Mast cells in human carotid atherosclerotic plaques are associated with intraplaque microvessel density and the occurrence of future cardiovascular events

Sanne Willems1*, Aryan Vink2, Ilze Bot3, Paul H.A. Quax4, Gert Jan de Borst5, Jean-Paul P.M. de Vries6, Sander M. van de Weg1, Frans L. Moll5, Johan Kuiper3, Petri T. Kovanen7, Dominique P.V. de Kleijn1,8,9, Imo E. Hoefer1, and Gerard Pasterkamp1

1Experimental Cardiology Laboratory (room G02.523), University Medical Centre Utrecht, Heidelberglaan 100, 3584 CX, Utrecht, the Netherlands; 2Department of Pathology, University Medical Centre Utrecht, Utrecht, the Netherlands; 3Division of Biopharmaceutics, Leiden/Amsterdam Centre for Drug Research, Gorklaeus Laboratories, Leiden, the Netherlands; 4Department of Cardiology, Leiden University Medical Centre, Leiden, the Netherlands; 5Department of Vascular Surgery, University Medical Centre Utrecht, Utrecht, the Netherlands; 6Department of Vascular Surgery, St Antonius Hospital, Nieuwegein, the Netherlands; 7Wihuri Research Institute, Helsinki, Finland; 8Interuniversity Cardiology Institute of the Netherlands (ICIN), Utrecht, the Netherlands; and 9Cardiovascular Research Institute and Surgery, National University Hospital Singapore, Singapore

Received 10 January 2013; revised 30 March 2013; accepted 10 May 2013; online publish-ahead-of-print 11 June 2013

See page 3681 for the editorial comment on this article (doi:10.1093/eurheartj/eht259)

Aims

Human autopsy, animal, and cell culture studies together have merged in a concept suggesting participation of mast cells (MCs) in the generation of atherosclerotic plaques. More specifically, these studies have suggested MC-induced intraplaque neovascularization as one mechanism by which MCs may render the plaques vulnerable. The present study was designed to assess the association between MC numbers and neovascularization in human atherosclerotic plaques, and to relate the abundance of plaque MCs to the occurrence of adverse cardiovascular events during the follow-up.

Methods and results

Atherosclerotic plaques of 270 patients suffering from carotid artery stenosis were stained for the presence of MCs (MC tryptase). Furthermore, during a follow-up of 3 years, cardiovascular-related endpoints were assessed in 253 patients. On average a high number of MCs were observed per plaque cross-section [median 108 (55–233) cells per section]. Plaques with high MC numbers revealed an unstable lipid-rich inflammatory phenotype and were associated with symptomatic patients. In addition, MC numbers were positively associated with microvessel density ($r = 0.416, P < 0.001$). Patients with high intraplaque MC numbers showed significantly more cardiovascular events during the follow-up (58/142 vs. 31/111 events, $P = 0.029$). In a multivariate analysis with correction for the main risk factors of cardiovascular diseases, MCs remained independently associated with adverse cardiovascular events ($P = 0.025$).

Conclusion

Mast cells are highly prevalent in human carotid atherosclerotic lesions and associated with plaque microvessel density. Furthermore, intraplaque MC numbers associate with future cardiovascular events.

Keywords

Mast cells • Unstable plaque • Intraplaque neovascularization • Atherosclerosis • Inflammation

Introduction

Acute clinical manifestations in patients suffering from atherosclerosis are usually caused by a plaque rupture followed by intraluminal thrombus formation. Unstable plaques that are prone to rupture are characterized by a large lipid core, a thin fibrous cap, intraplaque neovascularization, and a large inflammatory cell infiltrate composed of macrophages, T-cells and mast cells (MCs).1 Mast cells are inflammatory cells traditionally known for their role in allergy and in innate immune responses.2 Mast cells are filled with cytoplasmic secretory

