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Role of epicardial adipose tissue NPR-C in acute coronary syndrome

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HIGHLIGHTS

- Expression of NPR-C in epicardial adipose tissue (EAT) is lower in ACS compared to stable CAD or non-CAD patients.
- Lower NPR-C expression in EAT is associated with an increased risk of ACS.
- ACS patients have lower EAT UCP1 and PGC1α expression, and PGC1α levels are associated with NPR-C.
- Activation of p38 MAPK in EAT is lower in ACS patients and correlates with NPR-C.

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ABSTRACT

Background and aims: It has been suggested that epicardial adipose tissue (EAT) thermogenesis plays a role in coronary artery disease (CAD). Recent evidence indicates that natriuretic peptide receptors (NPRs) are critical for thermogenesis. We determined the expression and signaling of NPRs in EAT in the context of CAD progression and their association with brown fat-related genes, such as uncoupling protein 1 (*UCP1*) and peroxisome proliferator-activated receptor gamma coactivator alpha (*PGC1α*).

Methods: NPR-A, NPR-B and NPR-C mRNA and protein expression levels were analyzed in EAT and thoracic subcutaneous adipose tissue (SAT) from non-CAD (NCAD), stable CAD and acute coronary syndrome (ACS) patients. The associations of NPRs with thermogenic genes were also evaluated.

Results: The EAT of ACS patients showed lower NPR-C gene and protein expression levels compared with that of stable CAD or NCAD patients. *NPR-C* mRNA expression in EAT also decreased as the number of injured arteries rose, and correlated positively with left ventricular ejection fraction and EAT *PGC1α* mRNA expression. EAT *PGC1α* and *UCP1* gene expression levels also decreased in the ACS group. Linear and logistic regression models showed associations of EAT *NPR-C* mRNA levels with EAT *PGC1α* mRNA levels and the presence of ACS. Furthermore, the EAT of ACS patients showed reduced p38 mitogen-activated protein kinase (p38 MAPK) phosphorylation levels, which correlated positively with NPR-C protein levels.

Conclusions: The EAT of patients with ACS is characterized by decreased NPR-C, reduced *UCP1* and *PGC1α* mRNA expression levels and reduced activation of the p38 MAPK pathway. The associations among the expression of EAT *NPR-C* and ACS, and brown fat markers suggest that NPR-C may play a role in ACS and in the regulation of EAT brown-like fat features in humans.

1. Introduction

Epicardial adipose tissue (EAT) is a unique fat depot around the heart that shares a close anatomic proximity and vascular supply with the myocardium and coronary arteries [1]. Its accumulation around the heart, measured using various imaging techniques, has been associated with the onset and progression of coronary artery disease (CAD) in humans [2,3]. Mahabadi et al. [3] quantified EAT volume and the

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incidence of coronary events over a follow-up of eight years in 4093 participants in the Heinz Nixdorf Recall study. They found that the incidence of hard coronary events (cardiac death or nonfatal myocardial infarction) increased with the amount of EAT and that EAT volume remained independently associated with coronary events, even after adjustment for cardiovascular risk factors. It has been suggested that under pathological conditions, EAT dysfunction could negatively affect the heart in several ways: reactive oxygen species production, oxidative stress and macrophage activation, atherogenic and inflammatory environment, impaired thermogenesis or adipofibrokine production [4–6]. However, how EAT directly affects the pathological process of CAD or even acute coronary syndrome (ACS) still remains largely unknown.

Natriuretic peptides (NPs) are best known as the hormones secreted from the heart in response to cardiac and mechanical overload in order to regulate systemic blood pressure and maintain cardiovascular homeostasis [7]. NPs mediate their biological effects through a family of NP receptors (NP receptor [NPR] A NPR-A, NPR-B and NPR-C) [8]. Although NPR-A is considered the principal biologically active receptor of the NP family, the molecular signaling mechanisms of NPRs are not well understood. Activation of NPR-A and NPR-B generates the intracellular second messenger cyclic guanosine monophosphate (cGMP) and the subsequent activation of cGMP-dependent protein kinase 1 (also known as PKG1) [8]. NPR-C does not have guanylyl cyclase activity, and was initially considered as a clearance receptor with no signaling function [9], however, it is known that NPR-C is able to activate inhibitory G proteins and modulate intracellular transduction systems [10,11].

In addition to their chief function as cardiovascular hormones, NPs have emerged as potent metabolic hormones impacting on adipose tissue in which NPRs are expressed [7,12–14]. In 2000, Coralie Sengenès et al. demonstrated that physiological concentrations of NPs could stimulate lipolysis in isolated human adipocytes [15]. Consequent studies in humans reported that NPs could increase energy expenditure and fat oxidation [16]. More recently, studies by the Collins group have shown that NPs are also able to induce the expression of brown adipocyte markers such as uncoupling protein 1 (*UCP1*) and peroxisome proliferator-activated receptor gamma coactivator alpha (*PGC1α*) [12,17], suggesting that they may play a role in the "browning" of white fat cells and activate the thermogenic program.

A reduced function of brown adipose tissue in humans is closely associated with cardiovascular risk and compromised metabolic health [18]. Studies with mice have shown that brown adipose tissue activation mediates cardioprotection [19,20]. Our group and others have found that human EAT expresses genes as brown fat [21,22]. In line with a cardioprotective role of brown adipose tissue, we have also shown that decreased *PGC1α* expression in EAT is associated with a higher prevalence of coronary lesions [23]. Because NPs have recently been shown to induce brown fat markers in adipocytes [17], we hypothesized that a deregulated NPRs system in EAT may impact on EAT brown-like fat characteristics, and promote the progression of CAD.

In this study, we found that EAT *NPR-C* expression levels are associated with the presence of ACS and the expression levels of EAT *PGC1α*. EAT NPR-C mRNA and protein levels are lower in ACS patients compared with stable CAD or NCAD patients. We also showed a decrease in EAT *NPR-C* expression with angiographic extension of CAD. Finally, we also found a reduced expression of brown fat-related genes and a lower activation of the p38 MAPK signaling pathway in the EAT of patients with ACS, which might be in part due to reduced EAT NPR-C expression.

2. Patients and methods

2.1. Study population

This study included 45 patients who underwent coronary artery bypass graft surgery for ACS (n = 29) or stable CAD (n = 16) and 29 non-CAD patients (NCAD) who underwent surgery for aortic and/or mitral valve replacement. The ACS group was represented by patients with a diagnosis of unstable angina or acute myocardial infarction documented with electrocardiography, angiographic findings and laboratory markers, whereas the stable CAD group was represented by patients with angina symptoms that had been stable for some time, with angiographically documented coronary stenosis (50% or greater narrowing of diameter of one or more vessels). The NCAD group was characterized by coronary arteries with or without stenosis less than 50% in any vessel, requiring valve replacement but not coronary artery bypass graft. The elapsed time from the onset of symptoms to initiating coronary artery bypass graft procedure for patients with ACS was 10 days. Exclusion criteria were acute inflammatory disease, severe infective disease and/or cancer. All patients signed informed consent forms, and the study was reviewed and approved by the Ethics and Research Committee of Regional University Hospital (Malaga, Spain), and carried out in accordance with the Declaration of Helsinki.

