

# Carotid plaque rupture that results in stroke is accompanied by a transcriptome profile that is pro-inflammatory and thins the fibrous cap

Arnold Winfield

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## ABSTRACT

The cause of myocardial infarction and ischemic stroke is rupture of an atherosclerotic plaque. The paucity of information from plaques at the time of rupture contributes to the mystery surrounding the molecular mechanisms of rupture. Carotid plaques from patients undergoing carotid endarterectomy with high-grade stenosis and either (1) a carotid-related ischemic cerebrovascular event within the previous 5 days ('recently ruptured,' n=6) or (2) an absence of a cerebrovascular event were obtained. Ribosome-depleted total RNA was then sequenced from the carotid plaques. Plaque rupture was identified as the major contributor to sample variability (23.2%) by

principal component analysis, and transcripts linked to inflammation and extracellular matrix breakdown were more abundant in recently ruptured plaques. Differentiating the asymptomatic from the freshly burst plaques was accomplished using hierarchical clustering. Matrix metalloproteinases, interferon response genes, and transcripts for the function of immunoglobulins and B lymphocytes were also discovered to co-express in this analysis. The relevance of inflammation and the inhibition of proliferation and migration together with an increase in apoptosis were supported by analysis of the differentially expressed genes. This supports further research into the involvement of B lymphocytes and interferons in atherosclerotic plaque rupture and explains why the transcriptome of recently ruptured plaques is enriched with genes linked to inflammation and fibrous cap thinning.

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## INTRODUCTION

A thromboembolic event that causes ischemia is triggered by the rupture of atherosclerotic plaque. In the case of carotid arteries, clinical examples include acute neurological events such as transient ischemic attacks and stroke and acute coronary syndromes in the case of coronary arteries. An atherosclerotic plaque's growth is defined by a continuous inflammatory response that attracts T cells and macrophages made from monocytes. These generate inflammatory mediators that heighten the inflammatory response and encourage Vascular Smooth Muscle Cells (VSMC) to multiply and secrete collagen to create the fibrous cap. A rupture-prone thin cap fibroatheroma is produced in the late stages of atherosclerosis when the fibrous cap thins as a result of increasing VSMC death and matrix metalloproteinase production.

A strong, collagen-rich fibrous cap that is made up of sclerotic tissue,

VSMCs, and an intact endothelium lining covers an atheromatous core that is soft and rich in lipids. Thinner covering fibrous caps on ruptured plaques are penetrated by macrophages, T-lymphocytes, and B-lymphocytes. Further research is necessary to fully understand the molecular processes that transform asymptomatic plaques into those vulnerable to rupture and produce atheroemboli. The primary method for identifying the characteristics of a burst plaque is histological examination of tissue taken from patients who have experienced symptoms during the last 180 days. Even though these data are very useful, our earlier research suggests that plaque rupture causes observable brief changes in RNA expression. To identify the molecular pathways in play at the moment of rupture, we compared the transcriptomes of carotid plaques that were asymptomatic and those that had burst recently (within 5 days).

## RESULTS

### Patient population

11 individuals underwent CEA; 6 had an ischemic cerebrovascular

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Editorial Office, *Clinical Cardiology Journal*, Switzerland Correspondence: Arnold Winfield, Editorial Office, *Clinical*

*Cardiology Journal*, Switzerland, E-mail: [cardiology@clinicalres.org](mailto:cardiology@clinicalres.org)

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event within the previous five days, and 5 were asymptomatic. Carotid plaques were retrieved from these patients. Age, body mass index, lipid profile, serum creatinine, and estimated glomerular filtration rates did not significantly differ between the two patient groups. Additionally, both groups have comparable rates of drinking, smoking, and chronic renal disease. None of the participants had diabetes, although they were all hypertensive. Similar amounts of aspirin, statin, and clopidogrel were consumed by each group.