* Corresponding author. Tel: +31 887557155, Fax: +31 302522693, Email: s.willems-3@umcutrecht.nl

Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2013. For permissions please email: journals.permissions@oup.com
granules, which they exocytose upon activation. Such activated MCs are particularly abundant at the sites of atheromatous erosion or rupture in patients who have died of myocardial infarction. More recently, animal experiments have demonstrated that MCs are also actively involved in the initiation and progression of atherosclerotic disease. For example, more unstable atherosclerotic lesions were observed in ApoE−/− mice treated with MC activators. This unfavourable phenotype of plaque destabilization could be prevented by treatment with a MC stabilizer such as sodium cromolyn or tranilast. In line with these data, it was observed that MC-deficient mice were demonstrated to develop smaller lesions with a more stable phenotype.

One of the proposed mechanisms by which MCs may render the plaques unstable is their ability to induce neovascularization under normal and pathological conditions. Importantly, in autopsy studies it has been observed that MCs accumulate in neovessel-rich areas of atherosclerotic plaques. Moreover, it was demonstrated that the MCs situated near the newly formed vessels contained basic fibroblast growth factor, a potent pro-angiogenic factor. These data suggest that MCs are involved in the formation of microvessels in the atherosclerotic plaque and so accelerate plaque progression into an unstable plaque phenotype.

We recently showed that intraplaque neovascularization and intraplaque haemorrhage (IPH) in carotid plaques are predictive for cardiovascular events elsewhere in the human body. This underscores the need for improved understanding how intraplaque neovascularization renders atherosclerotic plaques prone to trigger atherothrombotic events in the atherosclerosis-prone segments of the arterial tree. Although it is known that the accumulation of inflammatory cells into the plaque largely contributes to the process of plaque destabilization, the size of the athero-express bank allows the identification of inflammatory cell types that are independently associated with intraplaque neovascularization. In the present study, we thus aimed to examine the association of local MC numbers with local plaque characteristics, such as plaque neovascularization, in a large number of human samples. As an association between local MCs and future cardiovascular events has not been investigated previously, we related plaque MC numbers with the occurrence of future cardiovascular events.

**Materials and methods**

**Study population and design**

The Athero-express is an ongoing longitudinal study in two Dutch teaching hospitals in Utrecht and Nieuwegein, the Netherlands. In this study, a total of 270 patients of the Athero-express biobank, undergoing carotid endarterectomy (CEA) were included. Indication for CEA was based on a total of 270 patients of the Athero-express biobank, undergoing carotid artery bypass, percutaneous coronary artery intervention, peripheral vascular surgery or angioplasty. Endpoints were validated by two members of the outcome assessment committee. If no consensus was reached, a third observer was consulted for the final judgment of the endpoint.

**Follow-up**

After CEA patients were followed up to 3 years by questionnaire. Primary outcome is defined as any cardiovascular event including (non-) fatal stroke, (non-) fatal myocardial infarction, sudden death, other vascular death, and any arterial vascular intervention that had not been planned at the time of inclusion (e.g. carotid surgery or angioplasty, coronary artery bypass, percutaneous coronary artery intervention, peripheral vascular surgery or angioplasty). Endpoints were validated by two members of the outcome assessment committee. If no consensus was reached, a third observer was consulted for the final judgment of the endpoint.

**Materials**

A total of 270 patients that underwent CEA were randomly selected for the present study with a ratio of 2:1 for control patients vs. patients who suffered from an event during the follow-up. Six patients were excluded from the study as the endarterectomy specimens were unsuitable for histological analysis, resulting in a total of 264 patients (events n = 89, controls n = 175). The carotid plaques used in this study were processed as described previously. In short, after surgical dissection the plaque was cut into segments of 5 mm. The segment with the largest plaque area was fixed in formalin and embedded in paraffin for histology. The two adjacent sections were frozen in liquid nitrogen and used for protein isolation. In addition, blood was drawn prior to the CEA procedure, and plasma was stored at −80°C.