2.2. Samples from patients who underwent open heart surgery

2.2.1. Adipose tissue collection

Human EAT biopsy samples (average 0.2–0.5 g) were taken near the proximal right coronary artery and subcutaneous adipose tissue (SAT, average 1.5–2 g) was obtained from the thorax. Thoracic SAT biopsies were taken 30–45 min after anesthesia and EAT biopsies were taken approximately 1 h after anesthesia. All the tissues were frozen immediately after surgery in liquid nitrogen and stored at −80 °C for RNA and protein isolation.

2.2.2. Blood collection

On the morning of surgery, peripheral venous blood was drawn into glass vacutainer tubes (BD vacutainer™, London, UK) to obtain serum samples. The tubes were left at room temperature for 20 min and centrifuged at 4000 g for 10 min at 4 °C before analysis.

2.3. Biochemical measurement

Serum levels of glucose, total cholesterol, triglycerides and high-density lipoprotein cholesterol were measured by standard enzymatic methods (Randox Laboratories Ltd., Antrim, UK). Low-density lipoprotein cholesterol was calculated from the Friedewald equation.

2.4. mRNA quantification

Relative mRNA expression was determined by TaqMan-based real-time PCR as detailed in the Supplemental Materials. TaqMan ID numbers are listed in Supplemental Table 1.

2.5. Western blot analysis

Protein expression levels in EAT and thoracic SAT were analyzed by Western blotting as described in the Supplemental Materials.

2.6. Statistical analysis

Data were analyzed using SPSS software, version 15.0 for Windows (Chicago, IL, USA), with figures generated using GraphPad Prism version 5.01. Statistical significance was set at $p < 0.05$. Details of the statistical analysis appear in the Supplemental Materials.

3. Results

Table 1 summarizes the demographic and clinical characteristics of the patients enrolled in the study. No differences were found between the stable CAD and ACS groups for demographic factors (sex, age and

Table 1
Characteristics of study participants.

	No CAD (n = 29)	Stable CAD (n = 16)	ACS (n = 29)	*p* value
Sex, (n, male/female)	15/14	14/2*	25/4*	0.004
Age (years)	64 ± 2	65 ± 3	65 ± 2	0.93
Body mass index (kg/m^2)	27.6 ± 1.2	28.9 ± 1.7	29.8 ± 1.5	0.51
Left ventricular ejection fraction (%)	60.1 ± 2.1	54.8 ± 4.6	50.5 ± 1.5*	0.014
Risk factors, (%)				
Type 2 diabetes	24	25	69*†	0.001
Dyslipidemia	39	62	68	0.08
Hypertension	59	69	86	0.06
Current smoking	28	50	34	0.32
Cerebrovascular accident	6.9	6.2	6.9	0.99
Treatment, (%)				
Aspirin	60	69	72	0.52
Clopidogrel	3.4	0	6.9	0.52
Statin	44	64	59	0.28
ACEI/ARB	30	27	54	0.07
Beta-blocker	72	64	68	0.79
Oral antidiabetic drug	14	25	52*	0.006
Insulin	3	6	17	0.18
Laboratory data				
Glucose (mg/dL)	103 (97–129)	102 (88–137)	130 (108–149)	0.12
Cholesterol (mg/dL)	161.4 ± 7.5	169.7 ± 9.1	150.9 ± 5.6	0.22
LDL-cholesterol (mg/dL)	93.8 ± 5.5	105.3 ± 7.8	89.4 ± 4.2	0.18
HDL-cholesterol (mg/dL)	40 (31–48)	39 (31–52)	33 (27–39)	0.13
Triglycerides (mg/dL)	124 (91–177)	173 (117–205)	171 (103–208)	0.09
Number of diseased vessels				
1		1	1	
2		4	0	
3		11	28	

Data are presented as mean ± SEM or median and interquartile range.
CAD, coronary artery disease; ACS, acute coronary syndrome; n, No. of subject; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker. LDL, low-density lipoprotein; HDL, high-density lipoprotein.
*$p < 0.05$ compared to NCAD, †$p < 0.05$ compared to stable CAD.

body mass index), left ventricular ejection fraction, consumption of medications, glucose and lipid variables and risk factors, with the exception of there being more subjects with type 2 diabetes mellitus in ACS. On the basis of angiographic analysis, 60.9% of the patients were characterized as having significant stenosis. There were 2 patients with 1-vessel disease, 4 patients with 2-vessel disease and 39 patients with 3-vessel disease.

3.1. The extension of coronary artery disease is associated with decreased expression of NPR-C in EAT

To investigate possible differential expression between ACS, stable CAD and NCAD, expression levels of *NPR-A*, *NPR-B* and *NPR-C* in EAT and thoracic SAT were evaluated (Fig. 1). No significant differences were found between the gene expression level of *NPR-A* and *NPR-B* in EAT of all groups of patients, whereas EAT *NPR-C* mRNA levels were significantly lower in ACS patients compared with NCAD patients ($p < 0.005$) or compared with stable CAD patients ($p < 0.05$) (Fig. 1A). When sex, age, body mass index, left ventricular ejection fraction and type 2 diabetes mellitus were included in a logistic regression model, we found that the association between lower EAT *NPR-C* mRNA levels and ACS remained statistically significant (OR 0.936 (0.879–0.996), $p = 0.037$, per a.u.), as shown in Table 2.

In thoracic SAT, *NPR-A* mRNA expression was significantly higher in stable CAD patients compared with NCAD patients ($p < 0.05$) but tended to decrease in ACS patients (Fig. 1A). SAT *NPR-B* mRNA expression was significantly higher in ACS patients compared with NCAD ($p < 0.05$), while SAT *NPR-C* mRNA expression was not statistically different (Fig. 1A). When adding sex, age, body mass index, left ventricular ejection fraction and diabetes mellitus in a logistic regression model, the association between SAT *NPR-B* mRNA expression levels and ACS remained statistically significant (OR 1.173 (1.037–1.327), $p = 0.011$, per a.u.), (Table 2).

There were no differences in the expression levels of any of the studied NPR genes in EAT and thoracic SAT between males and females or subjects with and without type 2 diabetes.

