Aggrecan - a new biomarker for acute type A aortic dissection

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Abstract

Acute type A aortic dissection (ATAAD) constitutes a life-threatening aortic pathology with significant morbidity and mortality. Without surgical intervention the usual mortality rate averages between 1-2 % per hour. Thus, an early diagnosis of ATAAD is of pivotal importance to direct the affected patients to the appropriate treatment. As a consequence, we investigated whether aggrecan (ACAN) is a potential biomarker for diagnosing ATAAD. Our investigation showed that ACAN mRNA expression is highest in aortic tissue from ATAAD patients. Mean ACAN protein concentration showed a significantly higher plasma concentration in ATAAD patients (38.59 ng/mL) compared to plasma of patients with thoracic aortic aneurysms (4.45 ng/mL), patients with ST-elevation myocardial infarction (11.77 ng/mL) and healthy volunteers (8.05 ng/mL). Cardiac enzymes like creatine kinase MB and cardiac troponin T showed no correlation with ACAN levels in ATAAD patients. Receiver-operator characteristics (ROC) curve analysis for ATAAD patients versus control subjects revealed an area under the curve of 0.947 and an optimum discrimination limit of ACAN plasma levels at 14.3 ng/mL with a corresponding sensitivity of 97 % and specificity of 81 %. According to our findings ACAN is a reliable potential biomarker in plasma samples to detect ATAAD with high sensitivity and specificity.

Keywords: aggrecan, acute type A aortic dissection, biomarker, ELISA

Introduction

Acute type A aortic dissection (ATAAD) is a life-threatening diagnosis which is associated with significant morbidity and mortality [1]. After onset of symptoms patients suffering from ATAAD have an associated mortality rate of 1-2% per hour without surgical intervention. However, a correct early diagnosis is complicated by the rare frequency of ATAAD. Furthermore, ATAAD shares similar symptoms with more common clinical presentations such as myocardial infarction, vascular embolization, gastric ulcer or acute back pain [2]. Hence, a clear and early diagnosis of ATAAD is crucial for immediate surgical intervention resulting in an improved survival rate for the patient. Therefore, the establishment of a specific and sensitive blood biomarker for diagnosing ATAAD would be the key to reduce the time period between symptom onset and the essential surgical treatment.

Within the last decades several biomarkers have been established in cardiovascular medicine. Especially creatin kinase MB (CK-MB) and cardiac troponins (cTnTs) have a long history for the specific and sensitive detection of myocardial infarction (MI) [3,4]. In addition, we have recently described MYBPHL as a reliable biomarker to specifically predict damage of atrial tissue [5]. Although preliminary data suggest a possible role of plasma biomarkers, like smooth muscle derived calponins and myosin heavy chain [6,7] or the fibrin fragment D-dimer [8] in early diagnosis of ATAAD, at present no reliable biomarker exists, to diagnose ATAAD with sufficient specificity and sensitivity.

Aggrecan (ACAN) is a multimodular proteoglycan which can make up to 10% of the cartilage [9]. ACAN plays a major role in bone and cartilage morphogenesis and several mutations have been identified in patients with short stature [10]. However, analysis of the proteoglycanome confirmed the presence of ACAN in the normal human aorta and also in aortic lesions of ATAAD patients [11].

Here we show that ACAN protein levels are significantly enhanced in plasma samples of ATAAD patients compared to samples from healthy controls. In addition, plasma ACAN levels of several clinical control cohorts stayed far beyond the values obtained in ATAAD patients and were similar to the concentration of healthy controls. Thus, our data suggest that ACAN may be a useful new biomarker for early diagnosis of ATAAD.

Methods

Blood samples and biopsies

Blood samples of ATAAD patients (n = 33) were collected between February 2017 and January 2020. Samples were drawn directly after admission and centrifuged at 2,000 x *g* for 10 min at 4°C. Plasma was partitioned in 200 µL aliquots and immediately stored at -80°C within 30 min after admission until further use. Basic demographic and clinical data for all patients are shown in Table 1. Plasma samples from all other experimental cohorts (Table I in the Data Supplement) were supplied by the KaBi-DHM. Human biopsies (skeletal muscle, fat tissue, left atrium, aortic tissue from ATAAD or coronary artery bypass graft patients, *Vena saphena magna* and *Arteria mammaria interna*) (Table II in the Data Supplement) were obtained during surgical procedures, directly snap-frozen and stored in liquid nitrogen until further use.