**Immunohistochemistry**

To assess MC numbers in the atherosclerotic specimens, plaque sections were pre-treated with pepsin buffer for 15 min at 37°C. A monoclonal mouse antibody against MC tryptase (dilution 1:400, Dako-Cytomation, Carpinteria, CA, USA) was used to stain MCs. Powervision poly HRP against mouse IgG (ImmunoLogic, Duiven, The Netherlands) was used as secondary antibody after which the tryptase staining was visualized with diaminobenzidine. Sections were counterstained with Haematoxylin. Total MC numbers were determined by counting all MCs present in a plaque cross-section at ×40 magnification. A subset was analysed by another independent researcher to assess intra-observer variability (Spearman correlation coefficient r = 0.947; P < 0.001). In 100 randomly chosen plaques, the number of degranulating MCs was determined. A degranulating MC was defined by a group of MC tryptase-positive extracellular granules in close proximity of each other or in close proximity of an MC. The percentage of degranulating MCs was assessed by counting the first 50 MCs observed by the researcher. This percentage was extrapolated to the total number of degranulating MCs per plaque section. The total plaque area (mm²) was measured using the analySIS 2.8 software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) to determine the distribution density of MCs expressed as numbers of MC/mm².

Consecutive sections were also stained for CD68 (macrophages), CD66 (neutrophils), and CD34 (endothelial cells). The Image-analysing software (Soft Imaging Solutions GmbH, Münster, Germany) was used to determine positive macrophage staining expressed as a percentage of the covered plaque area and to assess total neutrophil numbers. Microvessels were counted at three sites with most prevalent CD34
staining within the section (hotspots) and were expressed as average microvessel density per hotspot. The presence of IPH was assessed with Haematoxylin and Eosin (H&E) staining and fibrin (Mallory's phosphotungstic acid-haematoxylin). The size of the extracellular lipid core (atheroma) was assessed by the H&E and picrosirius red stain. The overall plaque phenotype was based on the percentage of confluent lipid areas of the total plaque area that were visually estimated (fibrous: <10% fat; fibroatheromatous: 10–40%; atheromatous: >40% fat).

For the double immunostaining of MCs and vessels pepsin pre-treated sections were incubated with the mouse anti-MC tryptase antibody (1:400) followed by Brightvision poly AP-anti-Mouse IgG (Immunologic) incubation and development with Liquid permanent red (DAKO, Glostrup, Denmark). Subsequently sections were boiled in citrate buffer; pH 6.0, incubated with a monoclonal mouse anti CD34 antibody (1:400; Immunotech, Marseille, France) followed by incubation with Brightvision poly AP-anti-Mouse IgG and developed with the alkaline phosphatase substrate kit III (Vector Labs, Burlingame, CA, USA). For the double immunostaining, no nuclear staining was used.

Quantification of mast cell tryptase levels in patient plasma
Plasma obtained before surgery was available from 135 (events n = 52, controls n = 83) of the selected patients. Baseline characteristics did not differ between these patients and the total group of studied patients (data not shown). Mast cell tryptase levels were determined in citrate plasma samples using an ImmunoCAP® 250 tryptase assay (Phadia AB, Uppsala, Sweden).

Quantification of RANTES, Eotaxin-1, monocyte chemotactic protein-1, and transforming growth factor-β1
Protein isolation on the adjacent segments to the culprit lesions was performed by a standardized method. In short, the segments were grinded in liquid nitrogen and dissolved in Tripure or Tris (Roche). Total protein concentration of each sample was quantified. Levels of monocyte chemotactic protein-1 (MCP-1), CCL-5 (RANTES), and CCL-11 (Eotaxin) were measured by the Multiplex Immunoassay (Biotool, Biorad Laboratories, Hercules, USA) in tripure protein isolates. Transforming growth factor-β1 (TGFB1) expression was quantified in Tris isolates by the Quantikine Human TGFB-1 Immunoassay (R&D Systems, Wiesbaden, Germany). Protein levels for all four chemoattractants were corrected for a total amount of protein within the segment.