To verify the mRNA expression data, we measured NPR-A and NPR-C protein levels by Western blot analysis from lysates of EAT and SAT. For the first time, we showed the protein bands of NPR-A (115 kDa) and NPR-C (62 kDa) in human EAT with significantly lower NPR-C levels in ACS patients compared with stable CAD or NCAD patients ($p < 0.05$ (Fig. 1B). Although in thoracic SAT, protein bands corresponding to NPR-C tended to increase in patients with ACS, this trend was not statistically significant (Fig. 1B). The relative ratio of NPR-A to NPR-C expression (NPR-A/NPR-C) seems to play a role in the function of adipose tissue [17,24]; however, no significant changes were found in gene expression and protein levels of NPR-A/NPR-C in EAT or SAT between all groups (Supplemental Figs. 1A and B).

We evaluated the mRNA expression of all NPRs in EAT and SAT for all patients in relation to the number of vessels with > 50% of stenosis (Supplemental Fig. 2). Patients with single- and 2- vessel disease were combined into one group due to the small sample size. No significant differences were found in *NPR-A* or *NPR-B* mRNA expression in EAT. It is worthy of note that *NPR-C* mRNA expression in EAT reduced with the number of injured vessels. Thus, patients with 3-vessel disease had significantly lower *NPR-C* mRNA levels than those with single- or 2-vessel disease ($p < 0.05$) and no vessel disease patients ($p < 0.005$) (Supplemental Fig. 2A). In stable CAD group alone, EAT *NPR-C* mRNA was also lower with the extension of coronary lesions (data not shown). In thoracic SAT, patients with 3-vessel disease had slightly higher *NPR-A* mRNA expression ($p = 0.044$) and higher *NPR-B* mRNA expression in thoracic SAT ($p < 0.05$) compared with no-vessel disease patients (Supplemental Fig. 2B).

In addition, EAT *NPR-C* mRNA expression positively correlated with left ventricular ejection fraction ($r = 0.489$, $p < 0.0001$) in all of the study patients.

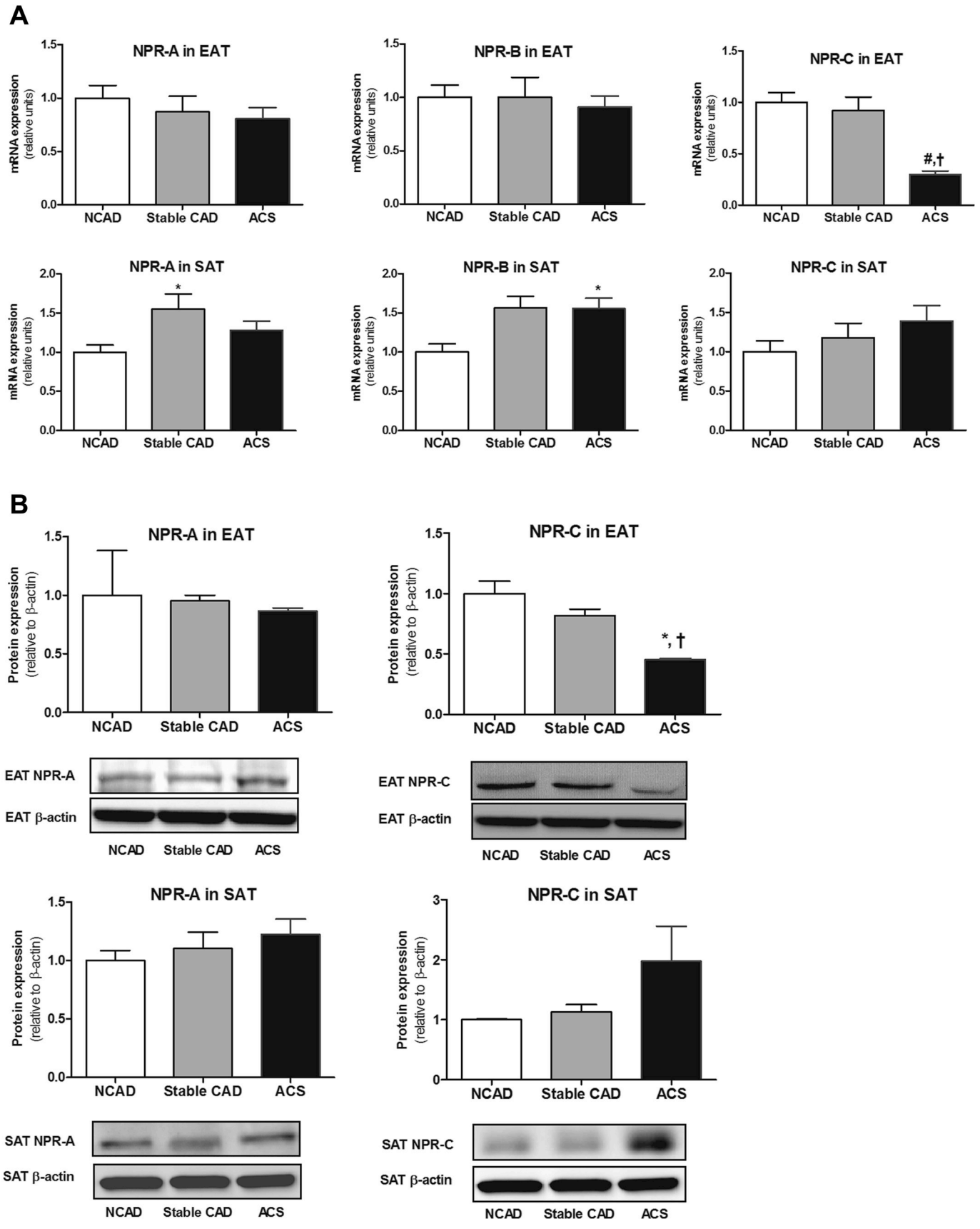

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Fig. 1. Expression of natriuretic peptide receptor C (NPR-C) in epicardial adipose tissue (EAT) is lower in patients with acute coronary syndrome (ACS).
(A) mRNA expression levels of *NPR-A*, *NPR-B* and *NPR-C* were measured by real-time PCR on EAT and thoracic subcutaneous adipose tissue (SAT) from non-coronary artery disease (NCAD), stable coronary artery disease (CAD) and ACS patients. Results are given as mean values of duplicates ± SEM. The expression of NPR-in NCAD patients was assumed to be 1. *$p < 0.05$ and $p \# < 0.005$ *vs.* NCAD; †$p < 0.05$ *vs*. stable CAD, one-way ANOVA followed by Bonferroni's test. (B) Box plots of the quantified expression of NPR-A and NPR-C are shown in the upper panel and representative blots are shown below. Data represents mean ± SEM of 3 independent experiments for each group. β-actin served as a loading control. *$p < 0.05$ *vs*. NCAD and †$p < 0.05$ *vs*. stable CAD.

Table 2
Relationship between EAT and SAT *NPR-A*, *NPR-B* and *NPR-C* gene expression and other variables with the presence of acute coronary syndrome.

