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BASIC SCIENCE

Human epicardial adipose tissue induces fibrosis of the atrial myocardium through the secretion of adipo-fibrokines

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Aims Recent studies have reported a relationship between the abundance of epicardial adipose tissue (EAT) and the risk of cardiovascular diseases including atrial fibrillation (AF). However, the underlying mechanisms are unknown. The aim of this study was to examine the effects of the secretome of human EAT on the histological properties of the myocardium.

Methods and results Samples of EAT and subcutaneous adipose (SAT), obtained from 39 patients undergoing coronary bypass surgery, were analysed and tested in an organo-culture model of rat atria to evaluate the fibrotic properties of human fat depots. The EAT secretome induced global fibrosis (interstitial and peripheral) of rat atria in organo-culture conditions. Activin A was highly expressed in EAT compared with SAT and promoted atrial fibrosis, an effect blocked using neutralizing antibody. In addition, Activin A levels were enhanced in patients with low left-ventricular function. In sections of human atrial and ventricular myocardium, adipose and myocardial tissues were in close contact, together with fibrosis.

Conclusion This study provides the first evidence that the secretome from EAT promotes myocardial fibrosis through the secretion of adipo-fibrokines such as Activin A.

Keywords Myocardial fibrosis • Epicardial adipose tissue • Atrial fibrillation • Atrial fibrosis • Activin A

Introduction

Recently, the epicardial adipose tissue (EAT) has emerged as an important factor in the pathogenesis of metabolic-related cardiac diseases. This ectopic fat depot, distinct from paracardial fat, surrounds the myocardium and coronary arteries and is in direct contact with cardiomyocytes and adventitia. The EAT is a metabolically active tissue that produces pro-atherogenic and pro-inflammatory adipokines.[1–4] In other adipose-tissue depots, a paracrine dialog between adipocytes, pre-adipocytes, endothelial, and inflammatory cells has been described. This results in the release of a myriad of pro- and anti-inflammatory molecules, leading to a low-grade inflammatory and pro-fibrotic environment.[5,6] The biological activity of the EAT is related to the severity of coronary artery disease (CAD),[4,7,8] the degree of cardiac hypertrophy,[9,10] and is enhanced in type-2 diabetic and obese patients.[11] In addition,

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the abundance of EAT is increased in patients with chronic atrial fibrillation (AF) or with atrial dilatation.[12–16]

To date, the mechanisms underlying the effect of EAT on the myocardium have been poorly studied. The fact that EAT is contiguous with the heart without fascia boundaries enables paracrine processes to occur, through the release of adipo-fibrokines. In this study, the hypothesis that adipose tissue secretes factors with pro-fibrotic properties was tested using an original organo-culture model of rat atria. We observed that the secretome from human EAT, but not from subcutaneous adipose tissue (SAT), has a marked pro-fibrotic effect on the rat atrial myocardium. This effect is mediated in part by Activin A, a member of the TGFβ family. Moreover, we confirmed in human myocardium that the adipose tissue is in close proximity with the myocardium, and is associated with an increased fibrosis.

Methods

An expanded version of the Methods is provided in the Supplementary material online.

Human adipose and cardiac tissue

Paired samples of EAT and SAT were collected from 39 patients undergoing routine cardiac bypass surgery. The SAT biopsies were taken from the parasternal region, while EAT samples were obtained from the left-interventricular groove before cardiopulmonary bypass (paracardial fat was not collected). The clinical parameters of patients are listed in *Table 1*. Twelve paired samples were dedicated for transcriptomic study (Group 1); 13 for the organo-culture experiments (Group 2), and 14 for the proteomic analysis of the conditioned media (Group 3). Samples of right ($n = 15$) or left ($n = 3$) atria were obtained for histological study from 18 patients. Human ventricle samples ($n = 5$) were obtained from autopsy. All clinical investigations were approved by the Ethical Committee of Pitié-Salpêtrière Hospital. All patients undergoing bypass surgery gave written informed consent after individual explanation.

Table 1 Patients characteristics

	Group 1	Group 2	Group 3
n	12	13	14
Age	60.1 ± 6.7	66.7 ± 8.8	62.5 ± 8.7
Sex (M/F)	10/2	8/5	11/3
BMI (kg/m^2)	26.5 ± 3.1	29.6 ± 6.6	26.4 ± 4.4
Diabetes (*n*)	5	5	6
Dyslipidemia (*n*)	10	9	11
Hypertension (*n*)	8	6	10
LEVF (<45%)	2	3	4

BMI, body mass index; LVEF, left-ventricular ejection fraction.
Group 1: 12 subjects whose EAT and SAT samples where used for transcriptomic analysis; Group 2: 13 subjects whose EAT and SAT samples were used for organo-culture experiments; Group 3: 14 subjects whose EAT and SAT samples were used for proteomic analysis.

Organo-culture of adult rat atria

To study the effect of adipose-tissue-conditioned medium, we developed an organo-culture model of adult rat atria. All experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health.

Rat cardiac fibroblast cultures

Atria-derived fibroblast cultures were prepared as previously described.[17]

Immunohistological analysis

Frozen atrial organo-culture tissue sections were used for immunohistological analysis of atrial fibrosis.

Multiplexed proteomics and enzyme-linked immunosorbent assay

Cytometric bead arrays were used to analyse protein composition of EAT- and SAT-conditioned media.

Statistical analysis

Values are expressed as mean ± SEM. The Gaussian distribution of all parameters was tested. Differences between variables were determined by the non-parametric Wilcoxon signed-rank test and the Mann–Whitney–Wilcoxon test for paired and unpaired data, respectively. Statistical tests were two-tailed. Correlative analyses were determined by Spearman statistical test. In all analysis, $P < 0.05$ was considered statistically significant. To study the relationship between clinical parameters and the composition of the EAT secretome, a multiple factor analysis was used [(Escofier and Pages, 1994, Multiple Factor Analysis (AFMULT package)].

