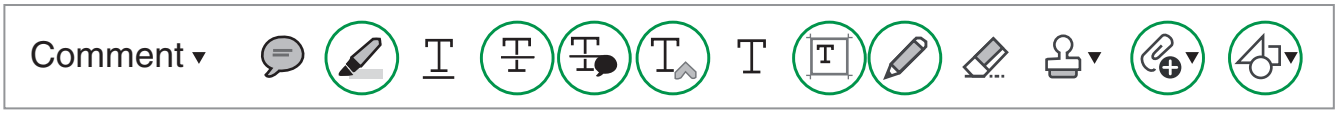
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Supplement: Surgical Treatment of Aortic Syndromes & CV Prevention

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SAGE

Studies on sporadic non-syndromic thoracic aortic aneurysms: 1. Deregulation of Jagged/Notch 1 homeostasis and selection of synthetic/secretor phenotype smooth muscle cells

Anna Chiarini[1], Francesco Onorati[2], Maddalena Marconi[1], Alessandra Pasquali[3], Cristina Patuzzo[3], Anna Malashichev[4], Olga Irtyega[4], Giuseppe Faggian[2], Pier F Pignatti[3], Elisabetta Trabetti[3], Ubaldo Armato[1] and Ilaria Dal Pra[1]

Abstract

Background: Sporadic non-syndromic thoracic aortic aneurysms (SNSTAAs) are less well understood than familial non-syndromic or syndromic ones. The study aimed at defining the peculiar morphologic and molecular changes occurring in the media layer of SNSTAAs [AQ4].

Design: This study was based on a single centre design.

Methods: Media layer samples taken from seven carefully selected SNSTAAs and seven reference patients (controls) were investigated via quantitative polymerase chain reaction, proteomics-bioinformatics, immunoblotting, quantitative histology, and immunohistochemistry/immunofluorescence.

Results: In SNSTAAs media, aortic smooth muscle cells numbers were halved due to an apoptotic process coupled with a negligible cell proliferation. Cystathionine γ-lyase was diffusely up-regulated. Surviving aortic smooth muscle cells exhibited diverging phenotypes: in inner- and outer-media contractile cells prevailed, having higher contents of smooth-muscle-α-actin holoprotein (45-kDa) and of caspase-3-cleaved smooth-muscle-α-actin 25-kDa fragments; in mid-media, aortic smooth muscle cells exhibited a synthetic/secretor phenotype, down-regulating vimentin, but up-regulating glial fibrillary acidic protein, trans-Golgi network 46 protein, Jagged1 (172-kDa) holoprotein, and Jagged1's receptor Notch1. Extracellular soluble Jagged1 (42-kDa) fragments accumulated.

Conclusions: In SNSTAAs, there is a relentless aortic smooth muscle cells attrition caused by the up-regulated cystathionine γ-lyase. In mid-media, synthetic/secretor aortic smooth muscle cells intensify Jagged1/NOTCH1 signalling in the attempt to counterbalance the weakened aortic wall, due to aortic smooth muscle cells net loss and mechanical stress. Synthetic/secretor aortic smooth muscle cells are apoptosis-prone, and the accruing thrombin-cleaved Jagged1 fragments counteract the otherwise useful effects of Jagged1/NOTCH1 signalling, thus hampering tissue homeostasis/remodelling, and aortic smooth muscle cells adhesion, differentiation, and migration.

Keywords

Thoracic aorta, aortic aneurysm, smooth muscle cell, cell signalling, gene expression, remodelling

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[1]Histology and Embryology Section, University of Verona Medical School, Italy
[2]Department of Surgical Sciences, University of Verona Medical School, Italy
[3]Biology and Genetics Section, University of Verona Medical School, Italy
[4]Federal Almazov Medical Research Centre, Russia [AQ1]

Corresponding author:
Ilaria Dal Pra, Histology & Embryology Section, University of Verona Medical School, Strada Le Grazie 8, Verona, I-37134, Italy.
Email ippdalpra@gmail.com