Variables	Odds ratio (95% CI)	*p* value
EAT *NPR-A* mRNA (a.u.)	0.993 (0.981–1.005)	0.28
Gender (male)	3.396 (0.468–24.648)	0.23
Age (year)	0.982 (0.898–1.073)	0.68
Left ventricular ejection fraction < 50 (%)	6.825 (0.107–42.07)	0.038
Type 2 diabetes mellitus	14.694 (2.260–95.539)	0.005
Body mass index (kg/m^2)	1.082 (0.948–1.235)	0.14
R^2 (Nagelkerke) = 0.511		
EAT *NPR-B* mRNA (a.u.)	0.927 (0.837–1.026)	0.15
Gender (male)	4.015 (0.530–30.406)	0.18
Age (year)	0.984 (0.900–1.077)	0.73
Left ventricular ejection fraction < 50 (%)	7.268 (1.175–44.938)	0.033
Type 2 diabetes mellitus	20.726 (2.930–146.630)	0.002
Body mass index (kg/m^2)	1.099 (0.959–1.260)	0.17
R^2 (Nagelkerke) = 0.551		
EAT *NPR-C* mRNA (a.u.)	0.936 (0.879–0.996)	0.037
Gender (male)	2.891 (0.342–24.396)	0.33
Age (year)	0.989 (0.898–1.089)	0.82
Left ventricular ejection fraction < 50 (%)	2.987 (0.417–21.408)	0.276
Type 2 diabetes mellitus	23.5 (2.739–201.606)	0.004
Body mass index (kg/m^2)	1.094 (0.952–1.257)	0.21
R^2 (Nagelkerke) = 0.604		
SAT *NPR-A* mRNA (a.u)	1.006 (0.999–1.014)	0.095
Gender (male)	3.954 (0.468–33.385)	0.21
Age (year)	1.035 (0.949–1.129)	0.44
Left ventricular ejection fraction < 50 (%)	16.425 (1.796–150.190)	0.013
DM2	7.881 (1.178–52.736)	0.033
Body mass index (kg/m^2)	1.173 (0.970–1.420)	0.10
R^2 (Nagelkerke) = 0.490		
SAT *NPR-B* mRNA (a.u.)	1.173 (1.037–1.327)	0.011
Gender (male)	4.400 (0.311–62.234)	0.27
Age (year)	1.057 (0.953–1.171)	0.29
Left ventricular ejection fraction < 50 (%)	42.850 (1.704–1077.34)	0.022
Type 2 diabetes mellitus	18.654 (1.134–306.761)	0.041
Body mass index (kg/m^2)	1.126 (0.932–1.360)	0.22
R^2 (Nagelkerke) = 0.570		
SAT *NPR-C* mRNA (a.u.)	1.013 (0.994–1.032)	0.19
Gender (male)	4.998 (0.556–44.970)	0.15
Age (year)	1.030 (0.949–1.117)	0.48
Left ventricular ejection fraction < 50 (%)	16.747 (1.891–148.308)	0.011
Type 2 diabetes mellitus	5.199 (0.861–31.410)	0.072
Body mass index (kg/m^2)	1.149 (0.949–1.390)	0.15
R^2 (Nagelkerke) = 0.465		

Result of logistic regression analysis.
a.u. arbitrary units; CI, confidence interval; EAT, epicardial adipose tissue; SAT, subcutaneous adipose tissue; NPR-, natriuretic peptide receptor-.

Our data indicate that reduced NPR-C expression in EAT is associated with ACS and angiographic extension of CAD.

3.2. NPR-C expression correlates with the expression of brown-like fat genes in human EAT

Because NPs have recently been demonstrated to induce the brown fat thermogenic program in adipocytes [17], we next investigated the relationship between the expression of NPRs and representative genes of brown adipocyte in EAT and SAT. Fig. 2A shows the mRNA expression of *UCP1*, *PGC1α* and *PRDM16* in EAT and thoracic SAT from NCAD, stable CAD and ACS patients. EAT *UCP1* mRNA expression was significantly lower in patients with ACS compared with NCAD patients ($p < 0.05$). EAT *PGC1α* mRNA levels were significantly lower in patients with ACS compared with NCAD patients ($p < 0.05$) or stable CAD patients ($p < 0.05$). *PRDM16* mRNA expression in EAT showed a downward trend in ACS patients, compared with NCAD or stable CAD, but this trend did not reach statistical significance (Fig. 2A).

It should be noted that EAT *NPR-C* mRNA expression levels positively correlated with EAT *PGC1α* and *PRDM16* mRNA expression levels in all subjects (Fig. 2B), even after adjusting for sex and diabetes status. Furthermore, *PGC1α* mRNA expression levels correlated with body mass index ($r = -0.294$; $p = 0.040$), HDL-cholesterol ($r = 0.373$; $p = 0.002$), left ventricular ejection fraction ($r = 0.347$; $p = 0.003$), and EAT *UCP1* mRNA expression levels (r = 0.251, $p = 0.032$). To strengthen the independence of these associations as predictors of EAT *PGC1α* gene expression, linear regression analysis was constructed, showing independent variables to be significant in the correlation, in addition to gender and diabetes status. Log-transformed values were used for the distribution of skewed variables, including *UCP1* and HDL-cholesterol. The variable which was associated with EAT *PGC1α* mRNA expression was EAT *NPR-C* gene expression (Supplemental Table 2). Neither *UCP1* nor *PGC1α* gene expression levels correlated with any NPR gene expression in thoracic SAT. These data suggest that classical thermogenic genes decrease in the EAT of patients with ACS, and that *NPR-C* gene expression relates to *PGC1α* gene expression in EAT.

3.3. Impaired NPR-C signaling in EAT could contribute to the extension of coronary artery disease

As NPs have been shown to enhance the expression of the brown fat gene in a p38 MAPK-dependent manner [17], we investigated p38 MAPK activation in EAT and SAT from the three groups of patients. Fig. 3A shows that activation of EAT p38 MAPK was significantly lower in patients with ACS compared with NCAD patients, as indicated by the double phosphorylation of p38 MAPK ($p < 0.05$). Although SAT p38 MAPK activation was significantly lower in stable CAD compared with NCAD patients ($p < 0.05$), an upward trend existed in patients with ACS (Fig. 3B). It should be noted that EAT NPR-C protein levels correlated with the activation of EAT p38 MAPK ($r = 0.720$, $p = 0.028$). No significant changes were observed in the expression of *PKG1* and *p38 MAPK* mRNAs between the three groups of patients in EAT (Supplemental Fig. 3A) or in SAT (Supplemental Fig. 3B).

Collectively, these data suggest that reduced EAT NPR-C expression in ACS is accompanied by a loss of EAT p38 MAPK pathway activation, which may lead to decreased brown-like fat gene expression in EAT.

