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Glycosylated apolipoprotein J in cardiac ischaemia: molecular processing and circulating levels in patients with acute ischaemic events

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Aim Using proteomics, we previously found that serum levels of glycosylated (Glyc) forms of apolipoprotein J (ApoJ), a cytoprotective and anti-oxidant protein, decrease in the early phase of acute myocardial infarction (AMI). We aimed to investigate: (i) ApoJ-Glyc intracellular distribution and secretion during ischaemia; (ii) the early changes in circulating ApoJ-Glyc during AMI; and (iii) associations between ApoJ-Glyc and residual ischaemic risk post-AMI.

Methods and results Glycosylated apolipoprotein J was investigated in: (i) cells from different organ/tissue origin; (ii) a pig model of AMI; (iii) *de novo* AMI patients ($n = 38$) at admission within the first 6 h of chest pain onset and without troponin T elevation at presentation (early AMI); (iv) ST-elevation myocardial infarction patients ($n = 212$) who were followed up for 6 months; and (v) a control group without any overt cardiovascular disease ($n = 144$). Inducing simulated ischaemia in isolated cardiac cells resulted in an increased intracellular accumulation of non-glycosylated ApoJ forms. A significant decrease in ApoJ-Glyc circulating levels was seen 15 min after ischaemia onset in pigs. Glycosylated apolipoprotein J levels showed a 45% decrease in early AMI patients compared with non-ischaemic patients ($P < 0.0001$), discriminating the presence of the ischaemic event (area under the curve: 0.934; $P < 0.0001$). ST-elevation myocardial infarction patients with lower ApoJ-Glyc levels at admission showed a higher rate of recurrent ischaemic events and mortality after 6-month follow-up ($P = 0.008$).

Conclusions These results indicate that ischaemia induces an intracellular accumulation of non-glycosylated ApoJ and a reduction in ApoJ-Glyc secretion. Glycosylated apolipoprotein J circulating levels are reduced very early after ischaemia onset. Its continuous decrease indicates a worsening in the evolution of the cardiac event, likely identifying patients with sustained ischaemia after AMI.

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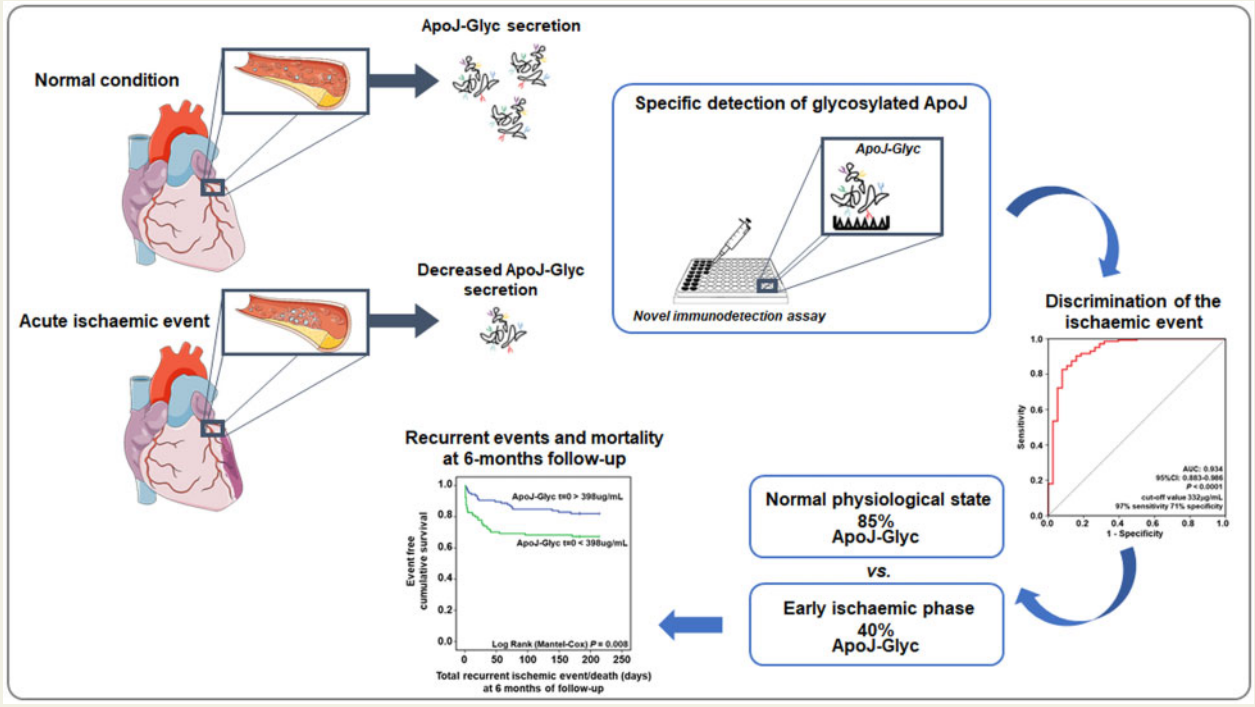
Graphical Abstract



Glycosylated apolipoprotein J as a novel marker of ischaemia. Under ischaemic conditions there is a decrease in the secretion of glycosylated apolipoprotein J. The quantification of specific glycosylated apolipoprotein J variants with a novel immunoassay discriminates the presence of a cardiac ischaemic event, indicating that glycosylated apolipoprotein J could be a novel marker of cardiac ischaemia.

Keywords Acute myocardial infarction • Apolipoprotein J • Clusterin • Acute myocardial ischaemia • Prognosis • Risk stratification

Translational perspective

This study highlights a potential role of glycosylated apolipoprotein J (ApoJ-Glyc) as a protein marker sensitive to the early ischaemic injury in acute myocardial infarction (AMI). Due to its rapid and specific changes in cardiac cells under simulated ischaemic conditions and the early decrease in its circulating levels, ApoJ-Glyc appears as a protein that, if further validated, could be used in mapping the initiation and progression of ischaemic events. Therefore, ApoJ-Glyc could represent a novel biomarker for the early detection of ischaemia with a potential added value in risk assessment post-AMI.

Introduction

The diagnosis and management of acute coronary syndromes (ACS) are based on clinical assessment, electrocardiogram (ECG) findings and troponin (Tn) levels, the most often used biomarker.[1,2] Due to their intracellular structural role, cardiac Tn are excellent markers of irreversible cell damage. Evidence has shown that the earliest Tn elevations are associated with cardiomyocyte apoptosis,[3] highlighting that some type of irreversible cell death is needed for Tn to be released. Troponin is elevated in many cardiovascular diseases, such as heart failure, aortic dissection, myocarditis, takotsubo cardiomyopathy, and atrial fibrillation among others,[4,5] making Tn a specific biomarker of cardiac injury. Undetectable levels of high-sensitivity Tn (hs-Tn) at admission, in patients presenting 1–3 h after the onset of chest pain, with an ECG without ischaemic changes may rule out an acute myocardial infarction (AMI) with a good sensitivity in one-third of the patients. However, there are some limitations in patients aged 65 years or less due to an increased risk of false-negative results.[5,6] Indeed, a final confirmatory test for safe discharge, such as exercise ECG or non-invasive stress testing, has been recommended in low-risk patients.[7,8] Still, the application of this protocol implies longer length of hospital stay, potentially unnecessary testing and higher costs, without clearly showing a direct benefit.[8,9] In this context, a specific biomarker of ischaemia, in combination with Tn, should allow

the rapid identification of the ischaemic event in those patients with undetectable Tn levels, unconclusive Tn/ECG results, or other criteria indicating a low probability of an event.