Introduction

According to recent data, the overall global death rate from aortic aneurysms increased from 2.49–2.78 per 100,000 inhabitants during recent decades.[1] Despite numerous studies on the pathological mechanisms and genetic basis underlying syndromic and familial non-syndromic thoracic aortic aneurysms (TAAs), little is still known about the sporadic non-syndromic thoracic aortic aneurysms (SNSTAAs).[1–5] The majority of the pathogenetic mechanisms, i.e. aortic smooth muscle cells (ASMCs) loss, apoptosis, and extracellular matrix (ECM) changes, occur within the diseased aorta's tunica media, configuring a progressive 'medial degeneration'.[1–8] The latter underlies various conditions (e.g. senile aorta, syndromic aneurysms, aortic dissections, etc.).[1–8] Still lacking are proteomic and genomic studies about mechanisms underlying SNSTAAs, as opposed to more common syndromic, atherosclerotic, bicuspid aortic valve-related and familial forms.[1–5] Biomarkers for SNSTAAs development, progression and rupture are missing. By using an integrated investigational approach, we aimed at identifying changes related to SNSTAAs which could clarify their pathophysiology. Therefore, herein we report the proteomic, transcriptomic and histologic changes detected in the tunica media of a selected SNSTAA group, as compared with corresponding data from heart donors without any individual, familial, genetic, inflammatory, infective or atherosclerotic history of TAAs.

Methods

Detailed descriptions of the methods used in this study are available in the Supplementary Material online.

Patients, surgery, aortic tissue sampling and processing

The Institutional Review Board approved the research protocol. All study participants provided written informed consent. Family history, clinical searching for specific findings in multiple organ systems (Ghent criteria), molecular genetic testing (FBN1, TGFBR1/2, COL5A1/2) and peripheral blood karyotype systematically excluded the syndromic nature of the aortic aneurysm (Marfan, Loeys-Dietz, Ehlers-Danlos, Turner).[9–12] Pre-operative echocardiography, intra-operative assessment and pathological examinations excluded bicuspid aortopathy and atherosclerotic distention. Further preoperative exclusion criteria were inflammatory, autoimmune and/or infectious diseases and previous thoracic trauma.

Between January 2014–December 2016, seven SNSTAA patients – out of 34 non-syndromic ascending aortic aneurysms – were enrolled in this study. Simultaneously, pretransplantation harvested aortas from seven heart donors (five deaths from car accident, two from subarachnoid haemorrhage) acted as normal reference controls.

Surgery was always performed through a median sternotomy under hypothermic (32–34°C) cardiopulmonary bypass, with membrane oxygenator and integrated arterial filter (Sorin Synthesis, Italy), combined with antegrade+retrograde hyperkalaemic cold blood cardioplegia. Baseline data are in Supplementary Material Table 1. Ascending aorta 'ring-like' specimens were collected soon after aortic cross-clamping. SNSTAA specimens were always sampled from the anterior wall, at the middle level of the aneurismal dilatation, whereas control specimens were from the anterior wall, at the middle of discarded ascending aorta. Each sample was then divided into three portions for transcriptomic, proteomic and microscopy/immunohistochemistry (IHC)/immunofluorescence (IF) studies.

Aortic media protein lysates prefractionation

Briefly, all media tissue samples were dissolved with isoelectrofocusing (IEF) denaturant buffer and homogenised using Ultra-Turrax (IKA, Germany). The resulting lysate were next pre-fractionated using the narrow pH range ZOOM-IEF Fractionator (pH 3.0–4.6, pH 4.6–5.3, pH 5.3–6.2, pH 6.2–7.0, and pH 7.0–10.0) (Invitrogen, UK) according to the suppliers' protocol.

Two-dimensional gel electrophoresis (2-DE) and proteomic analysis

Sample protein fractions were first separated according to p*I* range, then to M_r in a NuPAGE Novex 4–12% Bis-Tris ZOOM gel and visualised using a mass spectrometry (MS)-compatible staining, i.e. SimplyBlue SafeStain (Invitrogen), scanned and analysed using Delta2D software (version 4.0, Decodon, Germany). Because 87 soluble proteins were separated within 4.5–5.5 and 5.3–6.3 pH ranges in the 2-DE gels, the present study focuses on these two p*I* ranges.

The tryptic in-gel digestion and desalting steps, the MALDI-TOF/MS analysis, peptide mass fingerprinting (PMF) and bioinformatics were performed according to Chiarini et al.[13] [AQ2]

Western immunoblotting (WB)

Aortic media lysate proteins were separated by electrophoresis and subjected to WB analysis as previously described.[13]

Quantitative polymerase chain reaction (qPCR)

Total RNA from aortic media specimens (seven SNSTAAs and seven controls) was isolated by Trizol standard protocols. qPCR was prepared with SYBR Green reagents (Starbiotech, Italy) to analyse the expression of *JAG1, VIM, CTH, GFAP, NOTCH1* genes. For primers see Supplementary Material Table 2.