Statistics and data analysis
SPSS 20 was used for all analyses (SPSS, Inc., Chicago, IL, USA). Mast cell numbers are not normally distributed; non-parametrical testing was used to determine differences. The Mann–Whitney U test was used to obtain differences in MC numbers as a continuous variable for all common risk factors. For multiple groups testing, the Kruskal–Wallis test was used. The Spearman correlation coefficient was used to calculate relations of (degranulating) MC/mm² or tryptase plasma levels with all continuous variables in this study. Differences were considered significant with a two-sided P-value of <0.05. The receiver-operating characteristic (ROC) curve was used to determine the optimal cut-off value to divide the patients in two groups with low and high MC/mm². To determine the prognostic value of intraplaque MC/mm², the Cox-regression model was used. A multivariate analysis was performed to adjust for confounders. On the basis of the previous reports, we expected the number of MCs to associate with plaque vulnerability and increased risk for future cardiovascular events.

Results
Baseline characteristics
Table 1 depicts baseline clinical characteristics for the patients included in this study. The patient population represents a typical population of patients with vascular occlusive diseases. The mean age of the patients was 68, with a preponderance of males (71%). Moreover, the majority of patients was symptomatic (81%), suffered from hypertension (88%) and used statins (73%).

Mast cells and plaque characteristics
Mast cells were found to be distributed throughout the entire plaque. They were more abundantly present in the areas where also

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline characteristics of the patients in relation to mast cell numbers in carotid plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC/mm²</td>
<td>P-value</td>
</tr>
<tr>
<td>Age, mean years (SD)</td>
<td>68 (9)</td>
</tr>
<tr>
<td>BMI, mean kg/m² (SD)</td>
<td>26 (4)</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>188/264 (71)</td>
</tr>
<tr>
<td>Female</td>
<td>76/264 (29)</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>101/260 (39)</td>
</tr>
<tr>
<td>No</td>
<td>159/260 (61)</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>59/264 (22)</td>
</tr>
<tr>
<td>No</td>
<td>205/264 (78)</td>
</tr>
<tr>
<td>Statin use (%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>194/264 (73)</td>
</tr>
<tr>
<td>No</td>
<td>70/264 (27)</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>233/264 (88)</td>
</tr>
<tr>
<td>No</td>
<td>31/264 (22)</td>
</tr>
<tr>
<td>History myocardial infarction (%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>55/262 (21)</td>
</tr>
<tr>
<td>No</td>
<td>207/262 (79)</td>
</tr>
<tr>
<td>History vascular intervention (%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>100/264 (38)</td>
</tr>
<tr>
<td>No</td>
<td>164/264 (62)</td>
</tr>
<tr>
<td>Clinical presentation (%)</td>
<td></td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>49/264 (19)</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>215/264 (81)</td>
</tr>
<tr>
<td>Amaurosis fugax</td>
<td>41/264 (15)</td>
</tr>
<tr>
<td>TIA</td>
<td>117/264 (44)</td>
</tr>
<tr>
<td>Stroke</td>
<td>57/264 (22)</td>
</tr>
</tbody>
</table>

Data are presented as n (%) and median (IQR) unless otherwise indicated. r, Spearman’s rank correlation coefficient; SD, standard deviation; IQR, interquartile range; BMI, body mass index; TIA, transient ischemic attack.