Currently, there are no accepted biomarkers able to detect the initial ischaemic cell damage before it develops into cell death and necrosis; therefore, there is a need for further understanding of signals that may differentiate between ischaemia and necrosis.[10] Thus, the search has continued for biomarkers of ischaemia that may be translated into an improved management algorithm for patients in the early phase of myocardial ischaemia.[2,11–14]

In a previous proteomic study, we focused on the assessment of protein glycosylations and we described, for the first time, a shift in the glycosylation profile of apolipoprotein J (ApoJ; also known as clusterin) in the early phase of ischaemia (Supplementary material online, *Figure S1*).[15]

Here, we investigated, using cellular and preclinical experimental modelling, as well as performing clinical studies: (i) glycosylated ApoJ (ApoJ-Glyc) intracellular distribution and secretion in different cells and its changes during ischaemia; (ii) the early changes in circulating ApoJ-Glyc during acute myocardial ischaemia; and (iii) the relationship between ApoJ-Glyc levels and residual ischaemic risk post-AMI.

Materials and methods

For more detailed information, see Supplementary material online, *Materials and Methods*.

In vitro studies

In vitro cell culture experiments were used to investigate the influence of simulated ischaemia in the intracellular ApoJ forms and their secretion profile. Cells from different organ/tissue origin (cardiac, hepatic, endothelial, and vascular) were investigated (Supplementary material online, *Figure S2A*). The secretion pattern, glycosylation profile, and intracellular distribution of ApoJ-Glyc were analysed in normoxic, hypoxic (1% O_2), and simulated ischaemia (1% O_2 and acidic conditions by nutrient deprivation only with phosphate-buffered saline (PBS) at pH 6.4, as previously described)[16] conditions for 1, 2, 4, and 8 h (Supplementary material online, *Figure S2A*). Specifically, primary cultures of porcine ventricular cardiac fibroblasts (PVCFs), mouse atrial cardiomyocytes line HL-1, human liver cell line HepG2, human hepatic mesenchymal cells (Stellate), human umbilical vein endothelial cells (HUVEC), and vascular smooth muscle cells (VSMC) from coronary artery explants (without plaque) from heart transplant recipients with non-ischaemic (dilated) cardiomyopathy were expanded as previously described.[17,18]

Proof-of-concept in induced pluripotent stem derived human cardiomyocytes

Cellartis human induced pluripotent stem (iPS) cell line 22 differentiated to cardiomyocytes was used to further confirm the observed changes in ApoJ-Glyc secretion profile in cells of human origin. Differentiation was carried out following manufacturer's instructions and confirmed by western blot for troponin T (TnT) and Nkx-2.5 for each culture. After differentiation, cardiomyocytes were incubated for 4 h either in normoxia, hypoxia, or simulated ischaemia (following the same protocol as the one used with the other cell types).

Analysis of culture media

Glycosylated apolipoprotein J released to the culture medium was analysed by measuring ApoJ-Glyc with the lectin-based immunoassay. In this assay, no sample pre-treatment was carried out and, thus, crude cell culture medium was directly used for running the assay for the specific detection of ApoJ-Glyc variants. If ApoJ-Glyc concentration in the media was below 7.8 ng/mL [limit of detection (LOD) of the assay], the absorbance of the sample was similar to blank and no ApoJ-Glyc concentration could be obtained by extrapolation in the standard curve.

Glycosylated apolipoprotein J levels, using this immunoassay, were detectable only in PVCF, VSMC and iPS cells differentiatied to cardiomyocytes whereas, in the other cell types (HL-1, HepG2, Stellate, and HUVEC), the concentration in the medium was below the LOD.

To further confirm the presence of ApoJ-Glyc in the medium of all the cell types and to characterize the changes in the glycosylation profile of secreted ApoJ, peptide-*N*-glycosidase F (PNGase F) deglycosylation analysis was carried out in concentrated protein extracts of the medium of all the different cell types so that enough protein material was obtained to run a western blot analysis. Deglycosylation and western blot analysis were carried out as previously described.[15,19] In this assay, the relative amount of ApoJ-Glyc that was present in the sample is estimated based on the ratio between the ApoJ that is deglycosylated and the total ApoJ that is detected prior to running the deglycosylation.[15,19]

Analysis of cellular protein extracts

Total cell protein extracts were obtained and analysed by western blot analysis (Supplementary material online, *Figure S2B*).

Statistical analysis

Student's *t*-test was used for comparison between different conditions (normoxia, hypoxia, or simulated ischaemia) at each time point or between different time points within a specific condition.

In vivo studies in the porcine model of anterior ST-elevation myocardial infarction

We used a closed-chest pig model of ischaemia by coronary balloon occlusion [ST-elevation myocardial infarction (STEMI) model][20–22] to study the temporal dynamics of changes in circulating ApoJ-Glyc (decrease) and high-sensitivity troponin I (hs-TnI) (increase). This controlled animal model allows elucidation of the timeline of the protein signal changes following initiation of ischaemic injury. Pigs ($n = 32$; 8 animals/group) were randomly allocated to undergo 30, 60, 90, or 120 min of ischaemia using closed-chest balloon occlusion of the left anterior descending artery. Thereafter, animals were allowed to reperfuse and were maintained until 45 days after myocardial infarction, the time point at which they were sacrificed and scar size was assessed by TTC staining (Supplementary material online, *Figure S3*). Peripheral blood samples were obtained at baseline before ischaemia induction, from 15 up to 120 min post-ischaemia induction (AMI phase) and at 3 days, 2 weeks, 4 weeks, and 45 days post-AMI (recovery phase) for hs-TnI and ApoJ-Glyc assessments. A sham-operated group was run in parallel ($n = 8$).

Statistical analysis

Non-parametric Mann–Whitney was used for comparison between groups (sham and AMI) and Wilcoxon analysis for comparisons between the different time points within each specific group.

Clinical study

The study comprised two groups of patients.

Group 1: Patients who were admitted to the emergency room with a time frame from symptom onset to admission and initial blood sample collection of 2.5 h (1.9–3.2 h) {median [interquartile range (IQR)]} and showed negative conventional TnT levels at admission with a subsequent rise above the 99th percentile upper reference limit after the first blood sampling (early-AMI; $n = 38$; *Table 1*).

Inclusion criteria were ≥18 years of age, provision of signed informed consent, and diagnosis of AMI, consistent with the third universal definition of myocardial infarction,[13] in a subset of patients who met the following criteria: (i) admission to hospital within the first 6 h after the onset of chest pain with typical ischaemic symptoms; (ii) ST-segment elevation 0.2 mV in at least two contiguous leads; and (iii) negative conventional TnT levels at admission (excluding subacute myocardial infarction) with a subsequent increase to a level greater than the 99th percentile upper reference limit. Exclusion criteria were a previous documented or suspected myocardial infarction and antithrombotic treatment because of the onset of AMI before arriving to the emergency department and time of blood collection. Patients were admitted to the hospital between February 2004 and April 2005.

Freshly drawn venous blood samples from patients were collected at the moment of admission ($t = 0$). Samples were immediately processed and were aliquoted and stored at -80°C until ApoJ-Glyc analysis in batches.

Group 2: A more heterogeneous group of STEMI patients with a wide time span of ischaemic pain [median (IQR): 3.3 h (2.4)] and including patients with elevated hs-Tn at admission (group of patients with necrosis) whose clinical evolution was followed up for 6 months after event presentation ($n = 212$; *Table 2*).

Inclusion criteria were ≥18 years of age, provision of signed informed consent and diagnosis of STEMI based on the following criteria: first, ST-segment elevation in two contiguous leads of ≥0.25 mV in men below the age of 40 years, ≥0.2 mV in men over the age of 40 years, or ≥0.15 mV in women in leads V2–V3, and/or ≥0.1 mV in other leads (in the absence of left ventricular hypertrophy or left bundle branch block); and second, meeting the third universal definition of myocardial infarction criteria.[13] Exclusion criteria were patients transferred to the hospital after primary percutaneous coronary intervention (PCI) performed in another hospital, or absence of an acute coronary occlusion at the time of coronary angiography. Patients were admitted to the hospital between December 2011 and December 2014.