Results

Epicardial, but not subcutaneous, adipose-tissue secretome induces atrial fibrosis

The EAT was collected in patients with coronary insufficiency, a condition known to be associated with biologically active EAT[2,8] (*Table 1*). The secretome was obtained by harvesting the conditioned media of adipose-tissue samples maintained in culture as previously described.[18] EAT secretomes from two patients were pooled in order to obtain enough volume. Atrial organo-culture incubated for 1 week with EAT showed global fibrosis (*Figure 1A–F*). Picrosirius-red staining indicated a two-fold increase in fibrosis in EAT-treated atria (33.78% ± 1.33, $P = 0.001$) compared with control-treated ones (15.67% ± 0.78). The SAT-conditioned media did not induce fibrosis (15.33% ± 1.60) (*Figure 1J*). The atrial myocardium preserved its sarcomeric organization throughout culture (*Figure 1G–I*). Fibrosis was composed of collagen types I, III, and VI (Supplementary material online, *Figure S2A* and *B*). At higher magnification and using combined phase contrast and high resolution 3-dimensional deconvolution microscopy, collagen accumulation could be seen at the endocardial (peritrabeculae) and epicardial faces of the atria. Collagen accumulation was

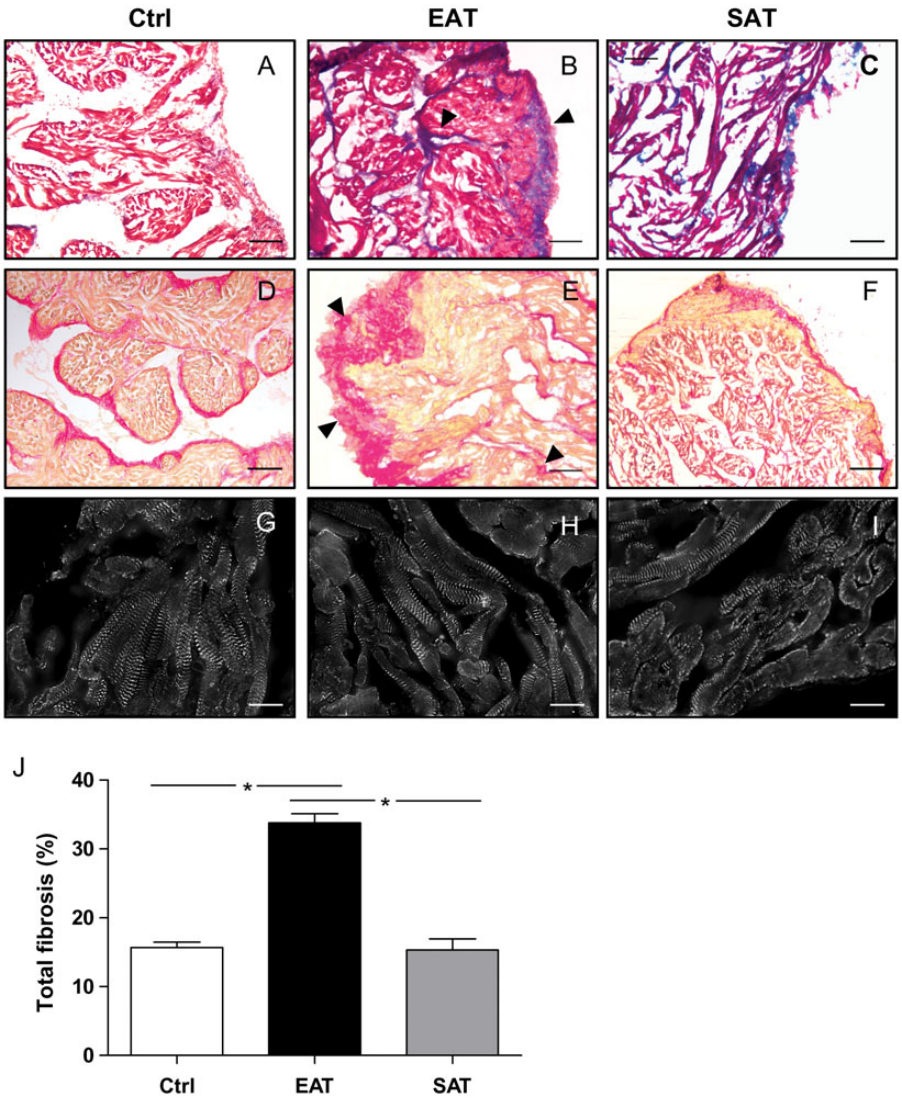

Figure 1 Epicardial adipose tissue induces atrial fibrosis. Masson's trichrome (*A–C*; collagen in blue) and Picrosirius red (*D–F*; collagen in red) were used to visualize fibrosis in rat atrial sections after incubation with epicardial (EAT), subcutaneous (SAT) adipose-tissue-conditioned media, or with control medium (Ctrl) (10× magnification, scale bar = 100 μm). Arrowheads show atrial fibrotic areas. (*G–I*) Sarcomeric α-actinin immunostaining of atrial sections confirmed that samples maintained their typical organization in organo-culture (60× magnification, scale bar = 10 μm). (*J*) Total fibrosis in atrial sections: data depict the amount of fibrotic area as a percentage of the total tissue surface (four separate experiments, $^*P < 0.05$).

also observed between myocytes (interstitial areas), and was more pronounced in EAT-treated atria (*Figure 2A* and *B*).

Fibroblasts are the primary source of extracellular matrix proteins. Indeed, collagen I gene expression was up-regulated in atrial fibroblasts cultured with EAT-conditioned medium for 4 days. The α-smooth muscle actin was also up-regulated in this condition, suggesting differentiation of fibroblasts into myofibroblasts (Supplementary material online, *Figure S3B*). Collectively, these results indicate that EAT secretome induces a global fibrosis of the atrial myocardium *ex vivo*.