Histological analysis

The numbers/tissue volume unit and the proliferating and apoptosing fractions of ASMCs were assessed via quantitative histology. The percentage $Ki67^+$ nuclear index was determined in tunica media cryostat sections previously stained with an anti-Ki67 antibody. Ratios $Ki67^+$/total nuclei were determined for each section under the microscope. The percentage fraction of active caspase-3^+ cells was similarly assessed. In each instance, means±standard error of the mean (SEM) of control and SNSTAA groups were calculated.

For IF, 7 μm thick cryostat sections from control and SNSTAA tissue were probed with primary antibodies against smooth-muscle-α-actin (SM-α-A). For IHC, 7 μm thick cryostat sections from control and SNSTAA aortic tissues were probed with primary antibodies against: trans-Golgi network protein46 (TGN46); vimentin (VIM); glial fibrillary acidic protein (GFAP); cystathionine γ-lyase (CTH); Jagged1 (JAG1); Notch1.

Statistical analysis

One-way analysis of variance (ANOVA) with post-hoc Holm-Sidak's test (Sigmastat 3.5, Systat Software, California, USA) was applied to the two groups of proteomic and transcriptomic findings. The statistical significance level was $p \leq 0.05$.

Results

ASMCs attrition and phenotype shift in SNSTAAs

SNSTAAs mean cell nuclei numbers/mm^3 tunica media tissue decreased vs controls (–51%, $p < 0.001$) (Figure 1(a)). The SNSTAAs' proliferating cell fraction assessed via Ki-67 antigen Nuclear Index (% $Ki67^+$ nuclei) was small (0.8%; Figure 1(b)), though doubling that of controls ($p < 0.001$, Figure 1(b)). Active caspase-3, an apoptosis executioner, was detected in 20% ASMCs in SNSTAAs vs 10% in controls ($p < 0.001$, Figures 1(c) and (d), right panels). Accordingly, in immunoblots active caspase-3 was 5.6-fold more copious in SNSTAAs than in control lysates ($p < 0.05$, Figure 1(d), left panels).

Thus, in SNSTAAs an inadequate rise in proliferative activity could not offset the falling ASMCs numbers resulting from an increased level of apoptosis.

As to SM-α-A, the predominant isoform in contractile ASMCs, both the 45 kDa SM-α-A holoprotein, of which the amount was similar ($p > 0.05$) in either group lysates, and a 25 kDa SM-α-A fragment, which by contrast increased in SNSTAA vs control lysates (Figure 1(e), left panels), were detected (+152%; $p < 0.03$). Accordingly, IF with a specific antibody showed that staining of total (holoprotein+fragment) SM-α-A was more intense in SNSTAA vs control sections (Figure 1(e), right panels).

Finally, concerning the increased synthesis/secretion of ECM constituents, the trans-Golgi network marker TGN46 was up-regulated in SNSTAA vs control lysates (+65%, $p < 0.01$) (Figure 1(f), left panels). IHC showed TGN46 expression was stronger – particularly by ASMCs – in the mid-media of SNSTAA vs control sections (Figure 1(f), right panels), denoting an ongoing Golgi apparatus hypertrophy indicating intensified synthetic/secretor activities.

2-DE analysis of aortic media protein lysates

Our present results focus on the 4.5–5.5 and 5.3–6.3 p*I* ranges as 87 soluble proteins were separated within them in the 2-DE gels. Supplementary Material Figure 1 displays typical 2-DE gels of aortic media proteins from control and SNSTAA lysates in these two p*I* ranges. The identified proteins, along with their index codes, peptide sequence coverage, total MOWSE scores, and theoretical/in-gel p*I*s and M_rs are listed in Supplementary Material Table 3. They mostly belonged to three classes: (a) cytoskeleton; (b) enzyme; and (c) cell signalling.

Immunoblot validation of proteomic results, correlated transcriptomics and IHC localization

Protein identification, location, expression and their mRNA levels were assessed via immunoblots, IHC and qPCR using the same tissue samples as for proteomics.

(a) Cytoskeleton. VIM protein levels – which were the predominant constituent of intermediate-sized filaments in ASMCs[14] – were lower in SNSTAA vs control protein lysates (–44%; $p < 0.05$), despite higher VIM mRNA levels observed via qPCR in SNSTAAs vs controls (+20%, $p < 0.03$) (Figure 2(a), left panels). IHC confirmed that VIM protein expression was reduced in SNSTAA vs control sections (Figure 2(a); right panels).