Among the 212 STEMI patients who were included, a total of 206 were reperfused (205 through PCI and 1 through emergent surgical revascularization). There was 1 patient in whom catheterization was not possible due to existing co-morbidities. In the remaining patients ($n = 5$), PCI was unsuccessful. Myocardial blush grade (MBG) could be assessed in 199 patients as an indicator of status of the microcirculation; among these, 129 had an MBG of 3, 46 had an MBG of 2, 21 had an MBG of 1, and 3 had an MBG of 0.

Freshly drawn venous blood samples were collected at the moment of admission ($t = 0$), and in a subset of 82 patients, an additional blood sampling was performed at 72 h post-admission. Samples were immediately processed and were aliquoted and stored at -80°C until the ApoJ-Glyc analysis was carried out in batches.

Table 1 Patients included in Group 1

	Early AMI patients ($n = 38$)	Controls ($n = 144$)	*P*-value
Age (years), mean ± SD	61 ± 13	63 ± 9	0.170
Females/males (*n*)	10/28	73/71	0.007
Risk factors (%)			
Dyslipidaemia	50	65	0.08
Diabetes mellitus	24	10	0.03
Hypertension	50	42	0.357
Tobacco smoking	39	24	0.05
Background medication (%)			
ASA	11	8	0.529
ACEI/ARB	18	28	0.211
Statins	18	51	0.0004
Beta-blockers	8	7	0.840
Calcium channel blockers	8	8	0.930
Killip class (%)			
I	87	—	—
II	10	—	—
III	0	—	—
IV	3	—	—

ACEI, angiotensin-converting enzyme inhibitors; AMI, acute myocardial infarction; ARB, angiotensin II receptor blockers; ASA, acetylsalicylic acid; SD, standard deviation.

Table 2 Patients included in Group 2

	STEMI patients with 6-month follow-up ($n = 212$)	*P*-value vs. controls	*P*-value vs. STEMI cohort 1
Age (years), mean ± SD	64 ± 13	0.358	0.123
Females/males (*n*)	49/163	<0.0001	0.669
Risk factors (%)			
Dyslipidaemia	46	0.0003	0.629
Diabetes mellitus	20	0.018	0.585
Hypertension	55	0.012	0.554
Tobacco smoking	24	0.995	0.04
Background medication (%)			
ASA	20	0.002	0.228
ACEI/ARB	39	0.027	0.012
Statins	30	0.0002	0.122
Beta-blockers	15	0.016	0.224
Calcium channel blockers	3	0.026	0.130
Killip class (%)			
I	51	—	
II	13	—	
III	7	—	
IV	29	—	

ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers; ASA, acetylsalicylic acid; SD, standard deviation; STEMI, ST-elevation myocardial infarction.

A group of 'healthy volunteers' without overt cardiovascular disease was used as a control group. Demographic and clinical characteristics of the control group and the AMI cohorts are shown in *Tables 1 and 2*.

The Ethical Committee of the Santa Creu i Sant Pau Hospital approved the project and the studies were conducted according to the principles of the Declaration of Helsinki. All participants gave written informed consent to take part in the study. Reporting of the study conforms to the STROBE guidelines.[23]

Statistical analysis

Non-parametric Mann–Whitney ($n = 2$) or Kruskal–Wallis ($n > 2$) tests were used for comparison between groups and Wilcoxon analysis for comparison between different time points (i.e. ApoJ-Glyc levels between the moment of admission and 3 days after in cohort 2). Bivariate correlations between variables were determined by Pearson correlation coefficients. χ^2 test or Fisher's exact test, when any of the expected values was <5, was used for categorical variables.

To determine the correlation between ApoJ-Glyc levels, anthropometric parameters (age, sex and body mass index), risk factors (diabetes, hypertension, dyslipidaemia, tobacco), background treatment, and biochemical parameters [total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), glucose and C-reactive protein (CRP) levels], we first performed bivariate analyses by correlation (for continuous variables) or by *t*-test (for categorical variables). This analysis allowed us to find the parameters that showed an influence on the levels of ApoJ-Glyc in each cohort of patients (variables with a significant *P*-value in the bivariate analysis in Supplementary material online, *Tables S1* and *S2*). Those variables were afterward included in a multiple linear regression analysis (stepwise selection of variables) to assess those that remained as independent factors for ApoJ-Glyc levels.

Receiver operating characteristic curves (to assess the discriminating power of selected variables) and Kaplan–Meier curves (to carry out survival analyses) were performed with IBM SPSS Statistics v19.0. A *P*-value of <0.05 was considered statistically significant.

Quantification of glycosylated apolipoprotein J levels

Glycosylated apolipoprotein J levels in samples from *in vitro* experiments (culture media), from the *in vivo* pig model (systemic levels), and from the two cohorts of patients (systemic levels) were measured with a novel lectin-based immunoassay that allows their specific detection and quantification (see Supplementary material online, *Material and Methods*). This immunoassay shows an intra- and inter-assay variability comparable to that of commercially available ELISAs to detect total protein levels (mean ± desvest: intra-assay variability: 3.8 ± 1.8; inter-assay variability: 8.05 ± 2.02; day-to-day variability: 8.1 ± 4.4; assessed in 9 samples with 10 replicates per sample in two independent plates and two non-consecutive days). The LOD is 7.8 ng/mL.

Results

Glycosylated apolipoprotein J secretion profile by isolated cardiac cells in culture under simulated ischaemic conditions

Porcine ventricular cardiac fibroblast subjected to simulated ischaemia showed a time-dependent decrease in the secretion of ApoJ-Glyc (*Figure 1A*) whereas hypoxia alone (O_2 deprivation) was not able to induce the same changes. The decrease was evident as early as in 1 h (16% vs. normoxia; $P < 0.0001$) and was progressive until 8 h of simulated ischaemia (38% vs. normoxia; $P < 0.0001$). After 8 h of simulated ischaemia, there was a 20% decrease in ApoJ-Glyc secretion when compared to the levels detected at 1 h (*Figure 1B*).

In contrast, no significant differences in the secretion levels of VSMC were found. HL-1, HepG2, Stellate, and HUVEC cells released very low levels of ApoJ-Glyc with a concentration that was below the LOD of the assay (<7.8 ng/mL).

To further confirm the presence of ApoJ-Glyc in the medium of all the cell types and to characterize the changes in the glycosylation profile of secreted ApoJ, PNGase F deglycosylation analysis was carried out in concentrated protein extracts of the medium of all the

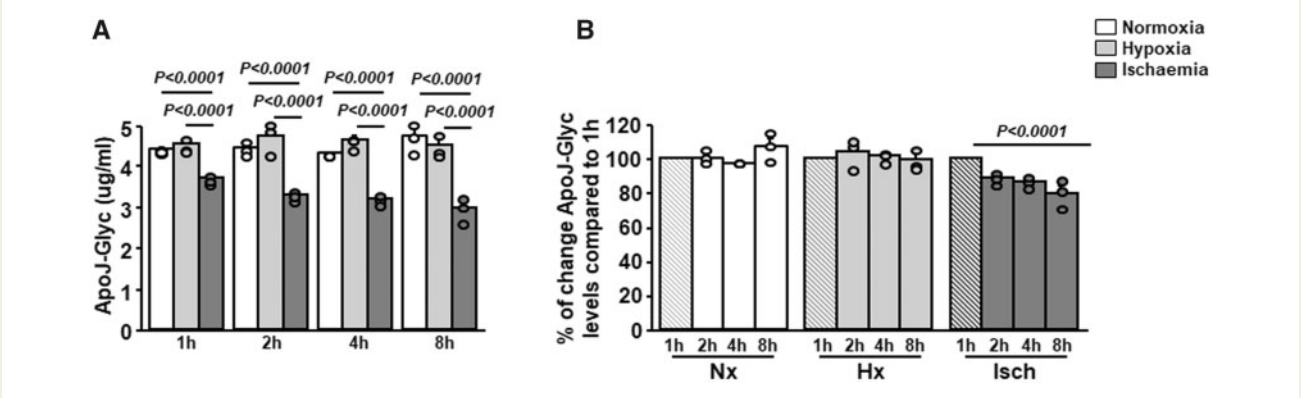


Figure 1 Changes in glycosylated apolipoprotein J secretion upon simulated ischaemia induction. Bar diagrams showing: (*A*) the time-dependent decrease in the secretion of glycosylated apolipoprotein J by porcine ventricular cardiac fibroblast cells subjected to simulated ischaemia that was not observed in cells subjected to hypoxia (*t*-test between groups in each time point) and (*B*) a 20% decrease in glycosylated apolipoprotein J release by porcine ventricular cardiac fibroblast after 8 h of ischaemia when compared to the levels detected at 1 h of simulated ischaemia (*t*-test between 1 and 8 h within each group). ApoJ-Glyc, glycosylated apolipoprotein J.