Protein expression in different heart regions

Protein concentrations in different heart regions had been previously determined by mass spectrometry [12].

Assessment of gene expression in human biopsies by qRT-PCR

Frozen biopsies were homogenized in 900 µL QIAzol lysis reagent for 30 sec using an Ultraturrax MICCRA D-8 (ART Moderne Labortechnik, Müllheim, Germany) and processed with the RNeasy Plus UniversalMini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendation. One hundred ng total RNA were reverse-transcribed into cDNA with M-MLV reverse transcriptase (150 U, Invitrogen, Carlsbad, CA), random hexamer primers (375 ng), dNTPs (10 mM each), 10 mM DTT and 1× first strand buffer in a final volume of 30 µL for 50 minutes at 37°C. The enzyme was inactivated for 15 minutes at 70°C. Gene-specific amplification of 1 µL cDNA was performed on a Quant Studio 3 (ThermoFisher, Dreieich, Germany) with 0.3 µM of each primer and Power SYBR Green Mastermix (ThermoFisher) using the following cycling conditions: 95° for 10 minutes to activate *Taq* polymerase, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The relative gene expression was normalized to *ACTB* (*b-Actin*) expression as the reference. The sequences of all primers are shown in Table III in the Data Supplement.

Measurement of ACAN, OGN, ITGA11 and LPYD in plasma samples by ELISA

Commercially available ELISA kits were used to determine the concentration of aggrecan (ACAN) (Cat.No. SEB908Hu, Cloud Clone Corp., Katy, TX), osteoglycin (OGN) (Cat.No. LS-F22608, LifeSpan Biosciences Inc.,

Seattle, WA) and integrin α 11 (ITGA11) (Cat.No. CSB-EL011863HU, Cusabio, Houston, TX) in plasma samples according to the manufacturers' instructions. In brief, all components and samples were brought to room temperature and 100 µL of undiluted plasma samples were added, processed and plates were read at 450 nm. In each assay a standard curve was included to determine the concentration in individual samples.

Statistical analysis

Data distribution was assessed using the Shapiro–Wilk test. Differences in gene expression were determined by the Mann-Whitney Rank Sum test or the one-way-ANOVA test. Significance of differences in ACAN protein concentrations for multiple groups was estimated by the Wilcoxon-Mann-Whitney, Kruskal Wallis or one-way-ANOVA test by all pairwise multiple comparison procedures (Dunn´s or Holm-Sidak Method). Significance is indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Values are presented as mean ± standard error of the mean (SEM), 95% confidence interval (CI) and fold change as appropriate.

Results

Selection of candidate genes to diagnose acute type A aortic dissection

ATAAD leads to a complete dissection of the physiological structure of the aortic wall [11] and may thus induce release of proteins into the circulation. In our previous work we have identified 8,699 proteins in the aorta of the human heart [12]. To limit the number of these possible candidates we conducted two approaches. Firstly, we selected proteins which were expressed most abundantly, but not necessarily restricted to aortic tissue. Secondly, we selected proteins which were preferentially expressed in aortic tissue compared to all other fifteen heart regions. Following these pre-selection criteria, we defined a list of 23 potential candidates (Figure 1A). Looking at the protein expression of the candidate markers across the sixteen regions of the human heart, several candidates showed high expression in the aorta and coronary arteries (Figure I in the Data Supplement), suggesting them as promising markers for vasculature. The concentration of ACAN protein, one promising candidate with a high specificity for arterial vessels across all sixteen regions of the human heart is shown in Figure 1B.