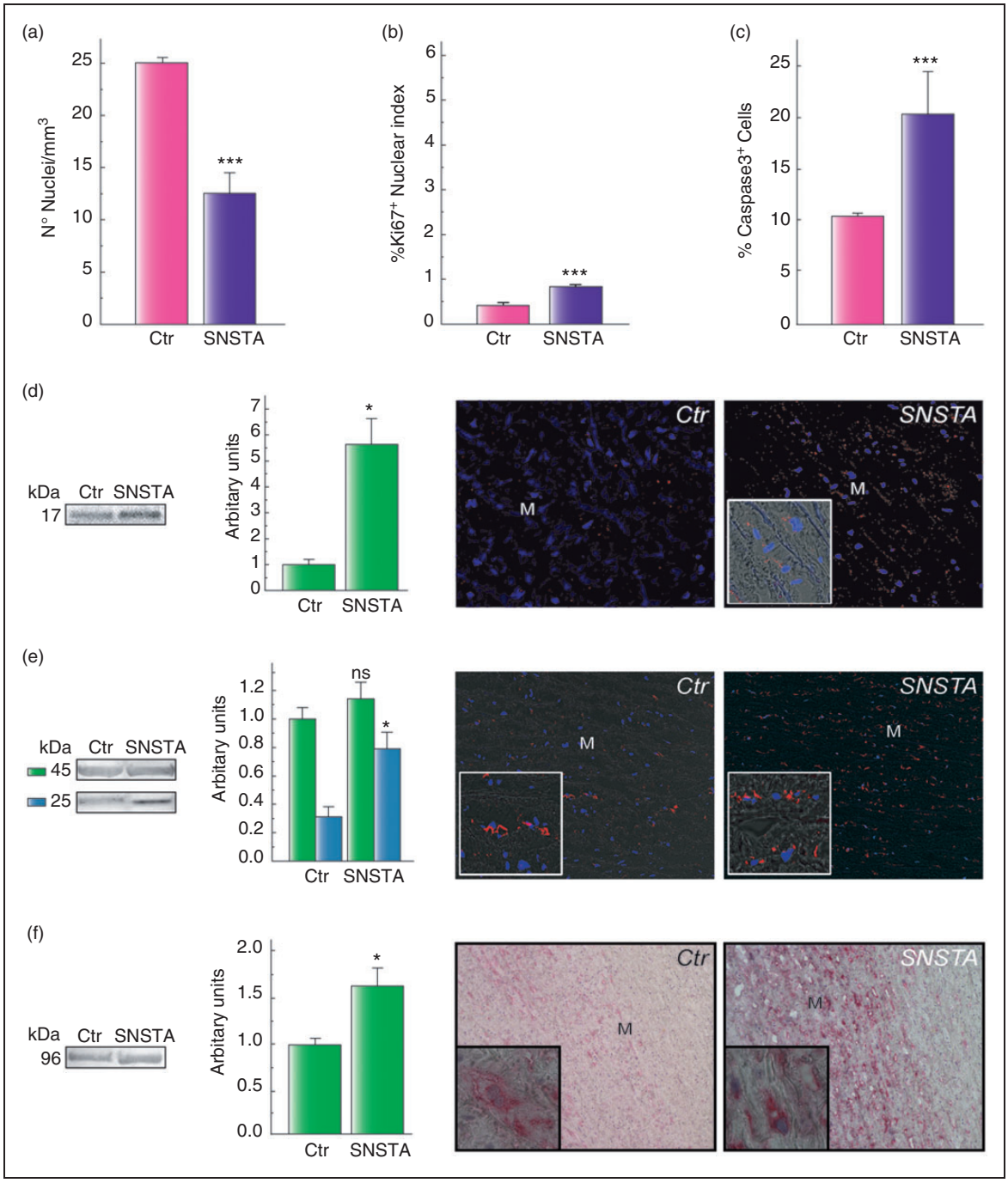


Figure 1. Aortic smooth muscle cells (ASMCs) attrition, via apoptosis and hindered proliferation, and shift to a synthetic/secretory phenotype simultaneously happen in sporadic non-syndromic thoracic aortic aneurysms (SNSTAAs). Bars are means±standard errors of the mean (SEMs). Levels of statistical significance are: ns: $p > 0.05$; *$p < 0.5$; ***$p < 0.001$. (a) Average numbers (N°) of nuclei/mm^3 of media tissue decrease in SNSTAAs vs controls (Ctr). (b) Proliferating cell fraction (% Ki67$^+$ Nuclear Index) was very low in Ctr and remained negligible in SNSTAAs. (c) Percentage active-caspase-3$^+$ cell fraction doubled in SNSTAAs vs Ctr. (d) Analysis of immunoblots from protein lysates (left panels) and of cryostat sections incubated with a specific antibody (right panels) revealed that cytoplasmic active-caspase-3 abounded more in SNSTAAs than in Ctr. (e) Left panels. SM-α-A 45 kDa holoprotein contents were similar in the two groups, but caspase-3-cleaved SM-α-A 25 kDa fragment levels markedly increased in SNSTAA. Right panels. Antibody-decorated total SM-α-A (holoprotein+fragment) abounded more in SNSTAA vs Ctr. (f) Left panels. Trans-Golgi network protein46 (TGN46) protein was up-regulated in SNSTAA vs Ctr. Right panels. TGN46 was more expressed by epithelioid-shaped ASMCs in the mid-media of SNSTAAs.
SM: smooth muscle.