Adipo-fibrokines secreted by human epicardial adipose tissue

The adipo-cytokines secreted by human EAT were identified using cytometric bead arrays and ELISA. The EAT secreted angiogenic

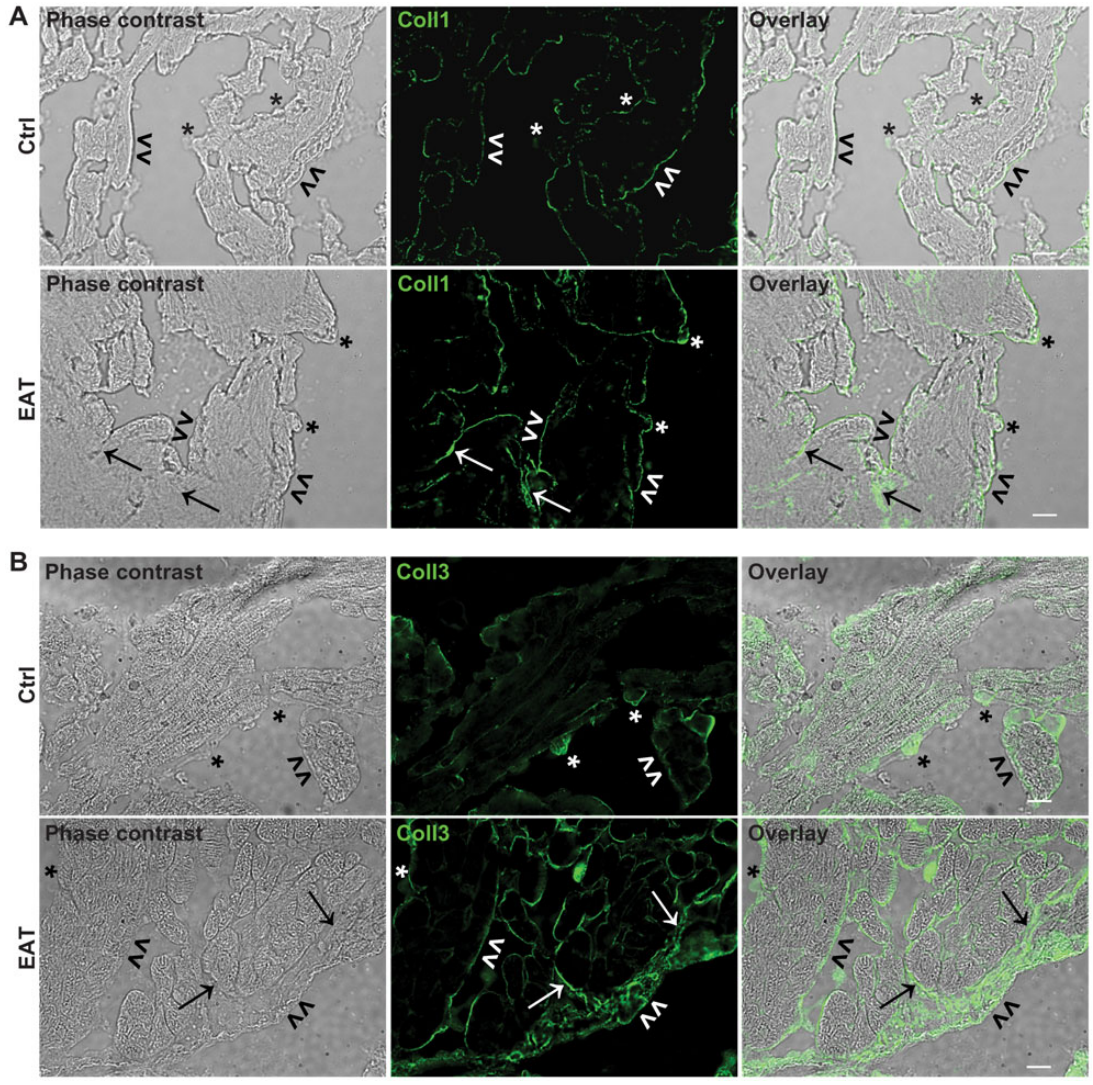

Figure 2 Distribution of fibroblasts and collagen fibres in rat atrial explants treated with EAT-conditioned medium. Collagen 1 (*A*) and 3 (*B*) immunostainings of rat atrial organo-cultures treated with control or/and EAT-conditioned medium. Arrow heads indicate perimyocyte (or peritrabecula) and epicardial collagen fibres, stars point fibroblast cells bodies. Note that collagen fibres are also found between two adjacent myocytes, deeply inserted into the tissue (arrows).

factors (VEGF and Thrombospondin-2) and matrix metalloproteinases (MMPs) such as MMP1, MMP8, and MMP9, more abundantly than SAT (*Table 2*). In addition, EAT expressed Activin A, a member of the TGF-β superfamily. In contrast, the level of TGF-β1 was unchanged between EAT and SAT, and other TGF-β members were not detectable. There was a three-fold increase in Activin A concentration in EAT-conditioned media (3.5 ± 0.8 ng/mL; ranging from 1.3 to 11 ng/mL, median concentration: 2.3 ng/mL) compared with SAT-conditioned media (1.3 ± 0.3 ng/mL; ranging from 0.3 to 3.6 ng/mL, median concentration: 0.8 ng/mL) (*Table 2*, $P = 0.0002$). This increase was also confirmed at the tissue level by immunostaining (*Figure 3E* and *F*). At the mRNA level, Activin A was higher in EAT than in SAT depots, although the difference did not reach significance (relative expression, 2.58 ± 0.65 vs. 1.15 ± 0.28; $P = 0.07$) (*Figure 3A*). In addition, Follistatin, the endogenous inhibitor of Activin A, was not enhanced in EAT resulting in an increase in Activin A/Follistatin ratio at both the transcriptional and protein levels (*Figure 3C* and *D*).