Next, we determined mRNA expression of all candidates in aortic tissue from ATAAD patients in comparison to aortic tissue from coronary artery bypass patients. Furthermore, we measured mRNA in left atrial tissue, venous and arterial vessels and we analyzed the expression in extra-cardiac tissues such as fat and skeletal muscle. Figure 2 shows the expression of four candidate genes: *HAPLN1* (hyaluronan and proteoglycan link protein 1), *ITGA11* (integrin α-11), *OGN* (osteoglycin) and *ACAN* (aggrecan). In all cases the mRNA abundance is highest in the aorta from ATAAD patients and it is significantly different to aortic tissue from coronary artery bypass graft patients (Figure 2). The mRNA expression of the remaining 19 candidates in these tissues is shown in Figure II in the Data Supplement.

ACAN protein concentration is enhanced in plasma of patients with acute type A aortic dissection

Our data on protein and gene expression prompted us to determine the protein concentration of ACAN, OGN and ITGA11 in plasma samples of ATAAD patients, obtained directly after the arrival at our hospital. For comparison, we analyzed plasma of healthy volunteers and patients who underwent minimally invasive, isolated mitral valve repair (MVR). Indeed, ACAN levels were significantly elevated, with a four to five-fold higher concentration compared to both control groups (Figure 3A). Mean plasma ACAN level was 50.16±5.43 ng/mL. Mean plasma levels of the healthy subjects (control) and MVR group were 10.33±1.42 ng/mL and 11.92±1.77 ng/mL, respectively. The levels of OGN were also significantly enhanced in ATAAD samples with a mean value of 25.34± 1.46 ng/mL compared to control and MVR samples with 17.65±2.58 ng/mL and 18.75±2.65 ng/mL, respectively. However, the difference between ATAAD patients and control groups was much smaller (Figure

3B). In contrast, ITGA11 values in plasma samples were lowest in the ATAAD group with 5.59±3.79 ng/mL and similar in the two reference groups (control and MVR) with 19.31±11.54 ng/µL and 22.84±17.88 ng/mL (Figure 3C). Thus, ACAN appears to be the most promising candidate to diagnose ATAAD.

ACAN plasma levels are not enhanced in patients with acute myocardial infarction and aneurysm

Next, we addressed the question whether elevated ACAN plasma levels are specific for ATAAD. To further substantiate our initial promising results, we increased the number of ATAAD patients (n=33). Using this extended cohort, we detected a significant almost 10-fold increase in plasma levels of ACAN in ATAAD patients with a mean plasma level of 38.59±4.08 ng/mL compared to samples from patients with asymptomatic chronic aneurysm of the ascending aorta with a mean value of 4.45±0.90 ng/mL (Figure 4). We next analyzed ACAN plasma levels of patients with acute ST-elevation myocardial infarction (STEMI), which may confound the correct diagnosis of ATAAD. Again, ACAN protein concentrations of ATAAD patients were clearly and significantly elevated compared to STEMI patients who showed a mean value of 11.77±1.89 ng/mL (Figure 4). In addition, ACAN protein levels in patients without coronary artery disease (N-CAD) are significantly lower compared to ATAAD patients, but not significantly different to healthy controls or STEMI patients. N-CAD group showed a mean value of 8.88±1.8 ng/mL (Figure 4). Mean value of the healthy control group was 8.05±1.38 ng/mL. The individual levels of CK-MB and cTnT and their correlation with ACAN for STEMI and N-CAD patients are shown in Figure III in the Data Supplement. Thus, ACAN protein levels of ATAAD patients in the circulation are significantly elevated compared to healthy controls and patients with important cardiac differential diagnoses, including MI, supporting the use of ACAN as a reliable and specific biomarker to detect ATAAD.

Association of ACAN plasma concentration with demographic parameters and severity of ATAAD