*p-value represents statistical analysis for asymptomatic patients vs. symptomatic patients (composed of amaurosis fugax, TIA, and stroke).
Microvessels were present (Figure 1C), i.e. in the deep layers of the plaque at the interface of the media (Figure 1A). In more unstable lesions, MC accumulations were also found in the shoulders of the plaques (Figure 1B). The median number of total MCs present was 108 (55–233) cells per plaque section (Table 2). Total MC numbers were associated with a plaque phenotype categorized in three groups: fibrous, fibroatheromatous, and atheromatous [79 (37–165), 124 (64–237), 139 (59–243) MC per plaque section, respectively, P = 0.008]. In addition, high MC numbers were associated with the presence of IPH: 70 (37–137) MCs in plaques without IPH vs. 130 (65–241) MCs in plaques with IPH (P = 0.001). Moreover, MCs did correlate positively with the percentage of the plaque area covered with macrophages (r = 0.156, P = 0.011) and with the number of counted neutrophils (r = 0.380, P < 0.001).

Degranulating MCs were also abundantly present in the plaques. Their numbers correlated with the percentage of plaque area-covered macrophages (r = 0.310, P = 0.002), but did not associate with any other characteristic of the rupture-prone plaque.

### Mast cells and intraplaque neovascularization

A strong significant correlation between MCs and intraplaque microvessel density was observed. A 2.4-fold increase in MC/mm² was observed comparing the first and fourth quartile of counted plaque microvessels (Figure 2). In addition, also on a continuous scale, microvessel density correlated significantly with total MC numbers (r = 0.416, P < 0.001; Table 2). A positive correlation with microvessel density was also observed for neutrophils and macrophages (r = 0.128, P = 0.045 and r = 0.133, P < 0.001, respectively, data not shown). Therefore, we examined whether a more general pan-inflammatory process would explain the observed association between MCs and microvessel density. In Figure 3, the average microvessel density is provided for four groups based on high or low amounts of MCs and macrophages (A) or MC and neutrophil numbers (B) using the median as a cut-off value. Plaques with high microvessel density were characterized by high MC numbers. The number of microvessels did not differ strongly between macrophage-rich and macrophage-poor cross-sections when

---

### Table 2 Mast cell numbers in carotid plaques with respect to the histological parameters of the plaques

<table>
<thead>
<tr>
<th>Plaque characteristics</th>
<th>MCs (n)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque phenotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrous</td>
<td>79 (37–165)</td>
<td>0.008</td>
</tr>
<tr>
<td>Fibroatheromatous</td>
<td>124 (64–237)</td>
<td></td>
</tr>
<tr>
<td>Atheromatous</td>
<td>139 (59–243)</td>
<td></td>
</tr>
<tr>
<td>Intraplaque haemorrhage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>130 (65–241)</td>
<td>0.001</td>
</tr>
<tr>
<td>No</td>
<td>70 (37–137)</td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>r = 0.156</td>
<td>0.011</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>r = 0.380</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Microvessel density</td>
<td>r = 0.416</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as Spearman's rank correlation coefficient (r) or median (interquartile ranges); MCs are presented as total number of MCs per cross-section (n).
controlled for MC numbers. Plaques characterized by a high abundance of both cell types did show a higher microvessel density. Similar results were observed for neutrophils.

Plasma tryptase levels correlated with the number of MCs and degranulating MC/mm² plaque (\(r = 0.243, P = 0.004\) and \(r = 0.363, P = 0.001\), respectively), but were not associated with microvessel density. Furthermore, no correlation was found between the number of degranulating MC/mm² and microvessel density. In addition, no association was observed for MCs and plaque protein levels of the MC chemoattractants RANTES (\(r = 0.041, P = 0.597\), Eotaxin (\(r = 0.126, P = 0.100\), TGF-β1 (\(r = 0.089, P = 0.239\), or MCP-1 (\(r = 0.044, P = 0.564\). In contrast, a positive correlation was observed between macrophage numbers and MCP-1 or RANTES plaque protein levels (\(r = 0.138, P = 0.001\) and \(r = 0.107, P = 0.008\), respectively).

