times 10^3$ μm$^2$ in transglutaminase inhibitor mice, $p = 0.01$, Fig. 4A and B).

In the low shear stress region, vessels showed outward remodeling associated with lesion development (Supplemental Table 1). This Glagov [16] remodeling is believed to preserve lumen diameter in the face of a growing plaque. Transglutaminase inhibition did not influence this remodeling. The oscillatory shear

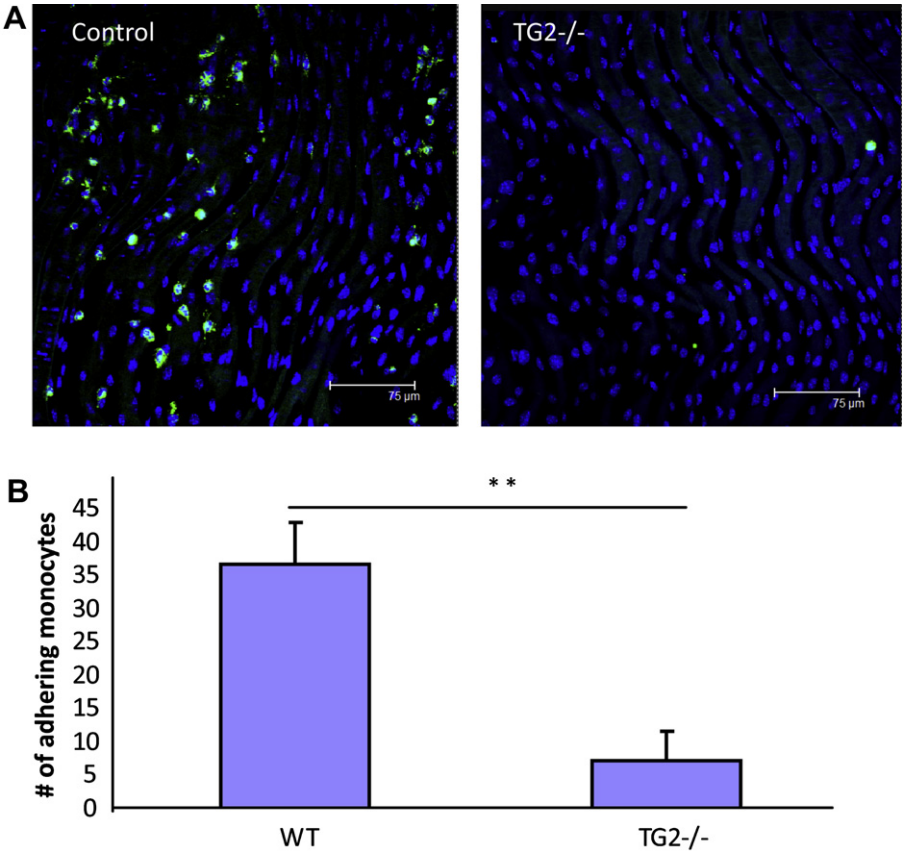


**Fig. 3.** Adhering monocytes/macrophages in the carotid artery lumen of TGM2 knockout mice and WT counterparts. A) En face staining for CD68 positive cells (green) adhering to the endothelium. Endothelial cell nuclei are shown by Dapi staining (blue). B) Quantification of the number of CD68 positive cells per field of view (0.14 mm$^2$). TGM2 knockout mice ($n = 5$) showed significantly less adhering monocytes/macrophages as compared to WT controls ($n = 5$). **Indicates ($p < 0.01$).