Table 2 Quantification of adipo-fibrokines secreted by human epicardial adipose tissue

	EAT (ng/mL)	SAT (ng/mL)	*P*-value*
Angiogenic factors			
Angiogenin	7.8 ± 1.5	9.4 ± 1.7	0.34
Endostatin	3.8 ± 0.5	3.4 ± 0.4	0.61
Angiopoietin	ND	ND	NA
Thrombospondin-2	2.6 ± 0.12	1.2 ± 0.15	<0.0001
VEGF-D	ND	ND	NA
VEGF	0.4 ± 0.02	0.09 ± 0.01	<0.0001
Remodelling factors			
Activin A	3.5 ± 0.8	1.2 ± 0.2	0.0002
Follistatin	0.54 ± 0.2	0.61 ± 0.1	0.73
TGF-β1	0.4 ± 0.2	0.7 ± 0.1	0.007
TGF-β2	ND	ND	NA
TGF-β3	ND	ND	NA
MMP1	10 ± 0.9	5 ± 0.6	0.0002
MMP2	2.6 ± 0.3	5.2 ± 0.3	<0.0001
MMP3	0.6 ± 0.1	4.1 ± 0.6	<0.0001
MMP7	ND	ND	NA
MMP8	1.3 ± 0.07	0.4 ± 0.03	<0.0001
MMP9	2.4 ± 0.2	0.6 ± 0.06	<0.0001
MMP12	ND	ND	NA
MMP13	0.09 ± 0.01	0.1 ± 0.01	0.03

NA, not applicable; ND, non-detectable.
*P-value for the difference between EAT and SAT, Mann–Whitney test.

Multiple factor and correlative analysis of individual secretome profile of EAT revealed a positive correlation between the level of Activin A and MMP8 (rho = 0.6689, $P = 0.00894$). Moreover, the composition of the secretome varied markedly between individuals; the level of Activin A being higher in patients with reduced left-ventricular function (LVEF < 45%) (*Figure 4A* and *B*).

Activin A promotes myocardial fibrosis

Given the known pro-fibrotic effect of Activin A,[19] we went on to test the hypothesis that Activin A is involved in EAT-induced atrial fibrosis. First, organo-cultures treated for 1 week with control medium supplemented with Activin A showed a marked fibrosis (2 ng/mL: 39.40% ± 3.9, $P < 0.001$; 5 ng/mL: 36.36% ± 2.04, $P < 0.001$) compared with controls (15.67% ± 0.78) (*Figure 5*). In addition, Activin A induced a *de novo* synthesis of collagen types I, III, and VI (*Figure 5D* and Supplementary material online, *Figure S4*). Collagen accumulation was observed at the endocardial and epicardial faces of the myocardium and between myocytes (*Figure 5D*). Secondly, when EAT-conditioned medium was pre-incubated with Activin A neutralizing antibodies, myocardial fibrosis was not observed (EAT+Activin A-neutralizing Ab: 11.83% ± 0.4; EAT+IgG: 24% ± 0.93; $P = 0.002$) (*Figure 6A–C*). Thirdly, EAT secretome and Activin A induced similar changes in atrial gene expression profiles (*Figure 7A*). Both EAT and Activin A increased the expression of TGF-β1 and β2 (*Figure 7B*). However, EAT-conditioned media did not induce additional Activin A expression in the atrial organo-culture, suggesting that EAT may be the primary source of Activin A (*Figure 7B*). These results indicate that atrial fibrosis induced by EAT is primarily mediated by Activin A.

Tight interaction between epicardial adipose tissue and human atrial myocardium

To study whether the presence of fat depots could be associated with fibrosis in human myocardium, histological analysis was performed in left ($n = 3$) and right atria ($n = 15$) as well as in ventricular myocardium ($n = 5$). *Figure 8* shows representative examples of