4. Discussion

We investigated the relationship between the expression and signaling of NPRs in EAT and thoracic SAT, and the progression of CAD in humans in a selection of patients with ACS, stable CAD and NCAD. The main results of this study show that patients with clinical ACS have a decreased expression of NPR-C at both the protein and mRNA levels in EAT, as well as a reduced activation of p38 MAPK and a lower expression of *UCP1* and *PGC1α* in EAT, findings that have not previously been reported. We also found a decrease in the mRNA levels of EAT *NPR-C* as the number of coronary artery lesions rose. Linear and logistic regression models highlighted significant associations of EAT *NPR-C* gene expression, EAT *PGC1α* mRNA levels and the presence of ACS. Collectively, these findings advance the field of EAT pathophysiology by positing an association between ACS and a reduced expression of

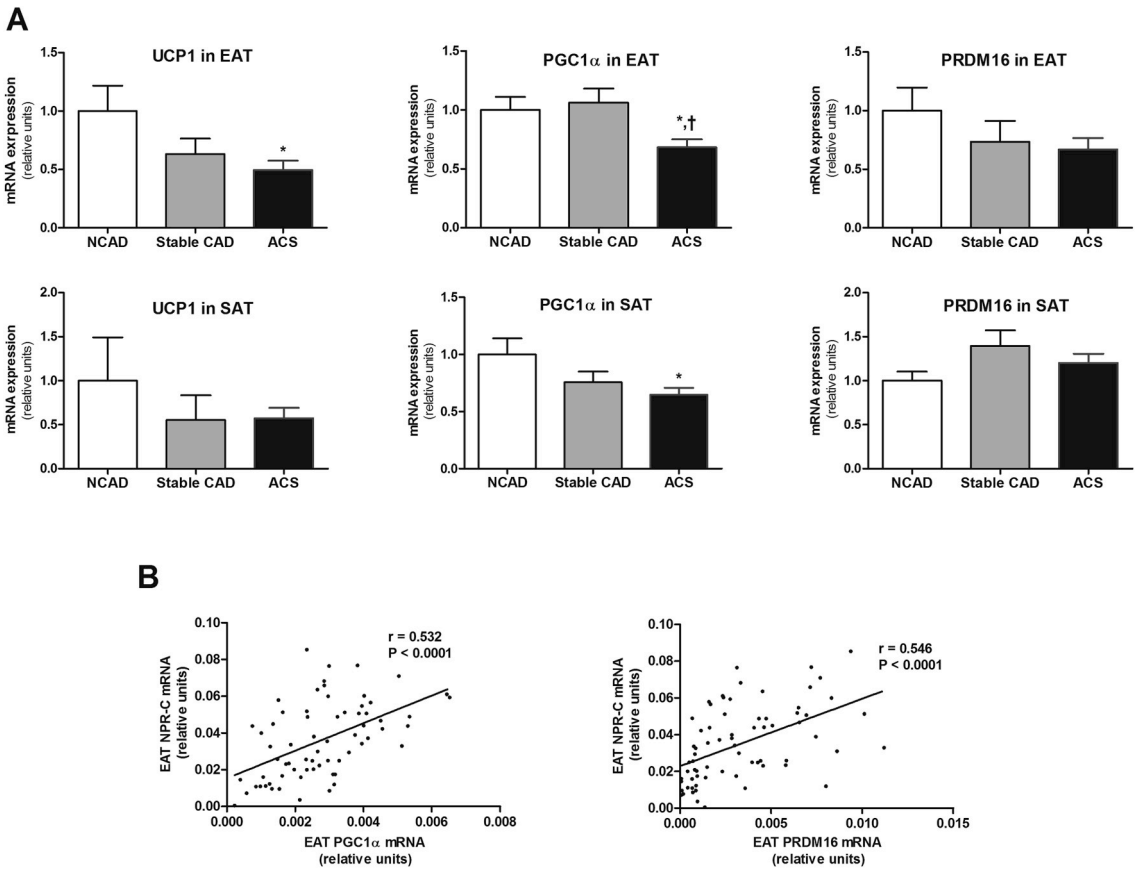

Fig. 2. Decreased mRNA expression levels of epicardial adipose tissue (EAT) brown-like fat markers in patients with acute coronary syndrome (ACS) and their correlation with *NPR-C* expression levels.
(A) Relative mRNA levels of UCP1, *PGC1α* and *PRDM16* were measured by real-time PCR in samples of human EAT and thoracic subcutaneous adipose tissue (SAT) taken from non-coronary artery disease (NCAD), stable coronary artery disease (CAD) and acute coronary syndrome (ACS) patients. Results are given as mean values of duplicates ± SEM normalized to cyclophilin A. *$p < 0.05$ *vs.* NCAD; †$p < 0.05$ *vs.* stable CAD. EAT and SAT UCP1 data were not normally distributed and were log transformed for the statistical analysis. (B) Correlations between *NPR-C* mRNA levels and *PGC1α* and *PRDM16* mRNA levels in EAT. Correlation coefficients and associated *p* values are shown on each plot.

genes as brown fat, independent of grade of obesity, through NPR-C.

EAT is the visceral adipose tissue which is in direct contact with the myocardium and coronary arteries. Given the close anatomic relationship in humans, where adipose tissue penetrates into the heart muscle, it is likely that alterations in EAT play an important role in CAD [5,6,25]. Clinical studies have shown that the amount of EAT is associated with the presence, progression or severity of CAD [2,3,26]. However, most studies have been limited to a comparison of patients with CAD vs. NCAD to elucidate the mechanisms of EAT in the progression of CAD [4,14,27], and only a few have been performed on a

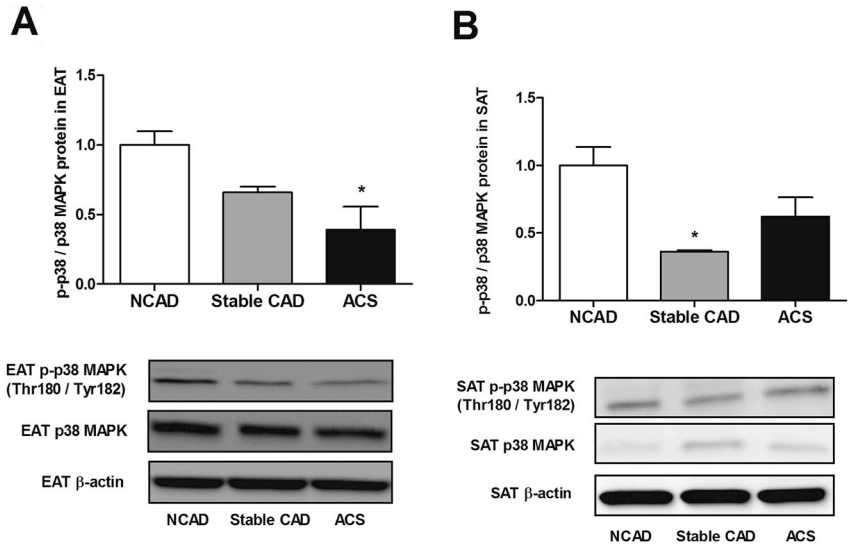

Fig. 3. Activation of p38 MAPK in epicardial adipose tissue (EAT) is lower in patients with acute coronary syndrome (ACS).
(A) Western blot was used to measure p38 MAPK (Thr180/Tyr182) phosphorylation. Quantitative bar graph and representative blots of p38 MAPK phosphorylation relative to total p38 MAPK in EAT and SAT from non-coronary artery disease (NCAD), stable coronary artery disease (CAD) and acute coronary syndrome (ACS) patients. Results represent mean ± SEM of 3 independent experiments for each group. *$p < 0.05$ *vs.* NCAD. Expression in the NCAD group was assumed to be 1.