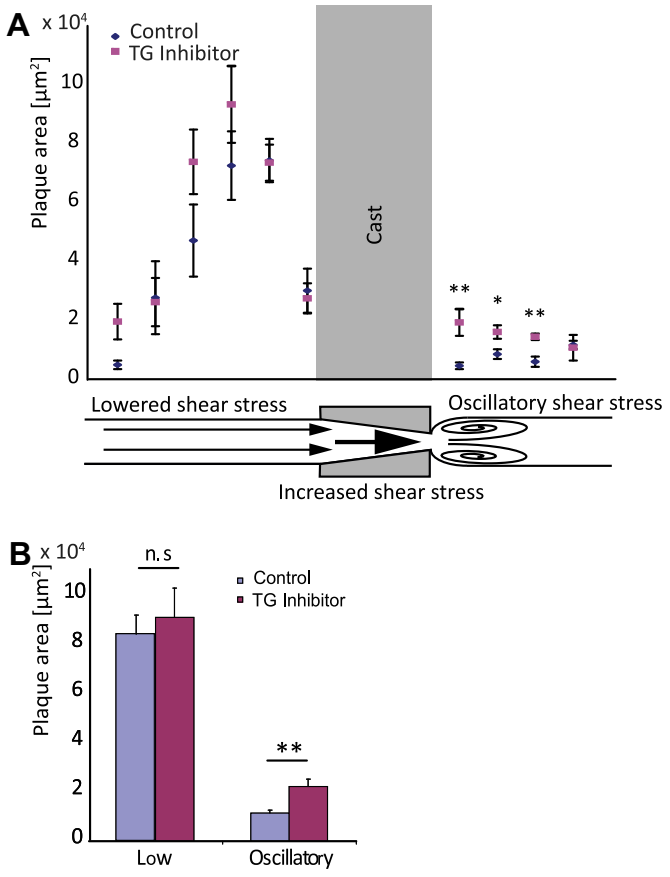


**Fig. 4.** Lesion size and role of transglutaminase depend on localization. A) Plaque area of control mice ($n = 8$) and mice receiving the transglutaminase inhibitor ($n = 8$) at several locations proximal and distal from the cast. B) Quantification of plaque areas based on sections with the largest lesion in low and oscillatory shear stress regions. Transglutaminase inhibition leads to larger lesions in the oscillatory shear stress region. * Indicates $p < 0.05$; ** Indicates $p < 0.01$. N.s. = not significant.

stress region did not show a significant increase in vessel diameter, both with and without the transglutaminase inhibitor.

Plaque composition was analyzed by staining cross-sections of the atherosclerotic carotid arteries for lipid content by Oil Red O (Fig. 5A and B). Lesions in the low shear stress region contained more lipid as detected by Oil Red O staining compared with the oscillatory shear stress region. After transglutaminase inhibition, atherosclerotic lesions in the oscillatory shear stress region contained markedly less lipids compared with control mice. In contrast, the transglutaminase inhibitor did not affect lipid content in the low shear stress area. High magnification images of the oscillatory shear stress region are shown in Supplemental Fig. 2.

Macrophages were detected by staining cross-sections for CD68 (Fig. 5C and D). No significant difference in macrophage content was detected between lesions developed in the low shear stress region, as compared to lesions in the oscillatory shear stress region. Transglutaminase inhibition leads to a pronounced reduction in macrophage accumulation in lesions that developed in the oscillatory shear stress region, but did not affect macrophage content in the low shear stress area.

The relative amount of smooth muscle cells in the lesions in the low shear stress region did not significantly differ from lesions in the oscillatory shear stress region (Fig. 5E and F). There was also no effect on smooth muscle content in the low shear stress area with transglutaminase inhibition. However, inhibition of transglutaminase resulted in increased smooth muscle cell content in lesions in the oscillatory shear stress region compared to control.

As we observed such a profound effect of transglutaminase inhibition on smooth muscle content in the oscillatory shear stress region, we further explored the effect of transglutaminase inhibition on cultured smooth muscle cells. This showed that opposite to the in vivo model, transglutaminase inhibition decreased smooth muscle proliferation in vitro (Supplemental Fig. 3A). As an increase in lesion smooth muscle content also could be a result of migration from the media, we also tested the effect of transglutaminase inhibition on smooth muscle migration in vitro. However, we observed that transglutaminase inhibition did not affect smooth muscle migration in a scratch wound assay (Supplemental Fig. 3B).

## 4. Discussion

The preferential localization of atherosclerotic lesions to areas of disturbed shear stress is well documented [1,17]. The involved sequence of events is not fully elucidated but includes locally increased oxidative stress, induction of a more pro-inflammatory and pro-coagulant endothelial phenotype [2], followed by infiltration of leukocytes and lipid uptake by macrophages. The importance of the current study is that it provides evidence that endothelial TGM2 is a key element in the initial events leading to disturbed shear-induced atherosclerosis. Thus, we established that TGM2 shows a relatively high activity during oscillatory shear stress in vitro and affects the expression of the chemotactic protein MCP-1. We then showed that after onset of oscillating flow in vivo in carotid arteries, far less monocytes bind to endothelial cells in TGM2 KO versus WT mice. Finally, chronic transglutaminase inhibition had major effects on the size and nature of atherosclerotic plaques at oscillating flow spots, rendering them larger but with a much lower macrophage and fat content. This spectrum of TGM2 effects was not observed under low laminar flow, pointing towards the involvement of TGM2 in atherogenesis in specifically oscillatory flow regions.