different cell types so that enough protein material was obtained to analyse ApoJ by western blot analysis (Supplementary material online, *Figure S4A*). In this analysis, the ratio of deglycosylated vs. glycosylated forms serves as an indicator of ApoJ-Glyc containing N-acetylglucosamine residues. Interestingly, simulated ischaemia, both in PVCF and HL-1, induced a 28% decrease in the deglycosylated/glycosylated ratio, highlighting reduced glycosylation of secreted ApoJ specifically in those cells of cardiac origin subjected to simulated ischaemic conditions (Supplementary material online, *Figure S4B and C*).

Intracellular glycosylated and non-glycosylated apolipoprotein J variants in isolated cardiac cells in culture under simulated ischaemic conditions

The analysis of intracellular ApoJ forms (Supplementary material online, *Figure S5* for representative western blot images in each cell type) revealed an influence of simulated ischaemia in the ratio between non-glycosylated ApoJ and ApoJ-Glyc variants, specifically in cellular types of cardiac origin (PVCF and HL-1). Both PVCF and HL-1 cells subjected to 8 h simulated ischaemia (O_2 deprivation and acidic conditions) depicted a shift in the abundance of non-glycosylated ApoJ vs. ApoJ-Glyc forms when compared to 1 h of simulated ischaemia (Supplementary material online, *Figure S6A and B*). Hypoxia alone did not induce these changes in intracellular ApoJ forms. The observed shift in the abundance of non-glycosylated ApoJ vs. ApoJ-Glyc forms indicates the accumulation of intracellular non-glycosylated variants over time in simulated ischaemic conditions. This difference was not observed in the other cell types (Supplementary material online, *Figure S6C–F*). This shift in the abundance of the different ApoJ forms is more evident when the ratio non-glycosylated vs. glycosylated variants is analysed (Supplementary material online, *Figure S4*). This shift is depicted by an inversion of the ratio after 8 h of simulated ischaemia specifically in PVCF and HL-1 cells compared to the other cell types in which the ratio remained negative in all the different conditions (Supplementary material online, *Figure S7*).

To confirm that the observed effect of simulated ischaemia on ApoJ-Glyc secretion was also evident on cells of human origin we run a proof-of-concept experiment on iPS cells that were differentiated to cardiomyocytes. After subjecting differentiated cardiomyocytes to 4 h of hypoxia and simulated ischaemia, we observed a significant decrease in the levels of ApoJ-Glyc secreted to the culture medium (*Figure 2A*). This decrease in the secretion of ApoJ-Glyc was mirrored by a decrease in intracellular ApoJ forms analysed by western blot being the decrease more evident in non-glycosylated variants (*Figure 2B–D*).

Early glycosylated apolipoprotein J decrease after initiation of ischaemia

In the pig model of STEMI, a decrease in serum ApoJ-Glyc levels was seen very early after ischaemia onset (reaching significance after

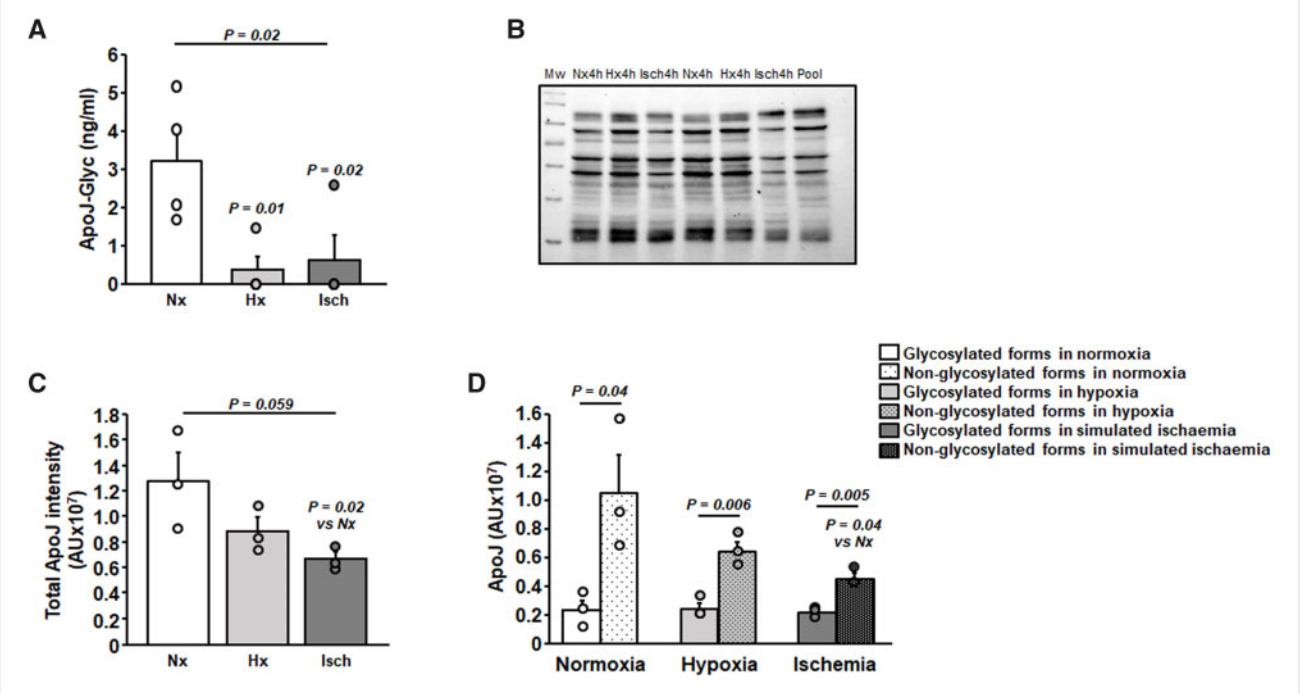


Figure 2 Proof-of-concept on induced pluripotent stem cells differentiated to cardiomyocytes. (*A*) Bar diagram showing the significant decrease in the levels of glycosylated apolipoprotein J secreted to the culture medium after subjecting differentiated cardiomyocytes to 4 h of hypoxia and simulated ischaemia (ANOVA and *t*-test between groups). (*B*) Representative western blot image of protein extracts of differentiated cardiomyocytes. Bar diagrams showing: (*C*) intracellular ApoJ forms total intensity and (*D*) glycosylated and non-glycosylated ApoJ variants (ANOVA and *t*-test between groups). ApoJ-Glyc, glycosylated apolipoprotein J.