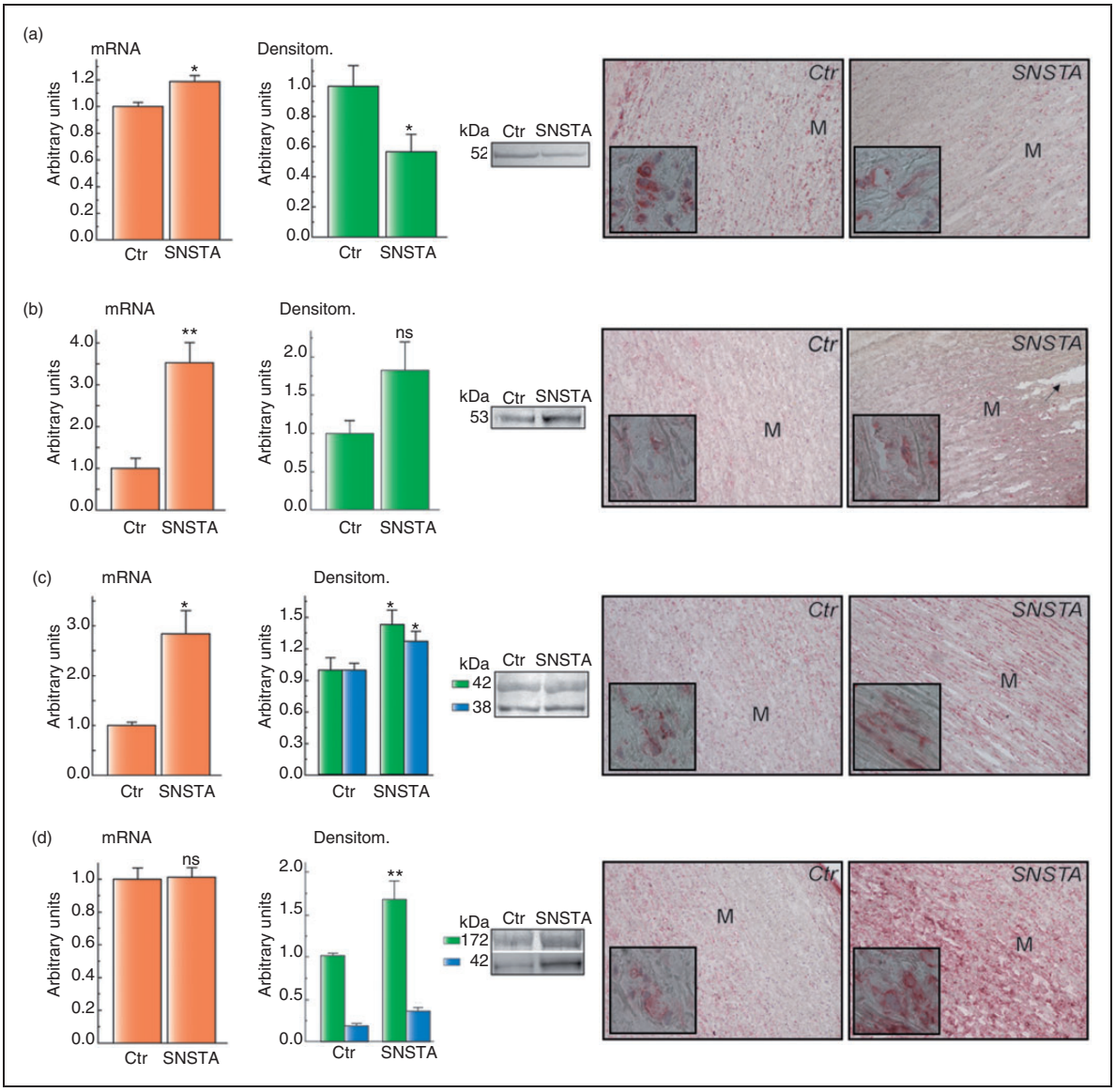


Figure 2. Changes in four different aortic smooth muscle cell (ASMC)-expressed proteins between sporadic non-syndromic thoracic aortic aneurysms (SNSTAAs) vs controls (Ctr). From left to right: quantitative polymerase chain reaction (qPCR) results; immunoblots plus densitometric assessments of the specific bands on whole sets of samples; immunohistochemistry (IHC), light microscopy pictures from cryostat sections. Bars are means±standard errors of the mean (SEMs). Levels of statistical significance: ns: $p > 0.05$; *$p \leq 0.05$; **$p < 0.01$. Optical magnifications: larger pictures, 20×; insets, 100×. (a) Left panels. Vimentin (Vim) mRNA expression was up-regulated but Vim protein levels were lower in SNSTAA vs Ctr. Right panels. Vim protein was less abundant in SNSTAA than Ctr cryostat sections. (b) Left panels. Glial fibrillary acidic protein (GFAP) mRNA expression was up-regulated, while GFAP protein levels increased distinctly in SNSTAAs vs Ctr. Right panels. Mid-media-resident ASMCs expressed GFAP protein more distinctly in SNSTAA than Ctr. (c) Left panels. Cystathionine γ-lyase (CTH) mRNA was up-regulated, as were both CTH protein isoforms, in SNSTAA vs Ctr. Right panels. IHC showed diffuse hyper-expression of CTH protein in SNSTAA vs Ctr. (d) Left panels. Though Jagged1 (JAG1) mRNA levels were similar, JAG1 172 kDa holoprotein was up-regulated in SNSTAA vs Ctr. The level of soluble JAG1 (42 kDa) protein fragment increased in SNSTAA vs Ctr. Right panels. Total JAG 1 (holoprotein+fragment) expression was weak in ASMCs of Ctr, being more abundant in the mid-media ASMCs of SNSTAA in IHC sections.
SM: smooth muscle.