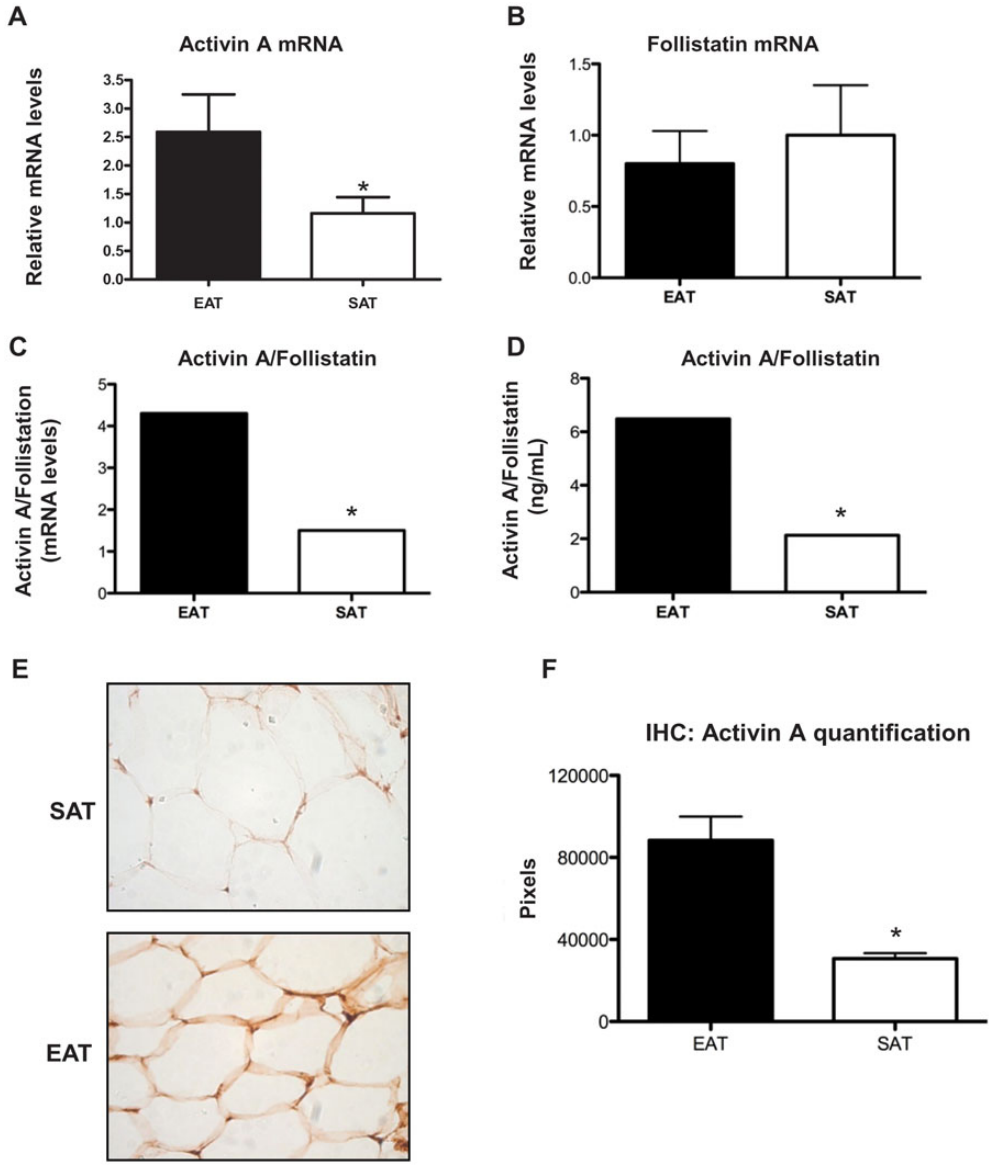

Figure 3 Activin A is increased in epicardial adipose tissue. (*A* and *B*) Activin A and Follistatin gene expression were measured in paired samples of EAT and SAT adipose tissue from 12 subjects (Study population: Group 1). All results are normalized vs. 18S gene expression levels. (*C* and *D*) Ratios of Activin A/Follistatin have been measured at the mRNA and protein levels. (*E*) Activin A immunostaining of paired samples of EAT and SAT adipose tissue from 12 subjects (Study population: Group 1). (*F*) Quantification of Activin A staining (signal intensity in pixels). The non-parametric Mann–Whitney–Wilcoxon test for paired and unpaired data was used. Data are represented by mean ± SEM. $*P < 0.05$.

dense fat tissue infiltrating the atrial and ventricular myocardium (*Figure 8A* and *B*). This tissue was composed of well-organized adipocytes surrounded by peri-cellular fibrosis as described elsewhere.[5] At a higher magnification, fibrosis could be seen at the interface between adipose and myocardial tissues (*Figure 8A* and *B*). A marked interstitial fibrosis of the neighbouring myocardium was also observed (*Figure 8B*). These results indicate that myocardial fibrosis and EAT can be associated *in situ* and support the possibility of a paracrine effect of the EAT secretome on the neighbouring myocardium.

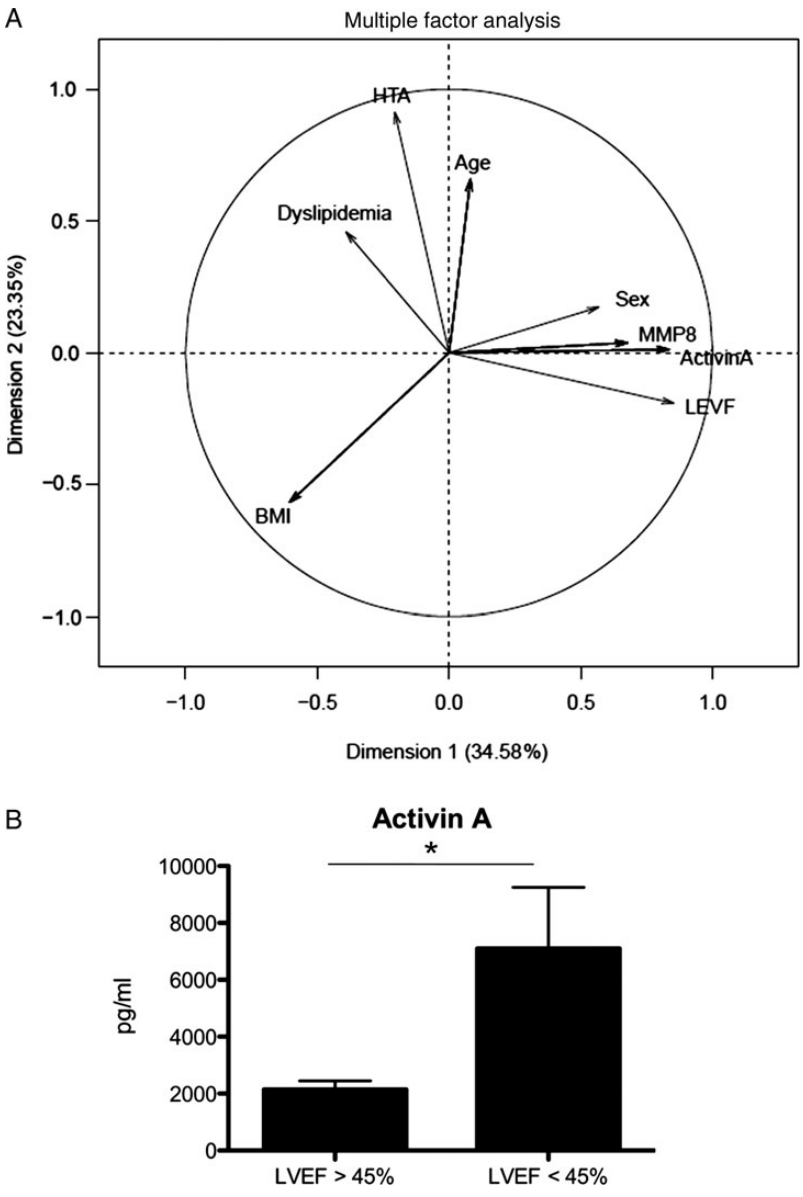

Figure 4 EAT secretome profile and clinical parameters. (*A*) Multiple factor analysis was used to establish the relation between Activin A, MMP8, and clinical parameters. (*B*) Histogram of Activin A level in patients with LVEF $>$ 45% ($n = 8$) and LVEF $<$ 45% ($n = 4$). The non-parametric Mann–Whitney–Wilcoxon test for unpaired data was used. Data are represented by mean $\pm$ SEM. *$P < 0.05$.

Discussion

This study constitutes the first evidence that the secretome from human EAT can induce fibrosis of the myocardium and that Activin A, an adipo-fibrokine, may be the mediator of this pro-fibrotic effect.