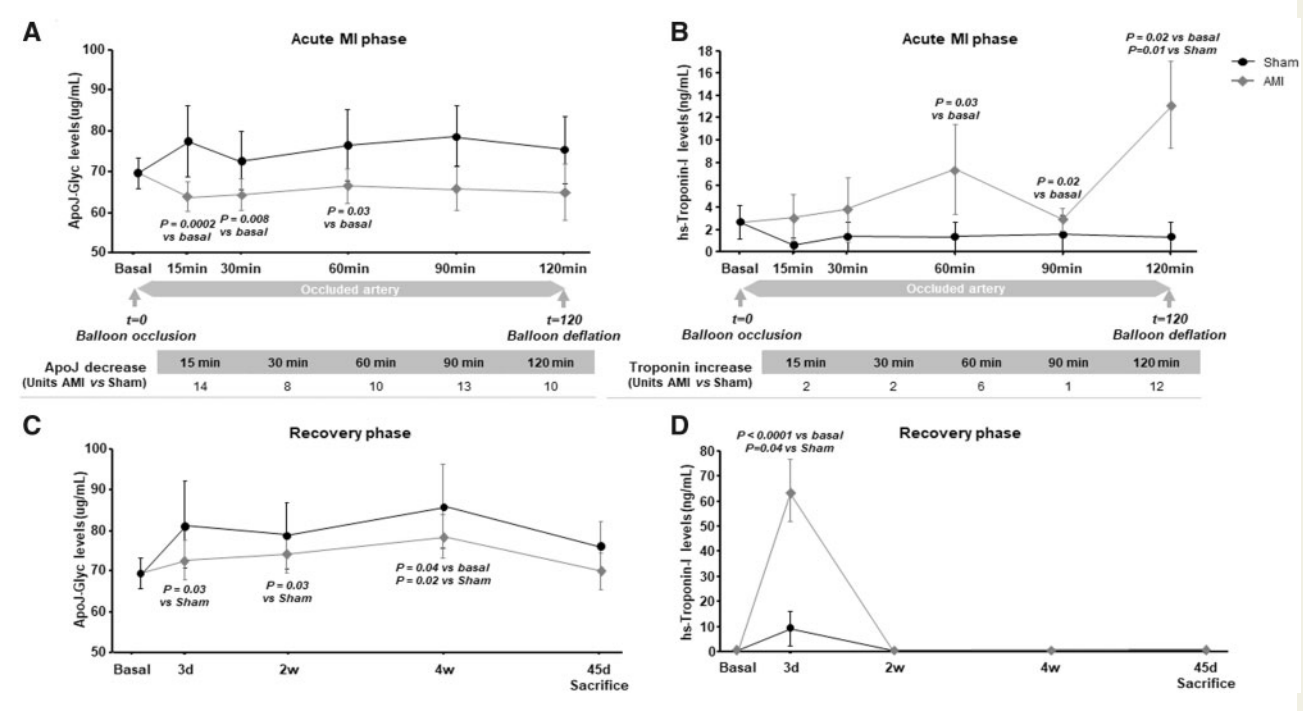


Figure 3 Glycosylated apolipoprotein J and high-sensitivity troponin I levels over ischaemia time in a controlled pig model of ST-elevation myocardial infarction. Acute myocardial infarction phase: (*A*) glycosylated apolipoprotein J levels declined rapidly after the onset of ischaemia ($t = 15$ min) and this effect was sustained up to 120 min (vs. mean basal values). (*B*) High-sensitivity troponin I levels remained low and significantly raised after 60 min of ischaemia. Recovery phase: (*C*) 3 days after myocardial infarction there was a recovery in glycosylated apolipoprotein J levels. (*D*) High-sensitivity troponin I levels showed the strongest increase 3 days after myocardial infarction with a decrease to baseline levels 2 weeks after the intervention. AMI, acute myocardial infarction; ApoJ-Glyc, glycosylated apolipoprotein J; hs, high sensitivity.

15 min) and was maintained during the ischaemic time ($P < 0.05$ vs. basal levels; *Figure 3A*). In contrast, hs-TnI levels showed a significant increase only after 60 min of ischaemia onset ($P < 0.05$ vs. basal levels; *Figure 3B*), time point in which signs of irreversible cardiac damage were evident as revealed by the detection of a significant scar in TTC staining (Supplementary material online, *Figure S3*). No significant correlation was observed between ApoJ-Glyc and hs-TnI levels ($R = 0.101$ $P = 0.058$). Three days after myocardial infarction, there was a recovery in ApoJ-Glyc levels (*Figure 3C*). High-sensitivity troponin I levels showed the strongest increase 3 days after myocardial infarction with a decrease to baseline levels 2 weeks after the intervention (*Figure 3D*).

Glycosylated apolipoprotein J in cardiac ischaemic events

Compared with controls, early AMI patients showed 45% lower ApoJ-Glyc levels on admission ($t = 0$) before any increase in necrosis markers (TnT and creatine kinase) [mean ± standard error of the mean (SEM): early AMI ($n = 38$): 264 ± 18 vs. C (cn = 144): 473 ± 6 μg/mL; $P < 0.0001$; *Figure 4A*]. The C-statistics analysis revealed that the measurement of ApoJ-Glyc levels showed a high discriminating value for the presence of myocardial ischaemia with an area under the curve (AUC) of 0.934 ($P < 0.0001$) and a cut-off value of 332 μg/mL with 97% sensitivity and 71% specificity (*Figure 4B*).

Multiple linear regression analysis, including those variables significantly associated with ApoJ-Glyc levels in the bivariate analysis (having an ischaemic event, sex, HDL-C, and CRP levels, and statin treatment; Supplementary material online, *Table S1*), showed that the presence of an ischaemic event remained as the only independent factor for ApoJ-Glyc levels in a first model with an *R* of 0.693 and a beta-value for unstandardized coefficients of -0.693 ($P < 0.0001$). In a second model, sex was also included as an independent factor for ApoJ-Glyc levels although with a beta-value for unstandardized coefficients of -0.139 ($P = 0.017$).

To further investigate ApoJ-Glyc in the clinical setting, we investigated a second independent group of patients. Specifically, ApoJ-Glyc levels were analysed in samples taken at admission in 212 STEMI patients (including *de novo* and secondary events) with different times of evolution of the ischaemic pain that included patients with positive hs-TnT detection at admission (group of patients with full-blown necrosis). ST-elevation myocardial infarction patients showed 15% lower ApoJ-Glyc levels at admission when compared with the control group (mean ± SEM: STEMI: 402 ± 8 vs. C: 473 ± 6 μg/mL; $P < 0.0001$; *Figure 5A*). *C*-statistics analysis revealed that the measurement of ApoJ-Glyc levels showed a discriminating ability for the presence of myocardial ischaemia with an AUC of 0.713 ($P < 0.0001$) and a cut-off value of 409 μg/mL with 80% sensitivity and 53% specificity (*Figure 5B*).

Glycosylated apolipoprotein J levels were significantly and inversely correlated with the duration of ischaemia (defined as time interval