cohort of patients with ACS [28,29]. We have previously shown that lower EAT *PGC1α* expression is associated with the extension of CAD and that *UCP1* expression in patients with CAD is 13-fold higher in EAT compared with thoracic SAT [23]. NPs have been shown to promote the transcriptional regulation of genes of brown adipocyte, such as *PGC1α* and *UCP1* [17]. Moreover, upregulation of NPR-A and NPR-B in the gonadal adipose tissue of mice has been associated with increased markers of browning [30]. Thus, the current study sought to investigate the relationship between NPRs and brown-like adipocyte gene expression in EAT in the context of CAD progression in a cohort of NCAD, stable CAD and ACS patients.

Initially, we found a decrease in both mRNA and protein levels of EAT NPR-C in ACS patients compared with stable CAD or NCAD patients, and there is a significant association between EAT *NPR-C* mRNA expression and the presence of ACS by multivariate logistic regression. To our knowledge this is the first study to report an association between EAT *NPR-C* expression and ACS. This association indicates that besides plasma NP levels [31], NPR-C signaling in EAT may influence ACS. We also showed that EAT *NPR-C* mRNA expression became significantly lower as the extension of coronary artery lesions increased: patients with 3-vessel disease showed lower *NPR-C* mRNA levels in EAT than those with 1- or 2- and no vessel disease. Since most ACS patients had 3-vessel disease and ACS was associated with lower *NPR-C* expression in EAT, the association found between lower EAT *NPR-C* and the extension of coronary lesions of all patients could be due to ACS. Even thought we found this association in the stable CAD patients, studying this association separately by CAD and ACS patients would be underpowered due to the small size of the sample. Therefore, the association between the expression of *NPR-C* in EAT and extension of coronary lesions needs to be interpreted with caution. It has previously been shown that EAT *NPR-C* mRNA expression in the CAD group is higher compared with the NCAD group [14]. These data appear to contradict our data. In that study, the authors compared angiographically confirmed patients without CAD to CAD patients but did not include patients with ACS. Moreover, the typical risk factors such as hypertension and obesity between the CAD and NCAD patients were significantly different in that study, as were disease states associated with deregulation of the NP system, including the *NPR-C* gene [10,24].

NPR-C is a receptor known to mediate NPs cellular response and to reduce NP plasma levels. In addition to cardiovascular effects in the heart and vasculature [10,11,32], recent studies argue for a critical role of NPR-C in modulating the metabolic effect of NPs in adipose tissue [17,33,34]. Mice lacking NPR-C have a reduced fat mass and higher gene expression of brown adipocyte markers in adipose tissue [17]. It is worthy of note that mice with adipose-specific deletion of NPR-C show increased expression of thermogenesis-related genes in brown, but not in white, adipose tissue, suggesting a depot-dependent function of NPR-C on adipose tissue [34]. In our study, we found that lower EAT *NPR-C* expression in patients with ACS was accompanied by a significantly lower expression of the selective marker for beige/brown adipose tissue *UCP1* and the important transcriptional coregulator *PGC1α*. Although not statistically different, EAT *PRDM16* gene expression was also lower in ACS patients. These results extend our previous work [23] by revealing that *UCP1* and *PGC1α* mRNA levels in EAT decrease with the severity of CAD, and suggest that the EAT brown-like fat characteristic might play a role in plaque stability. Importantly, the lower EAT *NPR-C* expression in patients with ACS was also accompanied by a marked decrease in EAT p38 MAPK phosphorylation, thus indicating a potential signaling defect, considering that p38 MAPK is recognized as a downstream molecular effector of the NPRs signaling pathway, as well as an upstream transcriptional mediator of *PGC1α* and *UCP1* [17,32,35]. In line with this, we found a positive correlation between EAT NPR-C protein and p38 MAPK activation levels. Furthermore, linear regression analysis showed an association between EAT *NPR-C* and *PGC1α* mRNA expression levels. Thus, reduced EAT NPR-C expression could impair the p38 MAPK signaling pathway, and lead to a decreased expression of fat brown-like genes. PGC1α is a key factor in the regulation of mitochondrial functional capacity and cellular energy metabolism [36]. Overall, our results suggest that lower EAT NPR-C expression could be associated with reduced thermogenic capacity and mitochondrial dysfunction in EAT [35,36]. Despite increasing the release of NPs by the heart in response to a failing heart [7,37], reduced EAT NPR-C expression might lead to dysfunctional epicardial fat surrounding the myocardium, and contribute to the progression of CAD. It is also possible that the loss of NPR-C in EAT might increase the production of reactive oxygen species, which has been shown to be associated with *trans*-differentiation from brown to white in the EAT of CAD patients [38]. Altogether, these findings suggest that EAT NPR-C plays an important role in ACS. Research with animal models is needed to establish whether reduced NPR-C in EAT is the cause of, or is merely associated with, ACS.

On the other hand, in accordance with the classical view of NPR-C being a clearance receptor for all three NPs [9], decreased *NPR-C* expression in EAT might contribute to keeping raised levels of NPs from circulation, as shown in patients affected with ACS [31,39]. An increase in NP levels is also reflective of left ventricular dysfunction [40]. We found a positive correlation between EAT *NPR-C* mRNA levels and left ventricular ejection fraction. Therefore, reduced EAT NPR-C expression in patients with ACS might suggest a lower clearance function with the grade of cardiac dysfunction, but also contribute to the "cardiac endocrine paradox" [37] through a diminished EAT responsiveness to NPs. In cardiac paradox, despite dramatic increases in NP concentrations as left ventricular dysfunction progresses, their effects become blunted. In our study, lower EAT *NPR-C* expression was associated with reduced *PGC1α* expression and lower p38 MAPK activation, suggesting epicardial fat dysfunction as a possible mechanism in the progression of CAD. In addition, considering that EAT represents only 1% of total body fat mass [41] it would be difficult for a decrease in EAT NPR-C expression levels to increase NP levels by a decreased rate of clearance.