Using an original atrial organo-culture model, it was possible to study directly the effects of the secretome of human adipose tissues on the myocardium, regardless of co-morbid factors associated with increased body fat. We observed that conditioned medium from human EAT, but not from SAT, obtained from patient suffering mainly from coronary insufficiency, rapidly induces marked myocardial fibrosis *ex vivo*. This may indicate that deep adipose tissues such as EAT have particular pro-fibrotic effects. Low-grade inflammation of adipose tissue is associated with the release of several adipokines and cytokines. For instance,

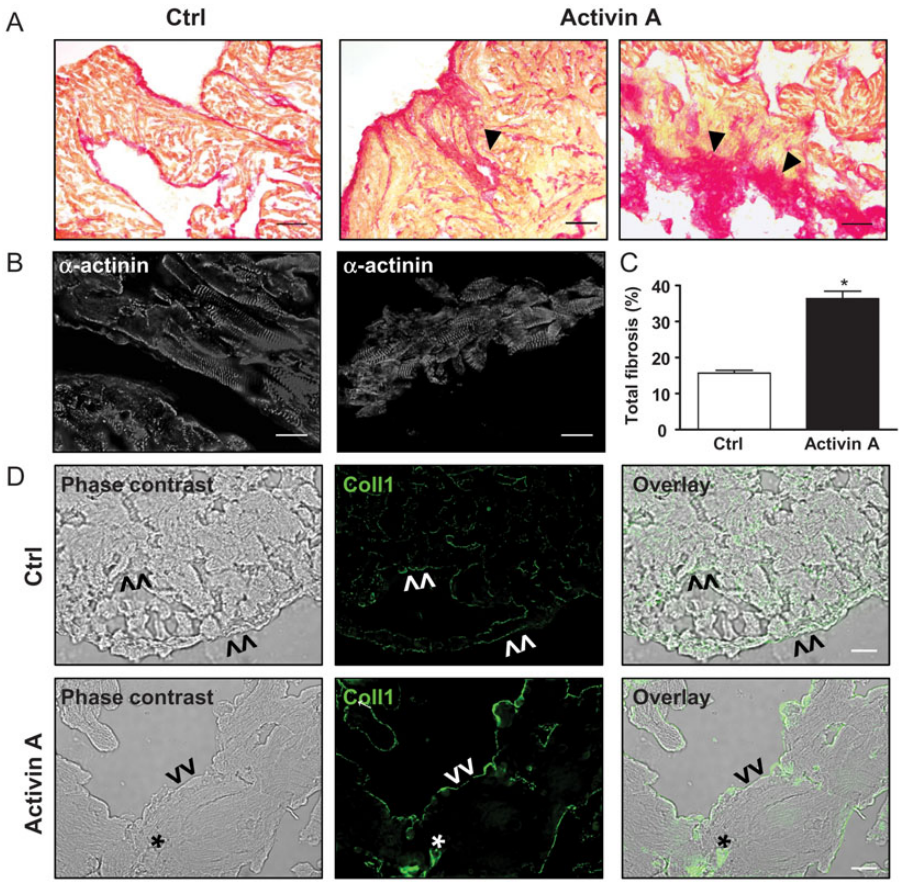

Figure 5 Activin A promotes atrial remodelling. (*A*) Picrosirius red was used to visualize collagen deposition in rat atrial sections after 1-week incubation with human recombinant Activin A (5 ng/mL, middle and right) or control medium (Ctrl, left). (*B*) Preservation of cardiac tissue characteristics after treatment with Activin A (left: Ctrl; middle: Activin A-treated). (*C*) Total fibrosis in atrial sections: data depict the amount of fibrotic area as percentage of the total tissue area. The non-parametric Mann–Whitney–Wilcoxon test for unpaired data was used. Data are represented by mean ± SEM. $^{*}P < 0.05$. (*D*) Distribution of fibroblasts and collagen fibres in rat atrial organo-cultures treated with Activin A. Immunostaining of collagen 1 in rat atrial sections after incubation with Activin A or control medium (Ctrl) (×60 magnification, scale bar = 10 μm). Data are representative of four separate experiments.

pre-adipocytes, which display strong similarities with fibroblasts, produce Activin A in macrophage-induced inflammatory microenvironment.[3] Activin A, a member of the TGF-β superfamily, is secreted by various cell types and exerts pleiotropic activities. For instance, Activin A has been involved in reproductive and stem-cells biology, inflammation, erythroid differentiation, and wound healing. Moreover, Activin A has pathogenic roles in lung, kidney, and liver fibrotic diseases.[19–23]

Here we found that Activin A is abundantly secreted by human EAT, a finding that was consistent with the recent study of Greulich *et al.*[11] on the EAT of type 2 diabetic and obese patients. The EAT of obese guinea pig also secretes Activin A.[24] We showed that supplementation of culture media with recombinant human Activin A reproduced the atrial myocardial fibrosis observed with EAT secretome, and that anti-Activin A antibody neutralized the pro-fibrotic effects induced by the EAT secretome. Taken together, these observations provide compelling evidence that Activin A is an important mediator of the pro-fibrotic effect of EAT on atrial myocardium. Other members of the TGF-β superfamily exhibit fibrotic properties.[25] Interestingly, both EAT-conditioned medium and Activin A induced the expression of TGF-β1 and -β2 in rat atria, which could indirectly contribute to the pro-fibrotic effect of Activin A. The high level of MMPs in EAT secretome is likely to contribute to extracellular matrix remodelling of the myocardium.[26] We found that Activin A and MMP8 from EAT secretomes were modulated in the same way. Of note, an up-regulation of MMP8 has been reported during heart failure.[27]