Next, we addressed the question whether the release of ACAN into the circulation might be affected by basic demographic parameters such as sex or age. However, neither sex nor age had a significant impact on ACAN plasma levels (Figure 5A and B). Mean ACAN plasma levels of female and male samples were 36.06±5.74 ng/mL and 40.69±5.80 ng/mL. For age association, the ATAAD samples were divided into five age groups. Mean ACAN plasma levels of the five age groups, organized from young to old, were 23.60±6.38 ng/mL, 44.49±7.94 ng/mL, 36.63±6.69 ng/mL, 41.28±8.37 ng/mL and 41.21 ng/mL (Figure 5B). Despite the considerably lower mean ACAN level of 23.60 ng/mL in the first group (40-49 years) compared to the mean ACAN levels of the other four age groups, there was no statistically significant difference of ACAN plasma

levels between all five groups according to the one-way-ANOVA test with a *p* value of 0.828. Furthermore, we considered whether extent of ATAAD, according to the De Bakey classification might be reflected by the ACAN concentration in plasma. However, there was no major difference between patients with De Bakey type I and II ATAAD (Figure 5C) with mean ACAN levels of 32.79±4.43 ng/mL and 36.27±6.51 ng/mL. Finally, we established kinetics of ACAN levels and the time period between onset of symptoms of ATAAD and the drawing of the blood samples. ACAN levels remained clearly elevated for up to 72 h after the onset without major differences at any time point (Figure 5D). ACAN plasma levels of the five time points in increasing order were 43.2±10.84 ng/mL, 34.0±6.67 ng/mL, 35.3±9.06 ng/mL, 53.2±12.39 ng/mL and 47.1±9.03 ng/mL (p=0.709).

ACAN detects acute type A aortic dissection with high specificity and sensitivity

We next evaluated the level of the already established clinical MI biomarkers, CK-MB and cTnT, in plasma samples of patients with ATAAD. For both markers, in the vast majority of samples, the values remained below the established clinical reference limit which defines myocardial cell damage (Figure 6A and C). In addition, no correlation between plasma levels of ACAN and CK-MB (Figure 6B) or cTnT (Figure 6D) was seen. All ATAAD samples with cardiac enzyme levels above the established clinical threshold suffered an involvement of the aortic root with presumably consecutive narrowing or obstruction of the coronary ostia. Thus, the increase of ACAN in the peripheral circulation of ATAAD patients apparently happens completely independent of both CK-MB and cTnT. Area under the curve on receiver-operator characteristics (ROC) curve analysis for all ATAAD patients (n=33) versus all control subjects (n=63) was 0.947 (Figure 7A). Based on the ROC curve analysis an ACAN concentration of 14.3 ng/mL in the plasma was the optimum discrimination limit, resulting in a sensitivity of 97% and a specificity of 81%. Only one ATAAD sample showed an ACAN concentration below this threshold (Figure 7B). Analyzing the ACAN levels in patients with cardiac complications (STEMI or aneurysms, Figure 7C and D) showed a specificity of more than 80%. In addition, in different experimental control groups (Figure 7E) a similar specificity was obtained. Thus, our data clearly support the potential of ACAN as a reliable biomarker in plasma samples to detect ATAAD with a high sensitivity and specificity.

Discussion

ATAAD is a very severe cardiovascular diagnosis occurring at a rate between 4 and 16/100 000 [13]. ATAAD patients are often hospitalized with concomitant co-morbidities which mask and complicate the diagnosis of ATAAD [2], demanding a high specificity for a reliable biomarker. In our study we have measured ACAN levels in peripheral blood of ATAAD patients. Our data clearly show that ACAN concentrations were significantly increased in plasma of ATAAD patients compared to plasma samples of healthy individuals and patients suffering from different cardiovascular disease.