GFAP, another constituent of intermediate filaments, is a marker of embryo-foetal, synthetic/secretor ASMCs.[15] GFAP mRNA expression was strongly up-regulated (+257%; $p < 0.007$). Although GFAP levels increased in SNSTAA immunoblots, the result (+81%; $p > 0.05$) was not significant due to a wider intra-group variability vs controls immunoblots (Figure 2(b), left panels). In SNSTAA mid-media ASMCs, IHC revealed

a GFAP protein expression which was stronger than in controls (Figure 2(b), right panels).

(b) Enzyme. Immunoblotting showed that two CTH (EC4.4.1.1) protein isoforms (38 kDa and 42 kDa) abounded more in SNSTAA vs control lysates (+28% and +42%; respectively; $p < 0.05$ in both instances). CTH mRNA expression was also upregulated (2.84-fold; $p < 0.02$) in SNSTAA vs control qPCR assays (Figure 2(c), left panels). IHC confirmed that CTH protein expression was stronger in SNSTAA ASMCs vs controls in the whole media (Figure 2(c), right panels).

(c) Cell signalling. Immunoblotting showed both JAG1 holoprotein (172 kDa) and extracellular soluble JAG1 42 kDa fragment abounded more in SNSTAA vs control lysates (+65%, $p < 0.01$ and +92%, $p < 0.05$, respectively). Conversely, qPCR revealed JAG1 mRNA levels were comparable in the two groups ($p > 0.05$) (Figure 2(d), left panels). By IHC, total JAG1 immunoreactivity was at its strongest level in mid-media ASMCs of SNSTAA sections, being weak in control ASMCs (Figure 2(d), right panels).

These findings justified the extension of our investigation to JAG1's receptor (namely Notch1),[16] even though its p*I* did not fall within the ranges presently examined. Accordingly, Notch1 mRNA levels were up-regulated (+119%, $p < 0.05$) just as were Notch1 protein levels (+138%, $p < 0.002$) in SNSTAA vs control samples (Figure 3). Overall, these results and the increase in JAG1 fragment suggested that the balance of JAG1/Notch1 signalling was also deregulated in SNSTAAs.

Discussion

Significant differences occur in cell survival, gene and protein expression in the media of SNSTAAs vs normal aortas.