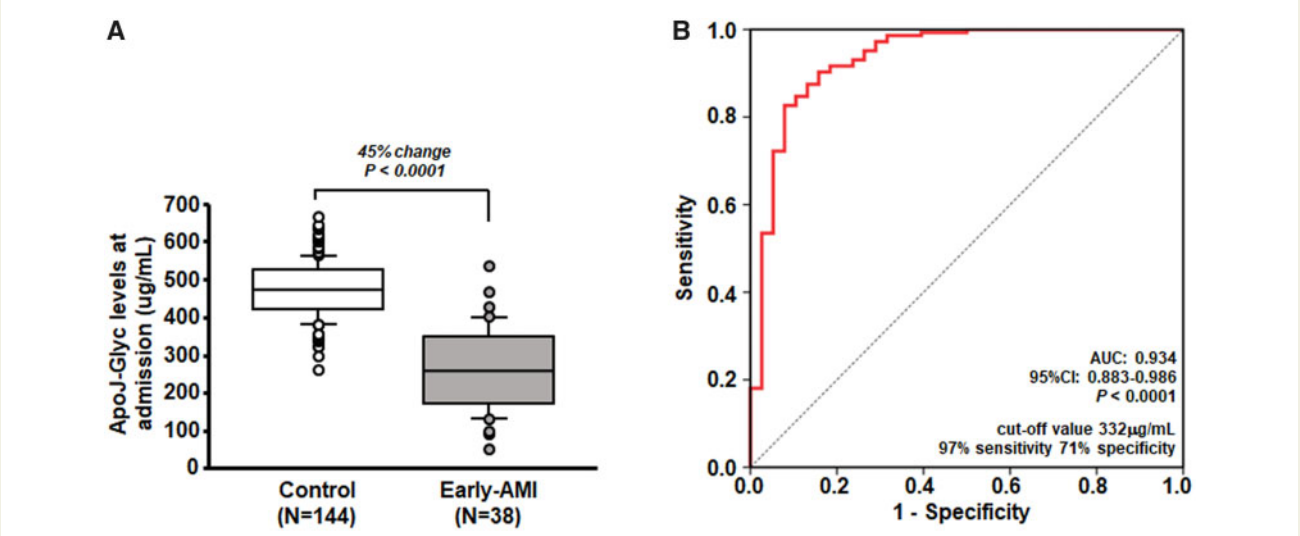


Figure 4 Glycosylated apolipoprotein J diagnostic value. (*A*) Box plot showing glycosylated apolipoprotein J levels in early acute myocardial infarction patients ($n = 38$) and in healthy subjects ($n = 144$). (*B*) Receiver operating characteristic curve showing the diagnostic value for the presence of myocardial ischaemia of glycosylated apolipoprotein J levels with an area under the curve of 0.934 ($P < 0.0001$) and a cut-off value of 332 µg/mL with 97% of sensitivity and 71% of specificity. ApoJ-Glyc, glycosylated apolipoprotein J; AUC, area under the curve.

between pain symptom onset and admission; $R = -0.259$ $P = 0.0003$; *Figure 5C*). In this second cohort of patients, multiple linear regression analysis including those variables significantly associated with ApoJ-Glyc levels in the bivariate analysis (having an ischaemic event, total cholesterol, LDL-C, HDL-C, and CRP levels, and hypertension; Supplementary material online, *Table S2*) also showed that the presence of an ischaemic event remained as the only independent factor for ApoJ-Glyc levels in a first model with an R of 0.326 and a beta-value for unstandardized coefficients of -0.326 ($P < 0.0001$). In a second model, total cholesterol levels were also included as an independent factor for ApoJ-Glyc levels although with a beta-value for unstandardized coefficients of -0.199 ($P = 0.001$). In a third model, CRP levels were also included as an independent factor for ApoJ-Glyc levels with a beta-value for unstandardized coefficients of -0.161 ($P = 0.001$). Nevertheless, in the three models, having an ischaemic event remained as the best predictor of ApoJ-Glyc levels.

Glycosylated apolipoprotein J in ST-elevation myocardial infarction patients after the acute event

ST-elevation myocardial infarction patients with a final TIMI flow grade of 0 or 1, which is known to be associated to a worse prognosis due to increased risk of mortality,[24] showed significantly lower ApoJ-Glyc plasma levels at admission ($t = 0$) than those STEMI patients with a final TIMI flow of ≥2 (*Figure 6A*). In addition, STEMI patients with cardiogenic shock showed 12% lower ApoJ-Glyc plasma levels at admission ($t = 0$) when compared to those who did not have cardiogenic shock ($P = 0.004$).

In a subset of 82 STEMI patients (for whom a blood sample was available 72 h after admission), ApoJ-Glyc levels were quantified in plasma samples obtained 72 h after admission (*Figure 6B*). Glycosylated apolipoprotein J showed, on average, a decrease with respect to levels at admission (mean ± SEM: $t = 72$ h: 331 ± 10 vs. $t = 0$: 402 ± 8 µg/mL; $P < 0.0001$; *Figure 6C*). If changes in ApoJ-Glyc levels were analysed individually, two different groups of patients were defined, namely those patients showing stable ApoJ-Glyc levels or even an increasing (recovery) trend and those showing a progressive decrease in ApoJ-Glyc levels ($P < 0.0001$; *Figure 6D*). The latter group showed a progressive decrease in ApoJ-Glyc levels and had higher Global Registry of Acute Coronary Events (GRACE) risk score values than the group showing stable ApoJ-Glyc levels ($P = 0.03$; *Figure 6E*).

Kaplan–Meier survival curve analysis revealed that those patients with ApoJ-Glyc levels below the median value of the STEMI group at admission showed a higher rate of recurrent ischaemic events (non-fatal AMI, unplanned revascularization for ischaemia after discharge, admission for heart failure or stroke) and mortality after 6 months of follow-up ($P = 0.008$; Supplementary material online, *Figure S8*; cut-off value median levels of STEMI patients at admission).

Discussion

Chest pain is one of the most common causes of hospital admission worldwide and represents a major diagnostic challenge. Early triage of patients with acute chest pain is crucial to optimize treatment and reduce healthcare costs. The heterogeneous nature of patients presenting with acute chest pain, together with the emerging importance of distinguishing between myocardial infarction and non-ischaemic myocardial injury,[25] has highlighted the need for the identification of novel pathways and pathological mechanisms to characterize ischaemic damage.