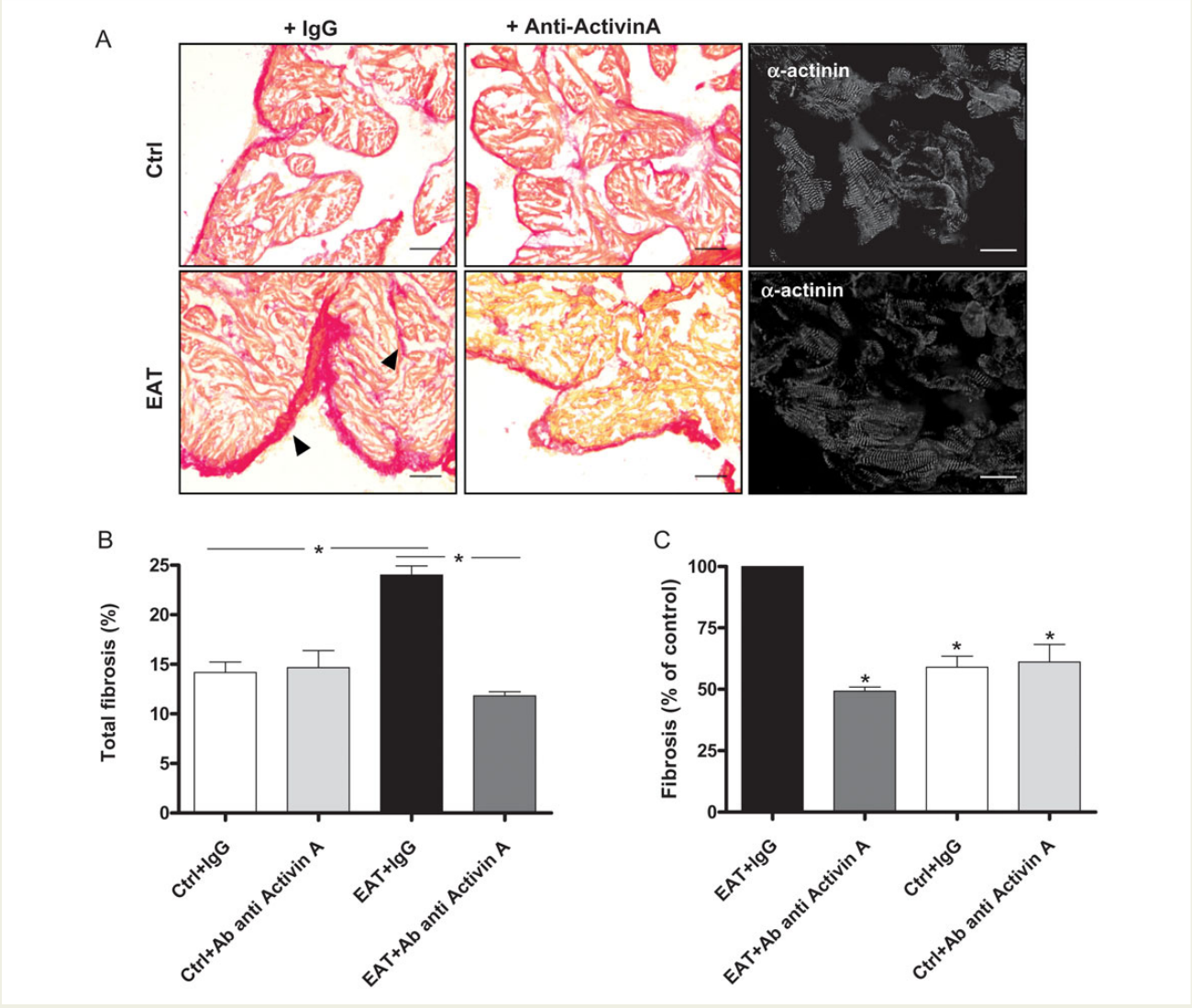

Figure 6 Neutralization of Activin A prevents atrial fibrosis. Atria were co-incubated for 1 week with epicardial adipose tissue (EAT)-conditioned medium or control medium (Ctrl) pre-incubated with Activin A-neutralizing antibody or IgG. (*A*) Fibrosis was visualized using Picrosirius red. Sarcomeric α-actinin immunostaining confirmed that the samples were well preserved (right panel). (*B*) Total fibrosis: data depict the amount of fibrotic area as the percentage of the total tissue surface. $^{*}P < 0.05$ (*C*) Data are expressed in the percentage of fibrotic area compared with EAT treatment taken as reference. The non-parametric Mann–Whitney–Wilcoxon test for unpaired data was used. Data are represented by mean ± SEM. $^{*}P < 0.05$ Data are representative of three separate experiments.

Additional cardiac effects have been reported for Activin A. For instance, Activin A (i) induces the expression of mediators involved in post-infarction healing in rats,[28,29] (ii) has anti-hypertrophic and anti-apoptotic properties on the myocardium exposed to ischaemia/reperfusion and pressure overload injuries,[30,31] and (iii) has a negative inotropic effect on adult rat cardiomyocytes.[11]

The lack of fascia between the EAT and the myocardium enables molecules secreted by the EAT to diffuse into the myocardium, in a paracrine-like manner. The close contact between adipose tissue and myocardium observed in human samples supports this possibility. It is also likely that cytokines secreted by the EAT accumulate in the pericardial fluid and contribute to the local effect of the EAT secretome on the heart.[32]

Pathological consequences and potential clinical significance

In the present study, we show that the EAT of all patients of the study expressed numerous adipo-fibrokines. Therefore, the question of whether EAT consistently exerts pathological effects on