We considered that TGM2 expression could be regulated by shear stress, and found that both laminar flow and oscillatory flow decreased TGM2 mRNA and protein levels. Thus, the increase in transglutaminase activity with oscillatory shear stress appears to be regulated entirely at the posttranslational level. A key regulator of transglutaminase activity in this respect may be nitric oxide (NO). High laminar shear stress is well known to stimulate NO release, whereas there is substantial evidence for ROS production under oscillating flow [18]. TGM2 is inactivated by NO through nitrosylation of thiol groups [19] which has recently been shown to be relevant for endothelial cell function [20,21] and small artery remodeling [22]. Thus, shear-induced NO release could inactivate TGM2 under healthy physiological conditions. In a situation of increased ROS production, NO could be quenched by ROS and thereby lead to activation of TGM2. A second possible link between ROS and TGM2 is an increase in intracellular calcium to concentrations that could enhance TGM2 activity [23]. While ROS-induced TGM2 activation is conceivable on the basis of such pathways, further research is needed to identify the exact mechanism. Shear stress was also shown to affect TGM2 secretion from endothelial cells [20], but whether this plays a role in the expression of MCP-1 and transendothelial migration of monocytes remains to be established.

We found that transglutaminase inhibition reduced the expression of MCP-1 under oscillatory shear stress. MCP-1 is upregulated in the in vivo cast model both in the area proximal to the cast as well as the area distal to the cast [24]. It is therefore unclear if endothelial MCP-1 expression is affected by transglutaminase inhibition only in the area exposed to oscillatory shear. Interestingly, a recent study using the currently used flow-modifying cast showed a preferential activation of NF-κB in