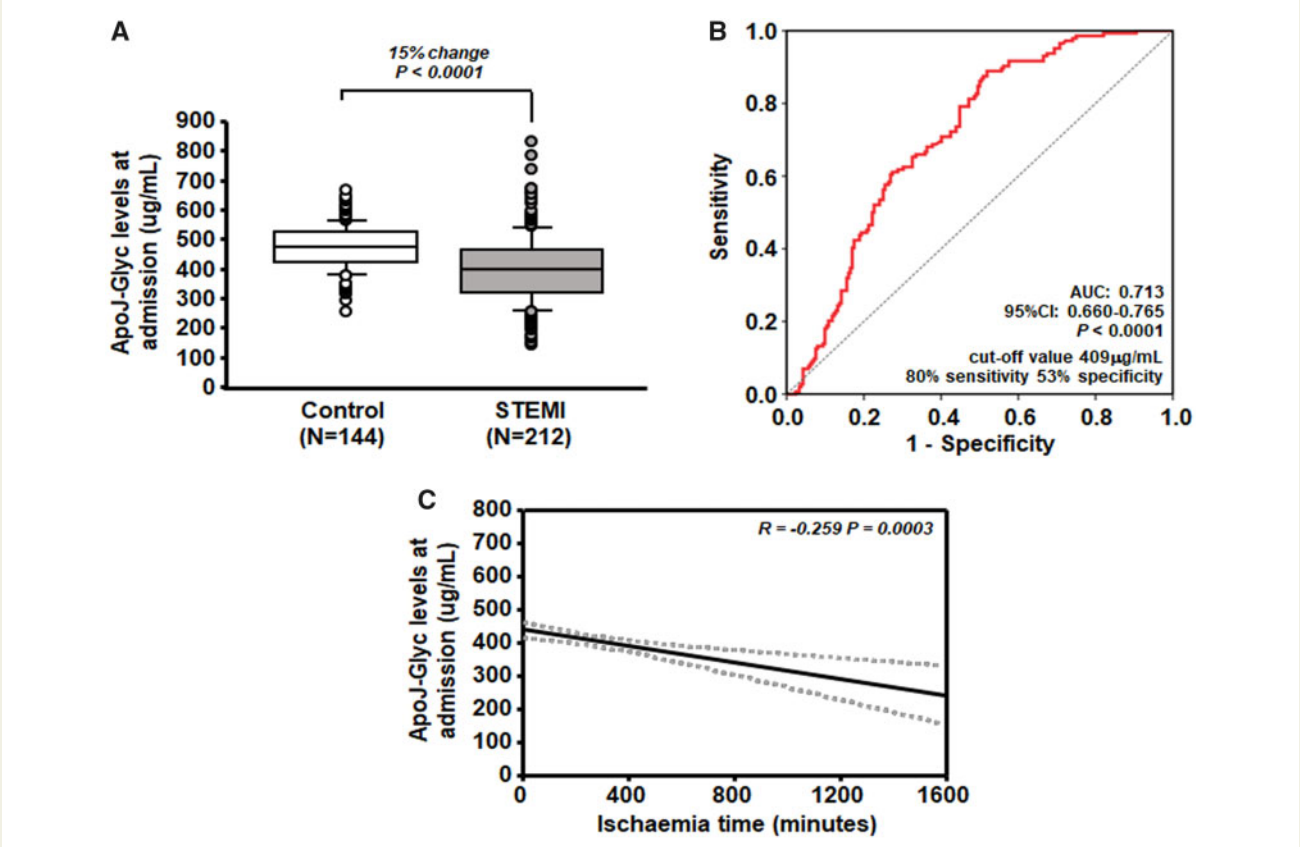


Figure 5 Glycosylated apolipoprotein J in ischaemia progression. (*A*) Box plot showing glycosylated apolipoprotein J levels in ST-elevation myocardial infarction patients at the moment of admission ($n = 212$) and in healthy subjects ($n = 144$). (*B*) Receiver operating characteristic curve showing the discriminating value for the presence of ischaemia of glycosylated apolipoprotein J levels with an area under the curve of 0.713 ($P < 0.0001$) and a cut-off value of 409 µg/mL with 80% sensitivity and 53% specificity. (*C*) Regression plot showing the inverse and significant correlation between glycosylated apolipoprotein J levels at admission and ischaemia time. ApoJ-Glyc, glycosylated apolipoprotein J; AUC, area under the curve; STEMI, ST-elevation myocardial infarction.

Glycosylated apolipoprotein J as an early sensor of myocardial ischaemia

In the present study, we have demonstrated, for the first time, that glycosylated ApoJ-Glyc forms are a specific and sensitive biomarker of myocardial ischaemia (*Graphical Abstract*). In a previous discovery study, we described a shift in the proteomic glycosylation profile of ApoJ, also known as clusterin, in the early phase of cardiac ischaemia.[15] Aberrant glycosylation has now been recognized as an attribute of many mammalian diseases, including hereditary disorders, immune deficiencies, neurodegenerative diseases, cardiovascular diseases, and cancer.[26] Therefore, protein glycosylation analysis has become a new approach for the discovery of novel cell damage pathways. Indeed, recent studies have suggested a relationship between differential glycosylation states of serum proteins and specific pathologies.[27,28]

Apolipoprotein J is a molecular chaperone with anti-oxidant and cytoprotective properties.[29] This apolipoprotein has different forms with different functions according to alternatively spliced variants, the presence of targeting sequences, the presence of disulphide bonds, and the presence of different post-translational modifications.[30] One of the most relevant post-translational modifications in ApoJ protein is glycosylation. There are different glycosylation states of ApoJ ranging from a partially glycosylated variant (60 kDa) to a fully glycosylated variant (75 kDa). This ability to change its glycosylation level is related to ApoJ sensitivity to cellular perturbations, such as the ATP and O_2 deprivation and their impact on cellular metabolism typical of ischaemia. Under normal conditions, fully ApoJ-Glyc has a functional role as a molecular chaperone.[31,32] Under oxidizing conditions, intracellular hypoglycosylated and retro-translocated ApoJ variants have shown an enhanced activity as molecular chaperones cooperating with other chaperones to fight against oxidative stress.[32,33] In the context of AMI, oxidative stress is precisely one of the pathophysiological concepts underlying myocardial ischaemia/reperfusion injury contributing to oxidative damage to proteins.[34,35] Our *in vitro* results, using cell cultures of different origin, show that simulated ischaemic conditions induce a shift in the glycosylation profile of ApoJ indicative of an accumulation of intracellular non-glycosylated variants. This accumulation could be related to the previously described enhanced chaperone activity of intracellular ApoJ variants in a highly oxidative milieu to help in the trafficking, binding, and/or refolding of denatured

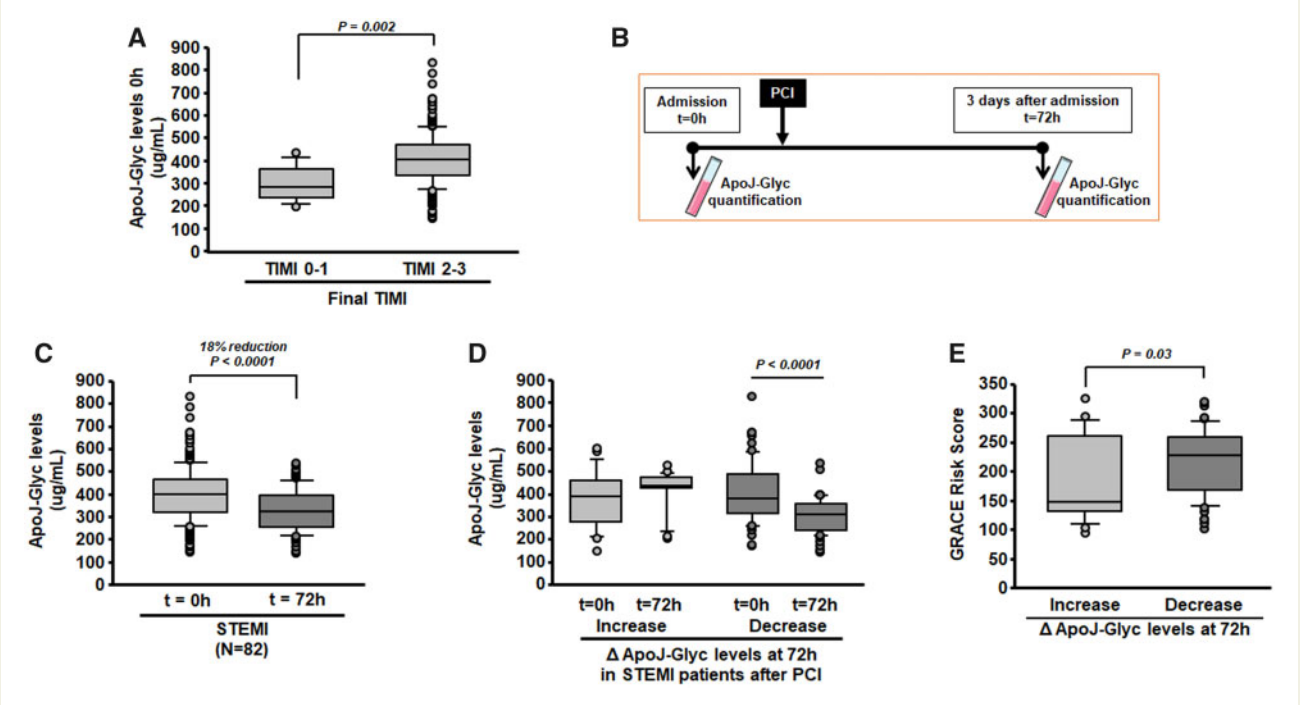


Figure 6 Glycosylated apolipoprotein J prognostic value. (*A*) Box plot showing the significant differences in glycosylated apolipoprotein J levels at admission in relation to the final TIMI flow grade. (*B*) Blood sampling in ST-elevation myocardial infarction patients at admission ($t = 0$) and 3 days after the admission time ($t = 72$ h) when patients had already undergone percutaneous coronary intervention. Box plots showing: (*C*) the progressive decrease in glycosylated apolipoprotein J levels 72 h after admission when compared to the moment of admission; (*D*) the different trends observed when changes in glycosylated apolipoprotein J levels were analysed individually, patients who showed stable glycosylated apolipoprotein J levels or even an increasing trend 3 days after the admission when compared to the admission time, and those showing a progressive decrease in glycosylated apolipoprotein J levels; and (*E*) the association between highest Global Registry of Acute Coronary Events risk score values and the decrease in glycosylated apolipoprotein J levels 3 days after admission in ST-elevation myocardial infarction patients. ApoJ-Glyc, glycosylated apolipoprotein J; GRACE, Global Registry of Acute Coronary Events; PCI, percutaneous coronary intervention; STEMI, ST-elevation myocardial infarction.