Although *NPR-B* mRNA expression has been shown in adipose tissue [13], to date no studies have investigated the expression of *NPR-B* in human EAT or thoracic SAT. When we examined the mRNA expression of all three NPRs in thoracic SAT, we found that higher *NPR-B* mRNA expression was associated with ACS by logistic regression analysis. Furthermore, we found that *NPR-B* mRNA significantly increased in the thoracic SAT of patients with 3-vessel disease. NPR-B has been shown to regulate adipogenesis in 3T3-L1 cells through binding to C-type NP [42]. There is emerging evidence that thoracic visceral adipose tissues may locally promote the development of atherosclerosis in the underlying coronary artery [43]. Thus, it is possible that NPR-B mediates a similar, yet to be discovered, NP function in thoracic SAT which needs further investigation.

NPs have been shown to enhance the brown fat thermogenic program in rodent and human subcutaneous adipocytes [17]. Although these effects have been attributed to NPR-A, and subsequent changes in cGMP, our data suggest that NPR-C could play a role in modulating the expression of brown-like fat genes in EAT. It is also possible that decreased EAT NPR-C expression in ACS patients may result in altered NP effects mediated by NPR-A and NPR-B in EAT. However, we found that *NPR-A* and *NPR-B*, as well as *PKG1* mRNA expression levels were unaltered in EAT with the severity of CAD, suggesting that reduced EAT brown-fat related gene expression may be attributed to impaired EAT NPR-C signaling in ACS patients. Whether the lower EAT NPR-C expression in ACS is also associated with alteration in the secretion of adipocytokines and a higher oxidative stress in EAT is still to be determined.

The study limitations include the cross-sectional study design, which did not allow inference of causal associations. The number of

enrolled patients was relatively small as it was a single-center study and only patients who underwent cardiac surgery were included. However, the results represent patients with ACS and may lay the foundations for future studies. Gender imbalance may have affected the results. The number of female gender patients was lower than those of male gender due to the low prevalence of CAD disease in the female gender and the nature of the single-center study. However, the difference in *NPR-C* expression between men and women was not statistically significant. The assessment of coronary angiographic findings was limited to visual interpretation, and angiography is a technique that detects only major coronary arterial lesions. Since the amount of EAT around the human heart is highly variable and dissecting too much EAT is not ideal, our sample size was also small, which limited the possibility of addressing multiple questions in a given sample. The NCAD subjects were not healthy persons. However, the only way we could obtain EAT samples was to use patients undergoing cardiac surgery. Our study did not measure NPs, however, the most important functional consequence in ACS might be the loss of NPR-C in EAT.

In conclusion, our study is the first demonstration that ACS is associated with a reduced expression of *NPR-C* in EAT. The EAT of patients with ACS is characterized by a decreased expression of NPR-C compared with either patients with stable CAD or individuals with angiographically normal coronary arteries. Reduced EAT NPR-C expression in ACS patients is also accompanied by a decrease in brown-like fat gene expression and a reduced phosphorylation of p38 MAPK in EAT. Furthermore, our results indicate that lower EAT *NPR-C* gene expression levels are associated with lower *PGC1α* expression levels and reduced activation of p38 MAPK in EAT.

In the clinical setting, pharmacological NPR-C activation in EAT could lead to the activation of EAT brown-like fat and protect the heart during hypothermia, ischemia or hypoxia. All current medications for ACS have side effects, and many of them interact with other medications. Thus, drugs that activate NPR-C might represent a new therapeutic opportunity to refine and improve existing therapies for ACS.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

IMS conceived the study and developed the experimental design. IMS and MMG performed all laboratory assays and participated in the analysis of data. CPM and GSE were responsible for obtaining the serum and adipose tissue samples of patients. DCC, CPM, GSE, JGD, and MJN got clinical information and assisted with analysis of the data. IMS, JGD, MJN participated in the interpretation of data. EDT performed the critical revision of the manuscript for important intellectual content. IMS wrote the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

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References

[1] G. Iacobellis, Local and systemic effects of the multifaceted epicardial adipose tissue depot, Nat. Rev. Endocrinol. 11 (2015) 363–371.
[2] K. Nakanishi, S. Fukuda, A. Tanaka, et al., Persistent epicardial adipose tissue accumulation is associated with coronary plaque vulnerability and future acute coronary syndrome in non-obese subjects with coronary artery disease, Atherosclerosis 237 (2014) 353–360.
[3] A.A. Mahabadi, M.H. Berg, N. Lehmann, et al., Association of epicardial fat with cardiovascular risk factors and incident myocardial infarction in the general population: the Heinz Nixdorf Recall Study, J. Am. Coll. Cardiol. 61 (2013) 1388–1395.
[4] K. Karastergiou, I. Evans, N. Ogston, et al., Epicardial adipokines in obesity and coronary artery disease induce atherogenic changes in monocytes and endothelial cells, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 1340–1346.
[5] S. Greulich, B. Maxhera, G. Vandenplas, et al., Secretory products from epicardial adipose tissue of patients with type 2 diabetes mellitus induce cardiomyocyte dysfunction, Circulation 126 (2012) 2324–2334.
[6] N. Venteclef, V. Guglielmi, E. Balse, et al., Human epicardial adipose tissue induces fibrosis of the atrial myocardium through the secretion of adipo-fibrokines, Eur. Heart J. 36 (2015) 795–805a.
[7] N.E. Zois, E.D. Bartels, I. Hunter, et al., Natriuretic peptides in cardiometabolic regulation and disease, Nat. Rev. Cardiol. 11 (2014) 403–412.
[8] L.R. Potter, Natriuretic peptide metabolism, clearance and degradation, FEBS J. 278 (2011) 1808–1817.
[9] N. Matsukawa, W.J. Grzesik, N. Takahashi, et al., The natriuretic peptide clearance receptor locally modulates the physiological effects of the natriuretic peptide system, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 7403–7408.
[10] S. Rubattu, S. Sciarretta, A. Morriello, et al., NPR-C: a component of the natriuretic peptide family with implications in human diseases, J. Mol. Med. (Berl.) 88 (2010) 889–897.
[11] R.A. Rose, W.R. Giles, Natriuretic peptide C receptor signalling in the heart and vasculature, J. Physiol. 586 (2008) 353–366.
[12] S. Collins, A heart-adipose tissue connection in the regulation of energy metabolism, Nat. Rev. Endocrinol. 10 (2014) 157–163.
[13] R. Sarzani, P. Dessi-Fulgheri, V.M. Paci, et al., Expression of natriuretic peptide receptors in human adipose and other tissues, J. Endocrinol. Investig. 19 (1996) 581–585.
[14] I. Shibasaki, T. Nishikimi, Y. Mochizuki, et al., Greater expression of inflammatory cytokines, adrenomedullin, and natriuretic peptide receptor-C in epicardial adipose tissue in coronary artery disease, Regul. Pept. 165 (2010) 210–217.
[15] C. Sengenes, M. Berlan, I. De Glisezinski, et al., Natriuretic peptides: a new lipolytic pathway in human adipocytes, FASEB J. 14 (2000) 1345–1351.
[16] M. Lafontan, C. Moro, C. Sengenes, et al., An unsuspected metabolic role for atrial natriuretic peptides: the control of lipolysis, lipid mobilization, and systemic nonesterified fatty acids levels in humans, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 2032–2042.
[17] M. Bordicchia, D. Liu, E.Z. Amri, et al., Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes, J. Clin. Investig. 122 (2012) 1022–1036.
[18] R.A. Takx, A. Ishai, Q.A. Truong, et al., Supraclavicular Brown adipose tissue 18F-FDG uptake and cardiovascular disease, J. Nucl. Med. 57 (2016) 1221–1225.
[19] J.F. Berbee, M.R. Boon, P.P. Khedoe, et al., Brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development, Nat. Commun. 6 (2015) 6356.
[20] R. Thoonen, L. Ernande, J. Cheng, et al., Functional brown adipose tissue limits cardiomyocyte injury and adverse remodeling in catecholamine-induced cardiomyopathy, J. Mol. Cell. Cardiol. 84 (2015) 202–211.
[21] L.M. Perez-Belmonte, I. Moreno-Santos, J.J. Gomez-Doblas, et al., Expression of epicardial adipose tissue thermogenic genes in patients with reduced and preserved ejection fraction heart failure, Int. J. Med. Sci. 14 (2017) 891–895.
[22] H.S. Sacks, J.N. Fain, B. Holman, et al., Uncoupling protein-1 and related messenger ribonucleic acids in human epicardial and other adipose tissues: epicardial fat functioning as brown fat, J. Clin. Endocrinol. Metab. 94 (2009) 3611–3615.
[23] I. Moreno-Santos, L.M. Perez-Belmonte, M. Macias-Gonzalez, et al., Type 2 diabetes is associated with decreased PGC1alpha expression in epicardial adipose tissue of patients with coronary artery disease, J. Transl. Med. 14 (2016) 243.
[24] Z. Kovacova, W.G. Tharp, D. Liu, et al., Adipose tissue natriuretic peptide receptor expression is related to insulin sensitivity in obesity and diabetes, Obesity 24 (2016) 820–828.
[25] M. Furuhashi, T. Fuseya, M. Murata, et al., Local production of fatty acid-binding