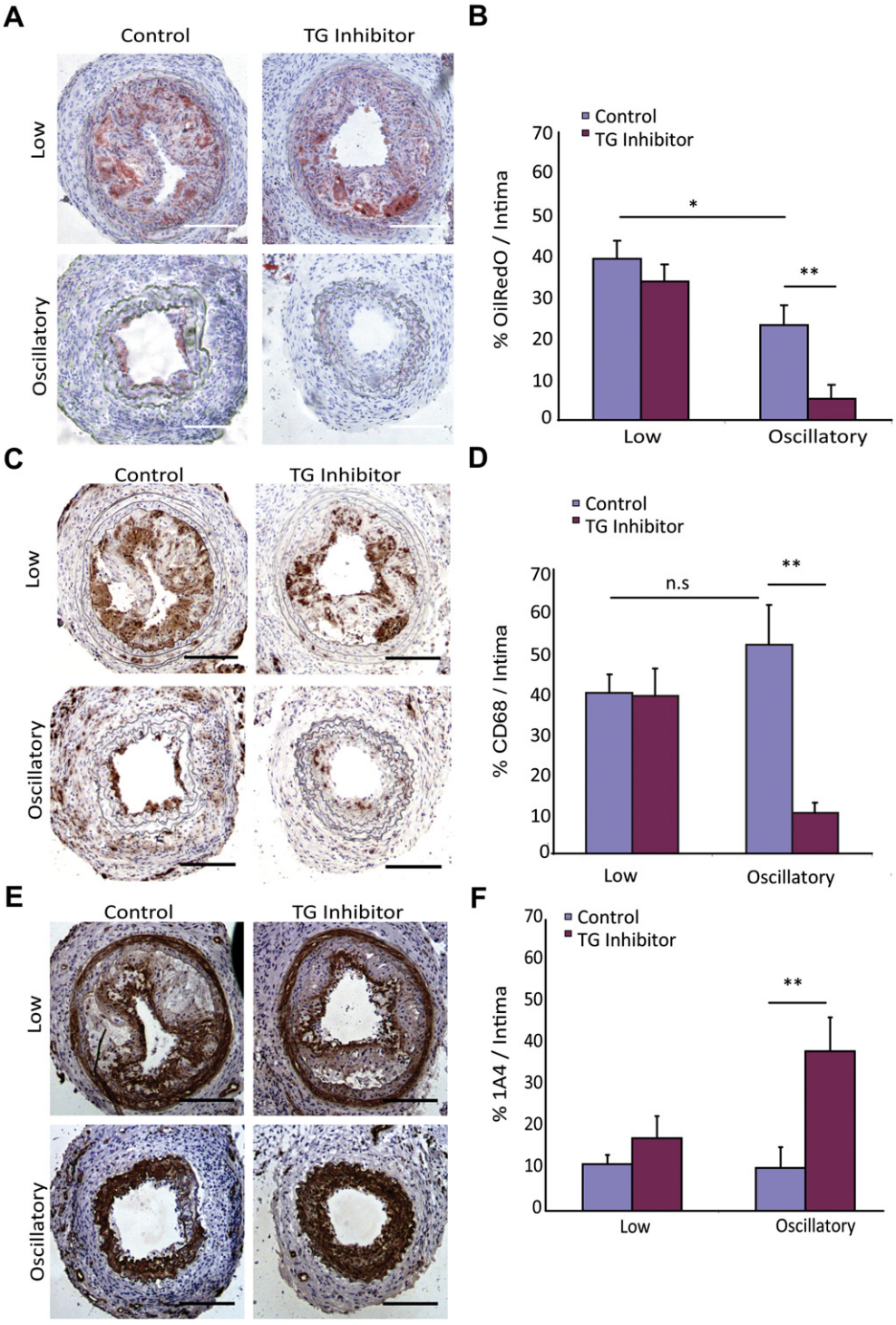


**Fig. 5.** Transglutaminase inhibition selectively affects lesion composition under oscillatory shear stress A) Oil Red O staining for lipids in lesions in the low and oscillatory shear stress regions for control mice ($n = 8$) and mice receiving the transglutaminase inhibitor ($n = 8$). B) Quantification of lipid accumulation by Oil Red O staining. Data are shown as fraction of the intima area. C) CD68 staining to detect macrophages D) Quantification of CD68 positive area. E) Smooth muscle actin staining (1A4). F) Area positive for 1A4 as fraction of the intima area. Scale bar = 150 μm. *Indicates $p < 0.05$; **Indicates $p < 0.01$. N.s. = not significant.

endothelial cells in the area distal to the cast, as compared to the area proximal to cast [25]. Work from several groups showed that TGM2 is essential in the prolonged activation of the transcription factor NF-κB in various cell types [26–28]. Whether TGM2 could act similarly in endothelial cells is currently unknown, but this would provide an attractive possible mechanism to explain the selective impact of transglutaminase inhibition during oscillatory shear. On the other hand, we found no changes in expression of three other tested NF-κB targets, the adhesion molecules E-selectin, ICAM-1, and VCAM-1. Future work is therefore needed addressing the mechanisms of TGM2-dependent MCP-1 activation in detail.

Transglutaminase inhibition profoundly changed the plaque phenotype in the oscillating flow region. The strong reduction in macrophage content is consistent with our finding that in TGM2 KO

mice, far less monocytes/macrophages bind to the endothelium in the first 48 h after onset of oscillating flow. The large reduction in lipid content is likely to be secondary to the impaired macrophage accumulation, since macrophages are responsible for most of the lipid uptake. An unexpected finding was that lesion size and SMC content were increased following transglutaminase inhibition. Additional experiments showed that transglutaminase inhibition did not affect smooth muscle migration in vitro, and actually decreased proliferation in vitro (Supplemental Fig. 3). The increase in smooth muscle content in vivo therefore appears a paradoxical result. However, this may also be an indirect consequence of decreased macrophage content, as several groups showed that activated monocytes/macrophages can induce smooth muscle apoptosis [29,30]. Thus, TGM2 inhibition could indirectly, through a reduction in macrophage content, increase smooth muscle survival.

The role of transglutaminases in atherosclerosis has been addressed before, but not in the context of disturbed flow. In a previous study we observed a reduction in macrophage content after transglutaminase inhibition in a model of femoral artery injury, induced by a non-restrictive perivascular cuff without changes in shear stress [13]. It thus seems that transglutaminases are activated in that model because of the injury. ApoE$^{-/-}$/TGM2$^{-/-}$ cross-bred mice were reported to have a decrease in lesion size, fibrosis and an increase in lesion macrophage content in one study [31], while others only found a mild reduction in lesion fat content in these mice [32]. Possibly, apart from differences in the anatomical locations that were studied and the hemodynamic profiles that are associated with this, the genetic background contributes to these apparently contradictory results, as TGM2$^{-/-}$ mice from different origin were used [31,32].

In the present study we focused on transglutaminase activity in endothelial cells and the subsequent implications for lesion development and composition. However, monocytes/macrophages are also known to express both TGM2 and the transglutaminase FXIII. It has been reported that monocytes show an increase in TGM2 expression upon attachment to the endothelium [33], which suggests that this may play a role in transendothelial migration. Thus, one could argue that in the current atherosclerosis model a direct effect of the inhibitor on monocytes could contribute to the results. However, this seems not very likely as the inhibitor was applied systemically, while the effects on plaque progression were only seen in the oscillatory shear stress region.

In conclusion, we showed that oscillatory shear stress increases endothelial transglutaminase activity as compared to laminar shear stress (7 dynes/cm$^2$). Inhibition of transglutaminase activity reduces MCP-1 expression in vitro. In vivo, TGM2 knockout mice show reduced monocyte/macrophage recruitment to sites of oscillatory shear stress. Inhibition of transglutaminase activity in vivo reduced both macrophage content and lipid content in plaques that developed in an oscillatory shear stress region. An adverse effect of the transglutaminase inhibitor however, was the increase in lesion size in this region. This was associated with an increase in the area occupied by smooth muscle cells. Taken together, these effects point out a beneficial effect of transglutaminase inhibition with respect to plaque composition in regions exposed to oscillatory shear stress.

## Conflict of interest

None declared.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2012.07.044.

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