**Mast cells and clinically relevant characteristics**

Patients entering the study with symptoms as amaurosis fugax, transient ischaemic attack, and stroke contained significantly more MCs in their plaques compared with asymptomatic patients [3.34 (1.75–5.87) vs. 2.12 (1.23–4.23) MC/mm², \(P = 0.016\); Table 1]. In addition, we observed a trend that males have more MCs in their carotid plaque compared with females [3.34 (1.64–5.68) vs. 2.56 (1.25–5.05) MC/mm², \(P = 0.073\)]. None of the other risk factors was associated with MC numbers.

**Outcome**

Eleven patients were excluded for the lack of relevant clinical data resulting in 253 patients of whom in 89 we observed secondary cardiovascular events. In 164 patients without any future events, a median MC/mm² of 2.83 (1.48–5.45) was observed. Events are subdivided in (non-) fatal stroke [3.33 (1.76–7.01), \(n = 32\), (non-) fatal myocardial infarction [3.68 (1.41–6.80), \(n = 19\)], sudden death, and other vascular death [3.38 (1.84–7.54), \(n = 20\)], and peripheral [3.30 (1.65–5.38), \(n = 34\)] and coronary interventions [3.61 (2.40–5.32), \(n = 19\)] that had not been planned at the time of inclusion.

The ROC curve analysis was used to reveal the optimal cut-off value of 2.75 MC/mm² plaque to classify the patients for future cardiovascular events (\(> 2.75 \text{MC/mm}^2\), \(n = 142\); \(< 2.75 \text{MC/mm}^2\), \(n = 111\)). Patients with a value \(> 2.75 \text{MC/mm}^2\) were associated with more cardiovascular events during the follow-up [58 (41%) vs. 31 (28%) events, hazard ratio (HR) = 1.62, 95% CI (1.05–2.51), \(P = 0.029\)]. To check for confounders, we added MC/mm² as a linear variable in a multivariate analysis with the most prominent risk factors for cardiovascular disease, i.e. gender, age, hypertension, smoking, diabetes, body mass index (BMI), and statin use. Mast cells remained significantly associated with adverse events [HR per SD = 1.23, 95% CI (1.03–1.47), \(P = 0.025\)]. Also other clinical factors that could potentially influence MC numbers, as other
Inflammatory co-morbidities and anti-inflammatory drug use, did not affect the relation of MCs and primary outcome.

In addition, higher tryptase plasma levels were observed in patients that had a secondary event [52 events out of 135 patients, 5.3 (4.2–6.9) vs. 4.5 (3.7–5.8) μg/mL, \( P = 0.046 \), Figure 4].

**Discussion**

Atherosclerosis is a chronic inflammatory disorder where numerous inflammatory cell types are known to be involved. Such cells can enter the plaque from the circulation through the endothelium and create an inflammatory environment in the vessel wall. A characteristic of a rupture-prone plaque that has gained attention of the scientific community is the presence of intraplaque vessels. In this study, we show that MCs associate with microvessel density in the plaque. It is known that not only MCs, but also other inflammatory cells, are capable of inducing new vessel formation, suggesting that an overall inflammatory environment is responsible for the formation of the neovessels in the plaque. We also observed that, besides MCs, macrophages, and neutrophils are associated with microvessel density. However, this study provides a sufficient sample size to show that the association between microvessel density and MCs is significant and independent of the presence of other inflammatory cell types. Particularly interesting is the finding that high MC numbers in the plaque were associated with a higher average microvessel density independent of the amount of macrophages and neutrophils. These data suggest that MCs might be important for plaque neovascularization. Nevertheless, when all cell types are present an even higher microvessel density is observed. A possible explanation might be that, as the plaque progresses, the number of inflammatory cells increases due to increased extravasation of the cells into the plaque via the newly formed microvessels. Of note, in this association study it remains an open question whether the MC numbers are a cause or a consequence of vessel formation, i.e. whether MCs are responsible for the induction of the new vessels or whether the MCs and other inflammatory cells enter the plaque via the vessels after their formation. Probably both mechanisms occur, as a low number of MCs are already present in the normal arterial wall and increase with plaque progression. In this study, we did not observe a correlation between known MC chemoattractants and MC numbers within the plaque, while for monocytes and their attractors a positive association was observed. This could be explained by the life span of MCs, which is long compared with other inflammatory cell types. Subsequently, chemokine levels at the moment of plaque excision may not reflect earlier MC recruitment. Additional research is needed to elucidate the underlying mechanism of MC migration towards the atherosclerotic plaque.