ACAN levels in ATAAD patients were elevated above our calculated threshold of 14.3 ng/mL based on the ROC curve analysis. In contrast, the ACAN plasma levels of the vast majority of patients with MI remained below this value. In addition, aneurysmatic alterations of the ascending thoracic aorta also did not result in increased ACAN plasma levels. Therefore, our study could definitely rule out ACAN as a possible screening marker for aneurysmatic thoracic aortic disease. In summary, it can therefore be said that secondary cardiovascular diagnoses did not influence the level of ACAN in plasma for specific diagnosis of ATAAD. Basic demographic parameters (age, sex), extent of the disease and the time between onset of ATAAD and hospitalization do not influence peripheral ACAN levels, suggesting that only the traumatic event of ATAAD would lead to ACAN release. Applying the optimum discrimination limit of 14.3 ng/mL, based on the ROC curve analyses, across our cohort of ATAAD patients, healthy probands and patients with other cardiovascular diagnoses (MVR, N-CAD) yielded a specificity of more than 97 % and a sensitivity of 81% when considering all of our experimental control groups. Even if we focused on clinical patients and excluded the healthy persons, we still ended up with a sensitivity of > 81%. Calponin and D-dimer have been proposed as diagnostic tools in ATAAD [6,8]. Comparing our results with these two markers we found a superior specificity of ACAN to discriminate ATAAD and MI (≈ 73%). Importantly, ACAN levels did not correlate with CK-MB or cTnT concentrations. Thus, a combination of ACAN with these markers might be beneficial to further increase the sensitivity. Therefore, the combined use of ATAAD and MI markers in an emergency setting should prompt the treating physician to run the appropriate, more invasive, and time demanding diagnostic test for definitive confirmation. Thus, unnecessary therapeutic delays will be prevented. The level of calponin increases in ATAAD but decreases beyond 12 h after onset [6]. In contrast, upon arrival at the hospital ACAN level stayed elevated and did not vary substantially for up to 72h after the onset of ATAAD. This might especially be crucial when ATAAD occurred before that time period.

Comparing the performance of ACAN with the existing markers like D-dimers and calponin [6,8] clearly underlines the superiority of ACAN. Still, the values for specificity and sensitivity do not completely reach the confidence of the well-established high-sensitive troponin assay for detection of MI [14].

Some limitations of our study must be mentioned. ACAN can make up a substantial part of cartilage and ACAN degradation is an important feature of osteoarthritis [15]. Though we can definitely exclude osteoarthritis in our patient cohort, it must be mentioned that this diagnosis could possibly confound the proper detection of ATAAD.

In summary, we have identified ACAN plasma levels as a reliable biomarker to detect the presence of an ATAAD. This marker reliably detected ATAAD patients in a very sensitive manner. At the same time, the biomarker showed a satisfying specificity which was not confounded by the presence of MI.

Declarations

Funding

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Conflicts of interest/Competing interests

The German Heart Center Munich is holder of a patent (EP-No 20 151 237.3) for: Diagnosis of an aortic dissection by detecting a specific biomarker in a blood sample. K.C. K., H. L., M. D., R.L. and M.K. are joint partners of the patent.

Ethics approval

Procedures and sampling were approved by the local ethics committee of the medical faculty at the Technical University of Munich (Project nos. 5943/13 and 223/18S).

Consent to participate and for publication

Human tissue biopsies and blood samples were obtained from the cardiovascular biobank at the German Heart Center Munich (KaBi-DHM). All samples in the KaBi-DHM were obtained with informed consent signed by all participants or probands prior to the inclusion in the study. All study procedures conformed to the ethical standards of the Declaration of Helsinki.

Availability of data and material

All data are presented in the text and the Supplemental Material.

Code availability

Not applicable

Authors' contributions

M.K. and R.L. designed the study and provided supervision. K.C.K., H.L., M.D., N.B., S.E. and K.K. performed experiments and analyses. S.D., S.D. and A.K. provided biospecimen and performed analyses. K.C.K. and H.L. drafted the manuscript. M.K. provided funding. All authors critically revised and finally approved the manuscript.

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Table 1 Baseline characteristics of patients with ATAAD

Characteristics (N=33)	No. (%)
age, mean (SD), y	65 ± 12.1
sex	
male	15 (45.5)
female	18 (54.5)
Stanford Classification	
type A	33 (100)
type B	0
DeBakey classification	
type I	16 (48.5)
type II	12 (36.4)
type III	0
unknown	5 (15.1)
prior thoracic aortic aneurysm	20 (60.4)
mean aortic aneurysm size	40.8 mm ± 23.5
left ventricular ejection fraction	
normal	31 (94)
slightly reduced	2 (6)
moderately reduced	0
severely reduced	0
coronary artery disease	6 (18)
peripheral arterial disease	1 (3)
carotid artery diseae	1 (3)
aortic valve disease	16 (48.5)
aortic stenosis	0
aortic regurgitation	16 (48.5)
bicuspid aortic valve	1 (3)
cardiovascular risk factors	
arterial hypertension	27 (82)
hyperlipidemia	10 (30)
diabetes mellitus	5 (15)
obesity	5 (15)
nikotine abuse	12 (36)
family disposition	5 (15)
COPD	1 (3)
chronic kidney disease	1 (3)
prior stroke	3 (9)
prior myocardial infarction	1 (3)
underlying syndrome	
Marfan	2 (6)
Ehlers Danlos	0
Loeys-Dietz	0
Turner	0
prior cardiac surgery	1 (3)
iatrogenic aortic dissection	1 (3)