#### **Hierarchical clustering of highly variable transcripts separates asymptomatic and recently symptomatic**

We carried out hierarchical clustering of the transcripts in the carotid plaques to more thoroughly study the changes in gene expression that take place with plaque rupture. The separation between the asymptomatic and newly ruptured plaque samples was achieved by clustering at least 400 of the most variable transcript samples. Intriguingly, two of the recently ruptured samples formed a cluster apart from the other samples, whereas the asymptomatic samples grouped closely together, indicating increased diversity in the newly ruptured plaque samples. Overall, the heatmap doesn't show any identifiable transcript clusters that are exclusive to ruptures that are asymptomatic or recent. The heatmap shows a cluster of transcripts related to immunoglobulins and B cell functions, whose expression is elevated in the newly ruptured group.

#### **Differential gene expression underlying increased atherosclerotic plaque vulnerability and rupture**

From atherosclerotic plaque samples of recently ruptured versus asymptomatic patients, a total of 422 DEGs were found. There were 23 considerably more transcripts overall, while 159 significantly fewer transcripts were produced. We thoroughly investigated important transcripts of interest to gain a better understanding of the molecular mechanisms behind the change from stable to susceptible atherosclerotic plaques. Increased expression of pro-inflammatory genes involved in the mobilisation and recruitment of leukocytes to the vessel wall was seen in recently ruptured atherosclerotic plaques. The chemokine receptor XCR1 and the neutrophil activation mediator CD177 were elevated in the group with recent ruptures. Additionally, the B cell function-related transcripts MZB1, several immunoglobulins, and transcripts linked to B cell activation and proliferation were also higher in the recently ruptured group (CD79A, SH2D3C, and ZAP70). Additionally, it was shown that samples of recently ruptured atherosclerotic plaques exhibited a change in transcript expression that might be related to the thinning of the fibrous cap via reduced VSMC proliferation and migration and increased apoptosis. SDC1 and SIK1 are linked to less VSMC migration and proliferation. Additionally, transcripts that hinder cell proliferation were expressed more frequently in the freshly burst plaques. In the recently ruptured samples, reduced expression of a promigration gene (*TMSB15B*) was also noted. Ras GTPase-activating proteins (*RASA4*, *RASA4B*, *ARHGAP4*, *HMHAI1*, and *SH3BP1*) are encoded by several transcripts, and they help to inactivate Rac1 and cdc42, which prevents proliferation and migration. Along with its anti-proliferative properties, *DOK3* is linked to an increase in apoptosis. A pro-apoptotic transcript (*TP63*) was found to be more abundant in recently ruptured samples, while a transcript encoding an apoptosis inhibitor was found to be less abundant (*MTRNR2L13*). A putative measure of carotid plaque vulnerability, *ADAMTS4*, which encodes a proteinase capable of degrading the extracellular matrix, was also elevated in the recently ruptured samples. In the freshly

ruptured samples, there was a substantial increase in the long non-coding RNA MIAT. According to this, MIAT is higher in ruptured carotid plaques than in stable ones. MIAT, however, increases proliferation and inhibits apoptosis.