Necrotic areas in atherosclerotic plaques did not contain MCs. Large plaques with avascular fatty areas would result in an undervalued number of MCs when expressed per mm². Therefore, in the histological analyses we expressed the number of the MCs/plaque.

Here, we show that plaques with high MC numbers show signs of increased plaque vulnerability. Previous studies demonstrated that this observation is also reflected in the association with the clinical outcome, since higher MC numbers were observed in the plaques of patients with unstable angina or symptomatic carotid artery disease. We confirm these observations in our patient cohort where we also noticed more MC in the plaques of symptomatic patients compared with asymptomatic patients.

The impact of MCs on atherosclerotic plaque progression has long been underestimated, as it was considered that MCs were only present in low numbers. Therefore, another important finding in this study is that MCs are highly prevalent in the atherosclerotic lesion: in some of the plaques total MC numbers of \( \approx 800 \) cells per section were detected, with an overall median of just \( >100 \) cells per section. Plaques often showed confluency of CD68-positive foam cells. The percentage of macrophages is, therefore, depicted as percentage of the covered area, because it was not always possible to quantify the individual cells. This makes comparison of absolute macrophage and MC numbers difficult in our cohort. It is though acknowledged that macrophages by far outnumber all other cell types in the advanced atherosclerotic plaques. Nevertheless, together with experimental data in the literature, our data suggest that the MC is a prominent inflammatory cell type accumulating in the atherosclerotic plaque during plaque progression.

We considered that particularly degranulating MCs would be responsible for inducing intraplaque neovascularization as they are frequently observed near the microvessels. However, we could not observe an association between degranulating MC numbers and microvessel density. Also, no association was observed between degranulating MCs and any of the other rupture-prone characteristics or future events. This might suggest that the induction of new vessel formation is more related to a regulated non-exocytotic release of pro-angiogenic growth factors rather than to the extent of an exocytotic release of countable granules, i.e. degranulation of activated MCs. In this study, we show that MC presence is associated with thrombus formation and IPH. It was hypothesized that MC components can
induce hyperpermeability or erosion of endothelial cells of the microvessels in the plaques eventually leading to IPH or thrombus formation.12,27 This is in accordance with the most important finding in our study, that plaque MC numbers associated independently with future cardiovascular events. In contrast, in the study of Hellings et al.,16 showing that microvessel density is also predictive for future events, neither macrophage nor neutrophil numbers were found to associate with the occurrence of secondary manifestations.

Interestingly, we also observed higher plasma tryptase levels in patients that experienced a secondary event. The tryptase levels correlated positively with the number of (degranulating) MCs in the plaque, implying that the presence of degranulating, i.e. tryptase-secreting MCs in a plaque is not only responsible for the local matrix degradation, but also representative for the systemic changes that might be responsible for the secondary manifestations. The correlation of intraplaque MC numbers with future events has never been reported previously; however, MC tryptase plasma levels have been tested as a possible biomarker for cardiovascular disease. Thus, in agreement with our data, elevated tryptase levels were observed in patients with substantial coronary heart disease with the highest levels in the subgroup with acute myocardial infarction.28 In addition, higher MC tryptase levels were observed in patients with significant coronary artery disease (CAD) defined by stenosis of over 50%.29 Moreover, in another study of the group of Deliargyris, tryptase levels were elevated in patients with CAD.30 Conversely, no differences in MC tryptase were observed in patients with acute coronary syndrome.31,32