Some studies reported unchanged[5] or increased[17] ASMC numbers in the media layer without ongoing apoptosis. Yet, ASMC apoptotic loss has already been observed in aortic dilatation.[17,18] Our data underscore a non-inflammatory apoptotic cell death – an inadequate compensatory proliferation does not offset – which consumes the ASMC population of the SNSTAA. In keeping with this are: (a) a reduction of ASMCs numbers/unit volume of media; (b) an increased activity of caspase-3 in protein lysates; (c) a doubled fraction of active-caspase-3$^+$ ASMCs as IF showed in cryostat sections; (d) an accumulation of caspase-3-cleaved SM-α-A 25 kDa protein fragments;[19] (e) a tiny fraction of proliferating ASMCs (as per the Ki67$^+$ Nuclear Index); and (f) a diffuse over-expression of hydrogen sulphide (H_2S)-producing CTH. Notably, CTH is a pyridoxal-5'-phosphate-dependent enzyme which catalyses the last step in the transulphuration pathway from methionine to cysteine releasing H_2S, other sulphur-containing metabolites, and pyruvate or α-ketobutyrate inside vascular and heart tissues.[20,21] Although formerly considered as a toxic gas,[22] H_2S at low concentrations is now believed to operate like a gaseous transmitter mediating plasmalemmal.[21,23] hyperpolarization, vasodilatation following ASMCs relaxation (via potassium $(K)_{ATP}$ channels), and cell proliferation However, heightened (>100 μM) H_2S levels hinder cell proliferation[24] and induce apoptosis in ASMCs.[25] To the best of our knowledge, this article for the first time evidences a hyper-expression of CTH in SNSTAAs, which is, from one side, likely to heighten the apoptotic cell death rate, and from the other side to severely hamper any compensatory cell proliferation. Attempting to offset detrimental changes in ECM,[1–4] a substantial fraction of the media-layer ASMCs shifts towards an embryo-foetal, apoptosis-prone, synthetic/secretor phenotype,[26,27] marked by VIM down-regulation[14] and by GFAP[15] and TGN46[28]

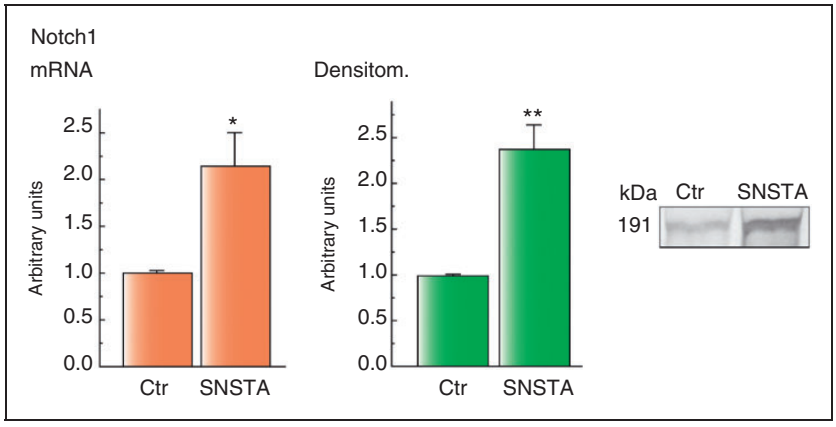


Figure 3. Notch1 mRNA and Notch protein expression were significantly up-regulated in sporadic non-syndromic thoracic aortic aneurysms (SNSTAAs) vs controls (Ctr). Levels of statistical significance: *$p < 0.05$; **$p < 0.02$.

up-regulation – the last indicating a Golgi apparatus hypertrophy. These epithelioid-shaped ASMCs discharge not only ECM constituents and elastic fibre precursors, but even multiple proteases, whose activation might further favour the cystic degeneration of aortic media.[29] Thus, ASMCs' increased synthetic/secretor activities might paradoxically favour ECM proteolysis, elastolysis, fibrosis and aortic wall rigidity. Differently from the SM cells of other body locations, ASMCs intermediate-sized filaments are predominantly made of VIM (conversely, desmin is virtually absent).[14] As our results indicate that the VIM levels and GFAP protein expression tended to be counter-regulated in SNSTAAs due to ASMCs shifting to a synthetic/secretor phenotype recalling their origin from the neural crest.[15] Likewise, GFAP was up-regulated, and VIM down-regulated, in ASMCs of dilated aortas associated with congenital aortic valve malformations. Partially in keeping with our findings, both VIM and SM-α-A were downregulated in ASMCs from aortic aneurysms cultured in vitro.[30] The remarkable intra-group variability of GFAP protein expression found in our study may result from *GFAP* gene-silencing in apoptotic cells.[31]