protein 4 in epicardial/perivascular fat and macrophages is linked to coronary atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 36 (2016) 825–834.
[26] P. Raggi, Epicardial adipose tissue as a marker of coronary artery disease risk, J. Am. Coll. Cardiol. 61 (2013) 1396–1397.
[27] A. Dutour, V. Achard, H. Sell, et al., Secretory type II phospholipase A2 is produced and secreted by epicardial adipose tissue and overexpressed in patients with coronary artery disease, J. Clin. Endocrinol. Metab. 95 (2010) 963–967.
[28] S. Langheim, L. Dreas, L. Veschini, et al., Increased expression and secretion of resistin in epicardial adipose tissue of patients with acute coronary syndrome, Am. J. Physiol. Heart Circ. Physiol. 298 (2010) H746–H753.
[29] D. Pedicino, A. Severino, S. Ucci, et al., Epicardial adipose tissue microbial colonization and inflammasome activation in acute coronary syndrome, Int. J. Cardiol. 236 (2017) 95–99.
[30] M.D. Neinast, A.P. Frank, J.F. Zechner, et al., Activation of natriuretic peptides and the sympathetic nervous system following Roux-en-Y gastric bypass is associated with gonadal adipose tissues browning, Mol. Metab. 4 (2015) 427–436.
[31] T. Omland, A. Persson, L. Ng, et al., N-terminal pro-B-type natriuretic peptide and long-term mortality in acute coronary syndromes, Circulation 106 (2002) 2913–2918.
[32] P. Madiraju, E. Hossain, M.B. Anand-Srivastava, Natriuretic peptide receptor-C activation attenuates angiotensin II-induced enhanced oxidative stress and hyperproliferation of aortic vascular smooth muscle cells, Mol. Cell. Biochem 448 (1-2) (2018) 77–89.
[33] O. Pivovarova, O. Gogebakan, N. Kloting, et al., Insulin up-regulates natriuretic peptide clearance receptor expression in the subcutaneous fat depot in obese subjects: a missing link between CVD risk and obesity? J. Clin. Endocrinol. Metab. 97 (2012) E731–E739.
[34] W. Wu, F. Shi, D. Liu, et al., Enhancing natriuretic peptide signaling in adipose tissue, but not in muscle, protects against diet-induced obesity and insulin resistance, Sci. Signal. (2017) 10.
[35] J. Robidoux, W. Cao, H. Quan, et al., Selective activation of mitogen-activated protein (MAP) kinase kinase 3 and p38alpha MAP kinase is essential for cyclic AMP-dependent UCP1 expression in adipocytes, Mol. Cell. Biol. 25 (2005) 5466–5479.
[36] B.N. Finck, D.P. Kelly, PGC-1 coactivators: inducible regulators of energy metabolism in health and disease, J. Clin. Investig. 116 (2006) 615–622.
[37] J. Diez, Chronic heart failure as a state of reduced effectiveness of the natriuretic peptide system: implications for therapy, Eur. J. Heart Fail. 19 (2017) 167–176.
[38] E. Dozio, E. Vianello, S. Briganti, et al., Increased reactive oxygen species production in epicardial adipose tissues from coronary artery disease patients is associated with brown-to-white adipocyte trans-differentiation, Int. J. Cardiol. 174 (2014) 413–414.
[39] A. Palazzuoli, A. Maisel, M. Caputo, et al., B-type natriuretic peptide levels predict extent and severity of coronary disease in non-ST elevation coronary syndromes and normal left ventricular systolic function, Regul. Pept. 167 (2011) 129–133.
[40] M. Bay, V. Kirk, J. Parner, et al., NT-proBNP: a new diagnostic screening tool to differentiate between patients with normal and reduced left ventricular systolic function, Heart 89 (2003) 150–154.
[41] J.M. Marchington, C.A. Mattacks, C.M. Pond, Adipose tissue in the mammalian heart and pericardium: structure, foetal development and biochemical properties, Comp. Biochem. Physiol. B 94 (1989) 225–232.
[42] T. Katafuchi, D.L. Garbers, J.P. Albanesi, CNP/GC-B system: a new regulator of adipogenesis, Peptides 31 (2010) 1906–1911.
[43] C. Hanley, K.J. Shields, K.A. Matthews, et al., Associations of cardiovascular fat radiodensity and vascular calcification in midlife women: the SWAN cardiovascular fat ancillary study, Atherosclerosis 279 (2018) 114–121.