## **DISCUSSION**

This study's RNA-Seq data shows that newly ruptured plaques have a distinctive transcriptome that includes anti-proliferative and inflammatory characteristics. According to the PCA analysis, rupture status is the main cause of the variation between samples. The hierarchical clustering showed that the plaques' transcriptomes also exhibit commonalities depending on their plaque state. This common expression profile, however, predicted, provides evidence that the development of plaque vulnerability is a dynamic process influenced by substantial changes in gene expression. In a related analysis, Papaspyridonos et al. collected CEA samples, analysed them macroscopically to categorise them as stable or unstable, and then utilised supervised analysis to find DEGs between these groups. 27 DEGs of interest were discovered in this investigation and were verified by PCR9. For 18 of these mRNAs, our investigation discovered Fold Changes pointing in the same direction as their study. These analogies imply that similar modifications to those observed in unstable plaques that have not necessarily ruptured are present in our sample. Many transcripts connected to the recently ruptured plaques, particularly B cell activity and type I interferon responses, were shown to be involved in inflammation when the transcripts underlying the clustering of the samples were examined. The fibrous capping of ruptured carotid plaques has been revealed to include B lymphocytes. Significant increases in transcripts linked to B cell function were seen in the newly ruptured plaques, which provided additional evidence from the analysis of the DEGs that B cells play a crucial role in plaque rupture. The role of B lymphocytes in atherosclerosis is depending on the subtype. B1a lymphocytes are atheroprotective because they lessen the size of the necrotic core. The B2 fraction, in contrast, may promote plaque development and susceptibility through increased apoptosis and the release of tumour necrosis factors. Even though there was an increase in the transcripts encoding several immunoglobulins in the freshly ruptured plaques, there was no such increase for the IgM transcript, which may indicate that the B2 subset predominates. Overall, these results show that B2 cells are crucial for the emergence of plaque vulnerability and rupture. Our findings show support for increased inflammation in addition to the B cells. Multiple cell types in atherosclerotic plaques, including B cells, can release type I interferons, which encourage the production of foam cells and intensify inflammation. A group of interferon response genes that were co-expressed were revealed using hierarchical clustering. *IFIT1* and *IFIT3* have been linked to reduced collagen deposition, pro-inflammatory polarisation of macrophages, and plaque susceptibility. In a mouse model of atherosclerosis, decreased atherosclerotic plaque development has been associated with loss of *OAS2* and *OAS3* expression. Increased cytokine signalling was suggested by both the PCA and hierarchical clustering. Overall, the information points to a rise in inflammation related to plaque rupture. In the hierarchical clustering, many matrix metalloproteinases were co-expressed, and transcripts involved in matrix disassembly were enriched in the newly ruptured group. In line with earlier studies, *ADAMTS4* levels were lower in the group that had recently ruptured. In a mouse model of atherosclerosis, loss of *ADAMTS4* is linked to increased collagen content and general plaque stability. The collagens (*MMP1*, *MMP8*, and *MMP13*),

(MMP9), elastins (MMP12), and fibronectin are only a few of the substrates that the matrix metalloproteinases co-expressed in the hierarchical clustering target (MMP7). In the recently ruptured plaques, several genes linked to reduced proliferation and migration were also elevated. Increased SDC1 is linked to differentiated VSMCs that are not proliferating, while SIK1 is linked to lessened vascular remodelling and was up in the ruptured samples. Several Ras GTPase-activating proteins were also upregulated. This protein family member has been linked to the emergence of plaque vulnerability. Additional transcripts indicate that at the time of rupture, there is a decrease in proliferation and an increase in apoptosis. The fact that this study detected RNA from entire artery homogenates should not be overlooked. Therefore, we are unable to link a particular alteration in RNA expression to a particular cell type. The observed variations in RNA expression may also be an indication of alterations in the plaque's biological make-up. Our data indicate that the freshly ruptured plaques had a rise in B cell infiltration. Additionally, the samples were taken 2 days to 5 days after the rupture, so they contain modifications brought on by the rupture's reaction. We are unable to separate the modifications that exist at the time of rupture from those that appear afterwards. Finally, more research is needed to demonstrate that the changes in transcript abundance we report here

are accompanied by changes in protein expression. To our knowledge, however, this is the first study to use carotid samples taken so near the plaque rupture for RNA-Seq. Studies that compare CEA samples frequently use symptomatic plaques that were taken more than 100 days after the rupture. In a previous study, we discovered that miR-221 and miR-222 are down-regulated in freshly ruptured plaques but that they are up-regulated to levels similar to those found in asymptomatic plaques in 7 days. Thus, these data probably contain RNA expression changes that were previously overlooked. Before carotid plaque rupture, "scarce" B cell infiltration was discovered through histologic investigations. Our findings suggest that B lymphocytes play a significant part in plaque rupture.

#### CONCLUSION

Overall, our findings paint a picture of notable modifications in the inflammation within the plaque associated with rupture, including an essential function for B cells. The loss of VSMCs and an increase in a wide range of matrix metalloproteinases are what cause the fibrous cap to thin out. Future investigations into RNA expression alterations that may be localised to certain cells or locations within the plaque will improve our comprehension of the molecular processes causing plaque rupture.