Besides tryptase, several other MC-derived components have also been associated with disease severity, and, in general, most studies underline the importance of MC mediators in inflammatory diseases. Taken together, we show here for the first time that intraplaque MC numbers and plasma MC tryptase associate with future cardiovascular events. However, the results of this clinical proof-of-concept study are not sufficient to suggest utilization of the above observations in a regular clinical setting for prediction studies. Nevertheless, the data do strengthen the hypothesis that the presence of MCs in advanced carotid plaques increases risk for secondary cardiovascular manifestations, possibly by inducing intraplaque neovascularization, and via matrix degradation, which may together increase the incidence of IPH and thrombus formation. However, extensive research is necessary before MC stabilizing agents can be considered as a possible therapeutic opportunity preventing clinical manifestations by plaque stabilization in the future. For example, animal experiments inhibiting MC activation by stabilizing agents should be performed to proof causality between plaque MCs and neovascularization.

In conclusion, we show that MC numbers in the carotid atherosclerotic plaque associate with future cardiovascular events. These data correspond with the strong association found between MC numbers and intraplaque neovascularisation, now evolving as an important characteristic of rupture-prone atherosclerotic lesions, which may trigger acute atherothrombotic complications in the vulnerable patients.

Acknowledgements
The financial contribution of the Netherlands Heart Foundation is gratefully acknowledged. Whiri Research Institute is maintained by the Jenny and Antti Wihuri Foundation. Petra van der Kraak-Homoe is acknowledged for technical support.

Funding
This research forms part of the Project P1.03 PENT of the research program of the BioMedical Materials institute, co-funded by the Dutch Ministry of Economic Affairs, Agriculture and Innovation.

Conflict of interest: none declared.

References
Aortic root rupture with giant thoracic haematoma: transient improvement after percutaneous closure with ASD-amplatzer device

Santo Ferrarello, Tiziano Moccetti, Jos C. Van den Berg, and Giovanni B. Pedrazzini*

Cardiocentro Ticino, via Tesserete, Lugano 6900, Switzerland

* Corresponding author. Tel: +41 91 8053178, Fax: +41 91 8053173, Email: giovanni.pedrazzini@cardiocentro.org

An 83-year-old man, known for previous coronary bypass surgery, was admitted to the emergency department with acute chest pain, poor haemodynamic stability, and superior vena cava syndrome. The CT angiography (Panel A) showed a rupture of the aortic root, 10 cm above the aortic valve, with the development of a large mediastinal haematoma (9.8 × 4.9 cm). Considering the extremely high surgical risk, we decided to attempt a percutaneous closure using an ASD-Amplatzer device 12 mm (St Jude Medical, St Paul, MN, USA). The large pseudo-aneurysm could be easily entered with a Multipurpose 6F catheter (Panel B). The Amplatzer-ASD was introduced by means of an 8F Torque View catheter and delivered across the aortic rupture under fluoroscopic guidance (Panel C). The CT angiography performed 12 h later demonstrated a well-positioned device with minimal residual perfusion of the false aneurysm (Panel D). Forty-eight hours after the procedure the clinical conditions of the patient worsened dramatically with the appearance of a massive left pleural effusion due to the spreading of the bleeding into the left thoracic cavity. Owing to severe multi-organ failure and despite inotropic support, the patient died few hours later in cardiogenic shock.

(Panel A) Angio-CT showing the aortic rupture (white arrow) and the large mediastinal haematoma (white-dotted arrows). (Panel B) Periprocedural fluoroscopy with 6 F Multipurpose into the haematoma and pig tail in the ascending aorta. (Panel C) ASD-Amplatzer 12 mm device across the aortic wall (white arrow). (Panel D) Angio-CT 12 h after the procedure with device (white arrow) stable across the aortic wall. Ao, aortic root; Ps-An, thoracic pseudo-aneurysm; LV, left ventricle.