ATAAD: Acute type A aortic dissection, SD: standard deviation, N: number of patients, No.: numbers, %: percent of patients, COPD: chronic obstructive lung disease, y: years

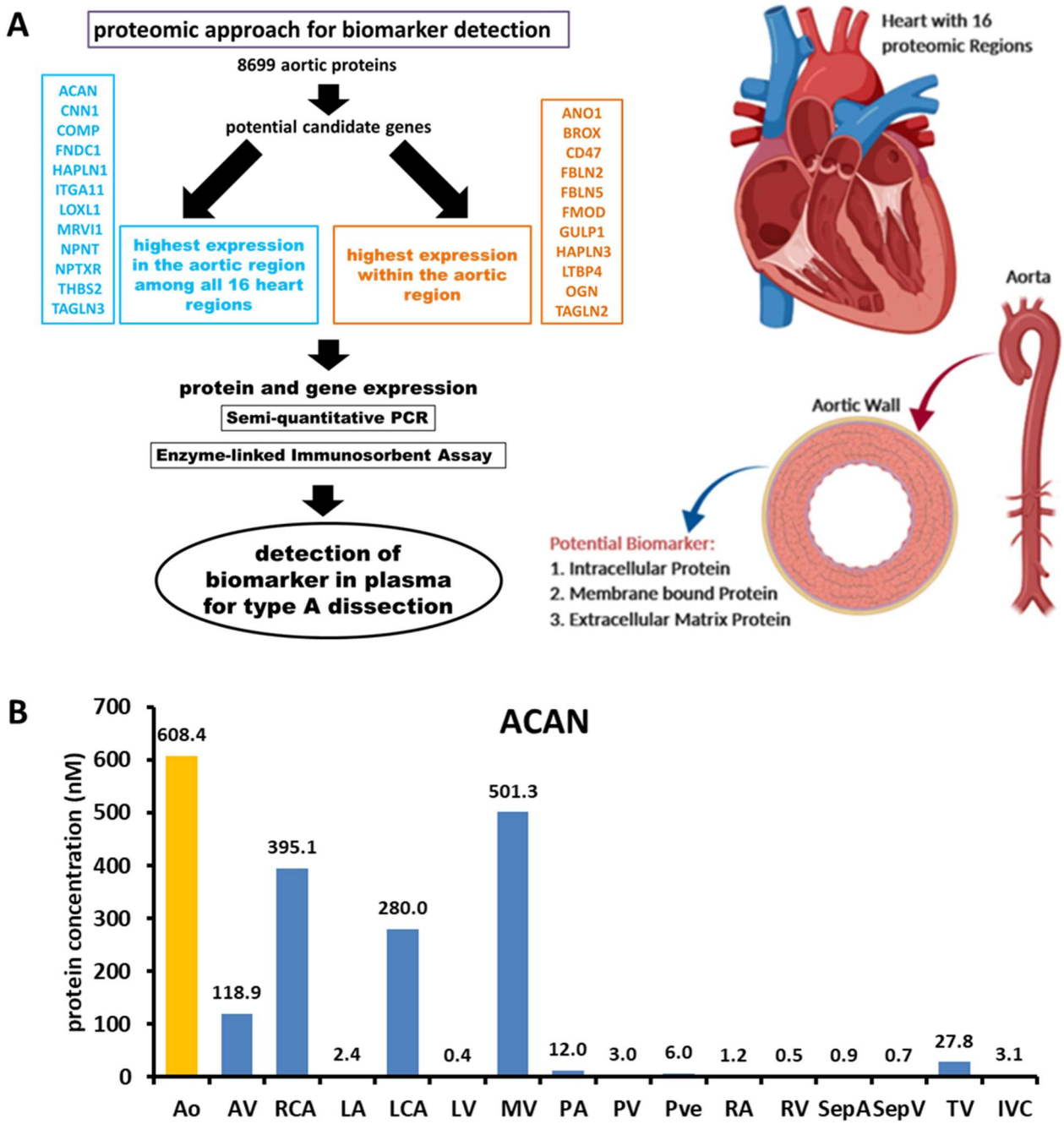
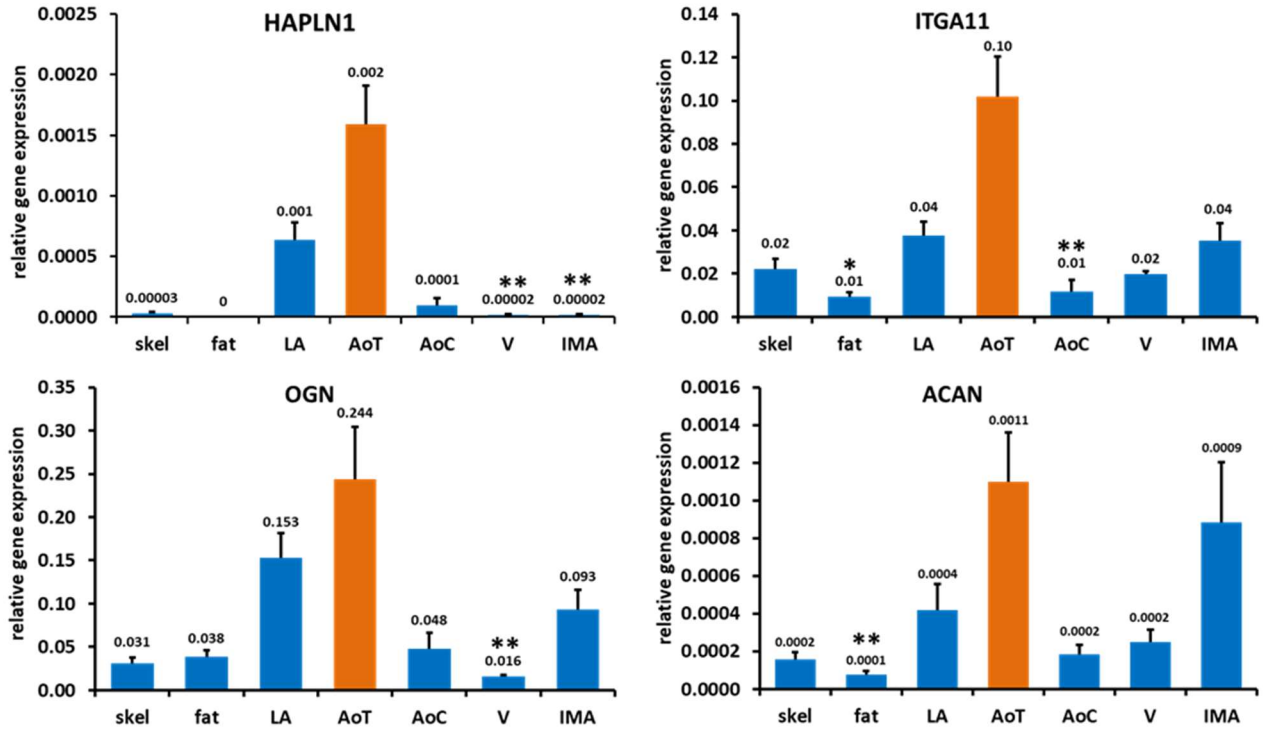


Figure 1. Workflow to identify candidate markers for acute type A aortic dissection patients and expression of selected candidate genes in human heart regions and surgical biopsies. (A) Strategy for selection and measurement of candidate biomarkers. **(B)** Protein expression of ACAN in different regions of the human heart. Ao: aorta, AV: aortic valve, RCA: right coronary artery, LA: left atrium, LCA: left coronary artery, LV: left ventricle, MV: mitral valve, PA: pulmonary artery, PV: pulmonary valve, Pve: pulmonary vein, RA: right atrium, RV: right ventricle, SepA: atrial septum, SepV: ventricular septum, TV: tricuspid valve, IVC: *inferior vena cava*.