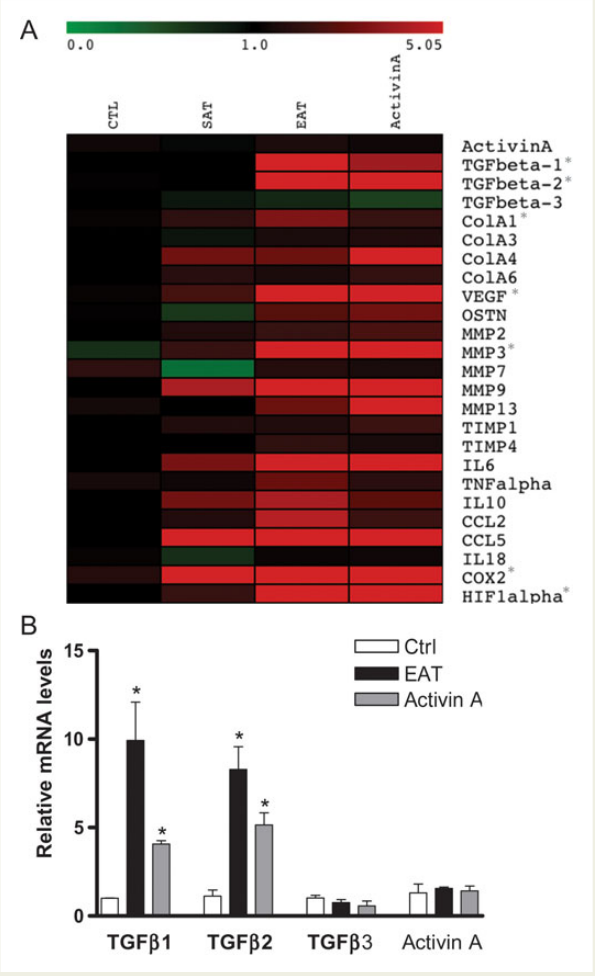

Figure 7 Treatments with EAT secretome and Activin A up-regulate genes involved in ECM remodelling. (*A*) Colour map representation of regulated genes in atria. Fold Expression < 0.8 (Green), $0.8 \leq$ Fold Expression < 1.5 (Black), and Fold expression ≥ 1.5 (Red). (*B*) Graphic representations of TGF-β family members and Activin A gene regulation in rat atrial organo-cultures. All results were normalized vs. 18S gene expression. Data are representative of six separate experiments. The non-parametric Mann–Whitney–Wilcoxon test for unpaired data was used. Data are represented by mean ± SEM. $^*P < 0.05$.

the neighbouring myocardium can be raised. Our observation of a pro-fibrotic effect of EAT secretome on the atrial myocardium does not fully answer this question as collagen accumulation also exists in physiological conditions, for example at the periphery of the trabeculae or in the interstitial space.

However, it is possible that some clinical conditions lead to more abundant and biologically active adipose tissue.[33] This argument is supported by our observation that Activin A and MMP8 levels measured in EAT varied considerably between patients and were increased in those with low left-ventricular function. It has been previously reported that Activin A expression is increased in serum after heart failure and correlates with its severity.[28] Greulich *et al.* recently reported that Activin A, angiopoietin-2, and CD14 are abundantly expressed in the EAT of obese patients with type 2 diabetes. In our study, Activin A levels were not different between diabetic and non-diabetic patients. This discrepancy could be explained by the inclusion, in our study, of both type 1 and 2 diabetic and non-obese patient populations. Taken together, the study from Greulich *et al.* and ours indicate that the biological activity of EAT can be affected by clinical conditions.

Recently, epidemiological studies have reported an association between the thickness of EAT and the severity of AF.[12–16] Imagery studies have shown that patients in AF are characterized by an increased volume of peri-atrial adipose tissue compared with controls.[16] This association is more pronounced in chronic AF, or when AF is associated with left-atrial dilation. It has been proposed that pro-inflammatory cytokines may diffuse from EAT into the adjacent myocardium and promote arrhythmogenesis.[12,14,16] Given the role of fibrosis in the substrate of AF,[34,35] our results could provide a mechanism for the relationship between EAT thickness and the risk of atrial arrhythmia.[12,14,16]

Conclusion

Activation of the renin–angiotensin–aldosterone system, Platelet-derived Growth Factor, Connective Tissue Growth Factor,[26,36–38] and local inflammation are well-recognized pathogenic factors of myocardial fibrosis. Here we provide evidence that EAT, through its capacity to produce and secrete adipo-fibrokines (pro-fibrotic molecules) such as Activin A and MMP8, could be a complementary mechanism contributing to the formation of myocardial fibrosis in a paracrine-dependent manner.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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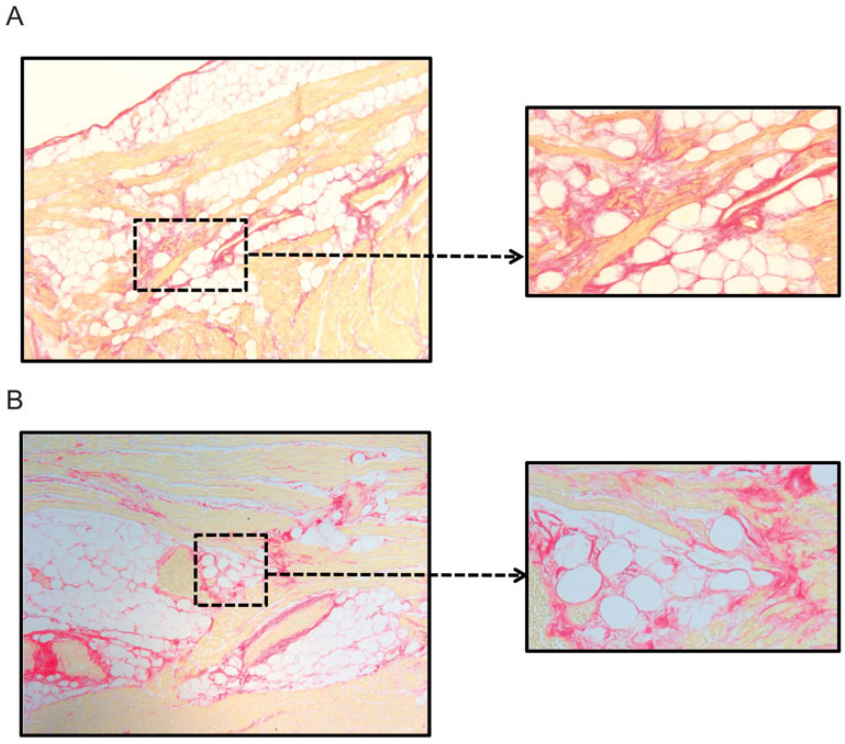

Figure 8 EAT infiltration in human myocardium is associated with myocardial fibrosis. Picrosirius-red staining of left atrial (*A*) and ventricular myocardial (*B*) sections. High magnification images show that adipocytes inclusions are associated with myocardial fibrosis (right panels).

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