intracellular proteins in the context of ischaemia. This change is mirrored by a decrease in the secretion of ApoJ-Glyc, specifically in cells of cardiac origin as confirmed by the proof-of-concept experiments in differentiated cardiomyocytes from iPS cells, appearing as the potential underlying cause behind the observed decreased circulating ApoJ-Glyc levels in patients with ischaemic events. Thus, under the initial ischaemic insult, the cell could be secreting lower levels of the glycosylated variant, before reaching the threshold of cell damage that triggers the apoptosis gene program, necrosis, and Tn release. Our results in the preclinical pig model of STEMI have demonstrated the early decrease in the secretion of ApoJ-Glyc upon the initiation of ischaemia that precedes, by ~1 h, the release of Tn (measured with a hs assay) into the bloodstream, highlighting a potential role as an early biomarker of ischaemia prior to the evidence of irreversible cell damage emerging. Importantly, ApoJ-Glyc changes were observed even when no signs of cardiac damage were evident by TTC (30 min post-AMI initiation).

Until now, studies analysing the potential role of ApoJ in the cardiovascular system have been based on the analysis of total protein levels[36,37] and/or animal models where ApoJ gene expression was knocked down or over-expressed,[38] being all of them scenarios in which the potential contribution of specific ApoJ protein variants could not be elucidated. Apolipoprotein J is represented by a series of different forms that differ in their intracellular processing and glycosylation state and are dependent on the tissue and the disease state.[39–41] Our results indicate that the specific shift in the glycosylation profile of ApoJ that we have observed in the context of ischaemia is specific for cells of cardiac origin, among the different cell types that we have tested. In fact, the proof-of-concept study in differentiated human cardiomyocytes has confirmed a significant decrease in ApoJ-Glyc release after 4 h of ischaemia.

Glycosylated apolipoprotein J and the residual ischaemic risk post-acute myocardial infarction

Interestingly, our study demonstrates that besides its change early after the onset of ischaemia, ApoJ-Glyc changes can be detected after the presentation of an acute ischaemic event. Indeed, one of the most important unmet medical needs in the context of ischaemic heart disease is the identification of biomarkers that could help improving risk stratification and to implement tailored treatments to reduce recurrent events. Nowadays, the most accepted tool for early risk stratification and prediction of recurrent ischaemic events and mortality in ACS is the GRACE risk score.[42] Conceivably, the

availability of sensitive and accurate biomarkers of ischaemia might improve current clinical risk stratification scores.[43]

The association between the progressive decrease in ApoJ-Glyc levels 72 h after admission and PCI and higher GRACE risk score values, points towards a potential role of ApoJ-Glyc in mapping an ongoing ischaemic process that could have a direct impact on a patient's prognosis. Indeed, increasing evidence has shown that in a proportion of patients, PCI does not achieve effective myocardial reperfusion due to the occurrence of coronary microvascular obstruction (MVO). This has led to the notion that MVO is another important independent predictor of adverse left ventricular remodelling that could be more predictive of major adverse cardiovascular events than infarct size itself.[44,45] In this context, having a reliable biomarker of ischaemia able to map the evolution of the ischaemic event, irrespectively of its origin, would be very useful to guide decision-making and treatment. In fact, our *in vitro* studies have revealed a time-dependent decrease in the secretion of ApoJ-Glyc that was evident as early as in 1 h and was progressive until 8 h of ischaemia, pointing to the potential added value of ApoJ-Glyc in detecting the evolution of the ischaemic process. Furthermore, the observed association between decreased ApoJ-Glyc levels and the worse outcome at 6-month follow-up after STEMI highlights the potential pathophysiological role of this protein in the clinical evolution of the disease. We have previously reported, for the first time, the association between the glycosylated status of another apolipoprotein, apolipoprotein A-I, and myocardial infarction.[19] The added value of ApoJ-Glyc points out to a potential role of this biomarker in the identification of high-risk patients at the moment of admission, representing a clear advantage in the triage of those patients with the highest need for advanced treatment and monitoring.

Limitations

A limitation of the study is that hs-TnT measurement was not available in the cohort of patients in Group 1 in which TnT levels were measured by conventional assays (as this was the assay that was clinically used at the time patients were included in the study) and unfortunately no blood samples were left to run the quantification of hs-TnT. However, in Group 2, hs-TnT measurements were available and we have seen that ApoJ-Glyc-reduced levels occur and are associated with clinical outcomes even in the context of elevated hs-TnT levels at admission. Furthermore, by using the pig model, we have been able to compare the kinetics of the changes between ApoJ-Glyc and hs-TnT. Further work is required to look at the behaviour of ApoJ-Glyc in different conditions where non-ischaemic myocardial injury occurs and Tn release is induced by mechanisms other than ischaemia,[3,46] to better clarify if ApoJ-Glyc decrease is specific for ischaemia. Nevertheless, it is important to stress that, if further validated, ApoJ-Glyc could be useful in complementing markers of myocardial injury.

In addition, due to the low numbers and the consequent lack of multivariable analysis, the survival analysis represents an early exploratory analysis that deserves further validation.

Conclusions and future research

We have identified, in proof-of-concept *in vitro* studies, in a preclinical STEMI model and in patients with myocardial ischaemia that ApoJ-Glyc is a promising novel sensor for the early detection of ischaemia with potential added value regarding risk assessment post-AMI (*Graphical Abstract*). Further studies are needed to test its performance in large prospective clinical validation trials in unselected patients with possible acute myocardial ischaemia and to fully assess its potential added value in the context of hs-Tn. Of particular importance could be the clinical value of ApoJ-Glyc in patients with suspected ACS and normal or inconclusive hs-TnT values and in the context of other possible mechanisms of cardiac ischaemia, such as non-obstructive coronary artery disease and functional disorders affecting coronary blood flow.

Supplementary material

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

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Conflict of interest: J.C., T.P., and L.B. are authors of a PCT Patent application to protect the use of ApoJ-Glyc as a biomarker of ischaemia. J.C., T.P., G.V., and L.B. are shareholders of a spin-off company (GlyCardial Diagnostics) related to this biomarker. J.C., L.B., and J.C.K. are employees/advisors of the spin-off company (GlyCardial Diagnostics) related to this biomarker. L.B. received institutional research grants from AstraZeneca; consultancy fees from Sanofi and Novartis; speaker fees from Lilly, Pfizer, and AstraZeneca. F.C. received speaker fees Amgen, AstraZeneca, Servier, and BMS and consultancy fees from GlyCardial Diagnostics. R.S. received institutional research grants/support from AstraZeneca, Cytosorbents, GlyCardial Diagnostics, and Thromboserin; consultancy fees and/or honoraria from Amgen, AstraZeneca, Bayer, Bristol Myers Squibb/Pfizer, Cytosorbents, GlyCardial Diagnostics, Haemonetics, HengRui, Idorsia, Intas, Medscape, PhaseBio, Portola, Sanofi Aventis, and Thromboserin. J.L.L.S. received grants and/or consultancy fees from Bayer, Pfizer, Menarini, Sanofi, Merck, Boehringer Ingelheim, Amgen, and GlyCardial Diagnostics. All other authors declared no conflict of interest.

Data availability

The data that support the findings of this study are available on request, in reasonable time and form, from the corresponding author.

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