In most multi-cellular organisms, during embryo-foetal development the highly conserved JAG1/Notch1 signalling pathway controls cell differentiation and fate, whereas it regulates homeostasis and stem cell maintenance in the adulthood tissue, through the binding of Notch1 with a specific extracellular ligand, e.g. JAG1 or Delta1.[16] Interestingly, mutations in *JAG/NOTCH* genes frequently occur in human diseases involving cardiovascular anomalies.[32] Although JAG1 mRNA expression levels did not differ in the two groups of our study, we found that JAG1 protein was significantly up-regulated in SNSTAAs, particularly in mid-media synthetic/secretor ASMCs. Notch1 mRNA and protein expression was also markedly increased in SNSTAAs. Moreover, by means of two different antibodies both directed against the extracellular domain of JAG1 protein we were able to identify the soluble JAG1 42 kDa protein fragment, the levels of which were similarly augmented in SNSTAAs. Recent evidence showed this extracellular 42 kDa JAG1 fragment is produced by an active thrombin, released from activated platelets within large TAAs of any aetiology.[33] Albeit its functions are not fully understood, the soluble 42 kDa JAG1 fragment inhibits JAG1/Notch1 signalling by acting as a dominant-negative ligand.[33] Additionally, the JAG1 fragment reduces cellular adhesion and migration, likely by preventing the formation of and/or destabilising focal adhesion complexes.[34] Hence, during tissue remodelling in vivo JAG1 holoprotein and JAG1 42 kDa fragment antithetically control cell-cell and cell-ECM interactions, cell adhesion and cell migration via the induction or prevention, respectively, of Notch1 signaling.[34] Finally, JAG1/Notch1 signalling also favours the differentiation of ASMCs towards a contractile phenotype.[35] Therefore, our findings seem to indicate that the attempt to boost JAG1/Notch1 signalling in SNSTAAs, via an up-regulation of JAG1 and Notch1 holoproteins, is at least partially frustrated by a concomitant hyper-production of the counteracting extracellular soluble JAG1 42 kDa fragment. Hence, this hindering of JAG1/Notch1 signalling would obstruct tissue remodelling, thus favouring the development of the SNSTAA through its negative role on cell-to-cell and cell-to-matrix interactions, and on ASMCs adhesion, contractile differentiation and migration. Notably, the unchanged (vs controls) SM-α-A holoprotein levels in our SNSTAA lysates indicate that the surviving ASMCs, mostly in the inner and the outer media zones (as shown in Figures 1(a) and (d)), had a greater amount of this predominant smooth-muscle-specific actin. Again, this upkeeping of the contractile phenotype would have been favoured by the reported intensified JAG/Notch signaling.[36]

In conclusion, the present study shows a set of unreported peculiar cellular and molecular features in a carefully selected group of sporadic non-familial SNSTAAs. Based on our finding, we can speculate that the tissue damage – elicited by persisting shear stresses and stretching forces – induces the hyper-expression of H_2S-producing CTH, via mechanisms presently not understood. Consequently, the production of H_2S would cause a steady fall in ASMC numbers, via an increased apoptotic rate and hindered cell proliferation. The declining availability of ECM constituents and elastic fibres, due to both ASMCs loss and increased local proteolysis, weakens the aortic wall resilience, favouring aneurismal development. Trying to up-keep the aortic wall structure, most mid-media ASMCs shift to an embryo-foetal, GFAP-positive, synthetic/secretor, apoptosis-prone phenotype, and strive to activate processes inherent to tissue remodelling via an intensified JAG1/Notch1 signalling. However, the relentless action of harmful mechanical forces, of proteases (MMPs) secreted from ASMCs or activated by MMPs (calpains) or released from platelets (thrombin) that destroy ECM components and fragment the elastic fibre network,[30,37] and the hindering of increased JAG1/Notch1 signalling by thrombin-generated extracellular soluble JAG1 42 kDa fragments, all frustrate any attempt at an effective repair.

Author contribution

AC, FO, GF, PFP, ET, UA and IDP: contributed to the conception and design of the study, contributed to analysis and interpretation, drafted the manuscript, critically revised the manuscript, gave final approval, agree to be accountable

for all aspects of the work ensuring integrity and accuracy. MM, AP and CP: contributed to the conception and design of the study, contributed to analysis and interpretation, drafted the manuscript, gave final approval, agree to be accountable for all aspects of the work ensuring integrity and accuracy. AM and OI: contributed to the conception and design of the study, critically revised the manuscript, gave final approval, agree to be accountable for all aspects of the work ensuring integrity and accuracy.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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