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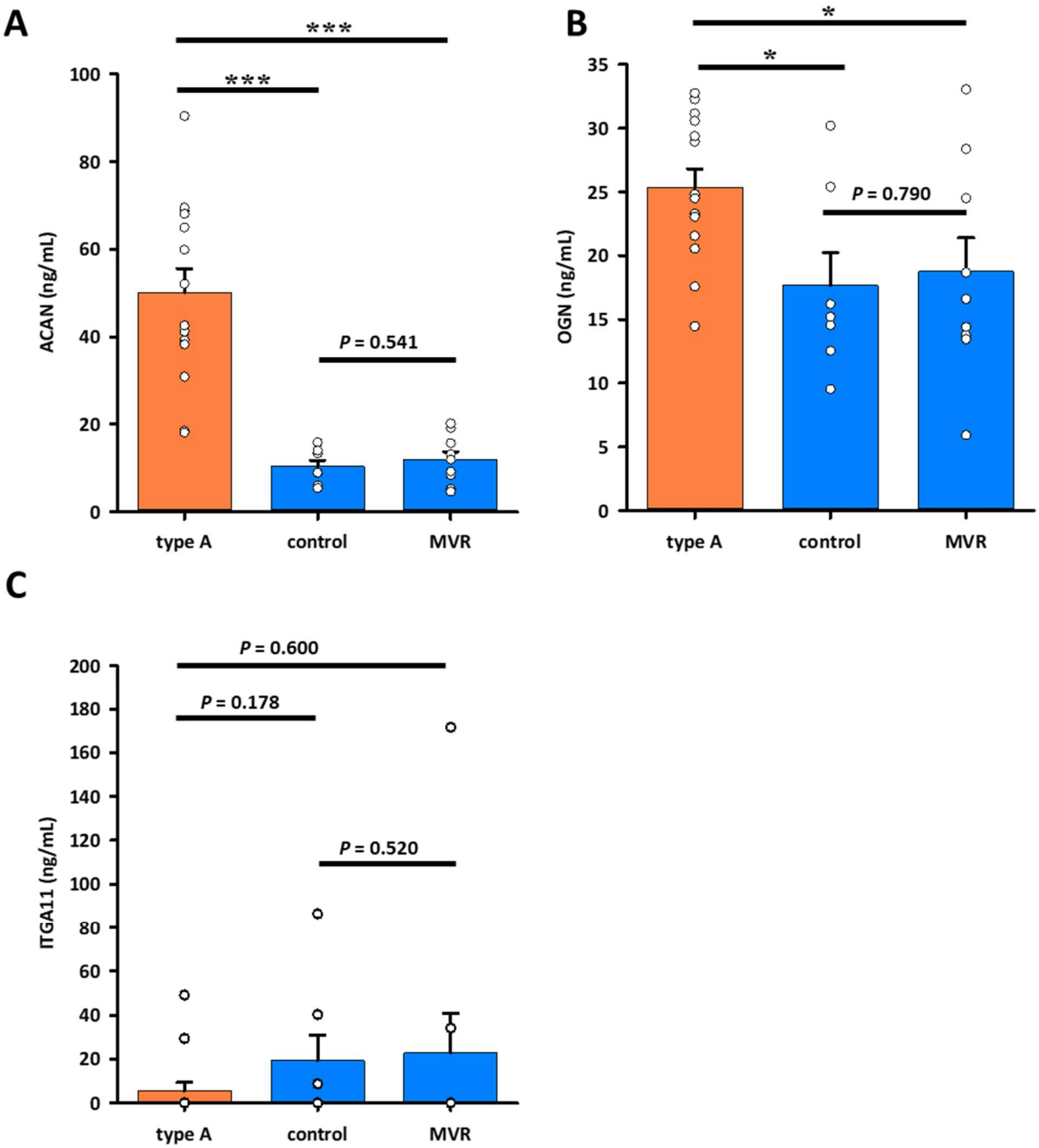

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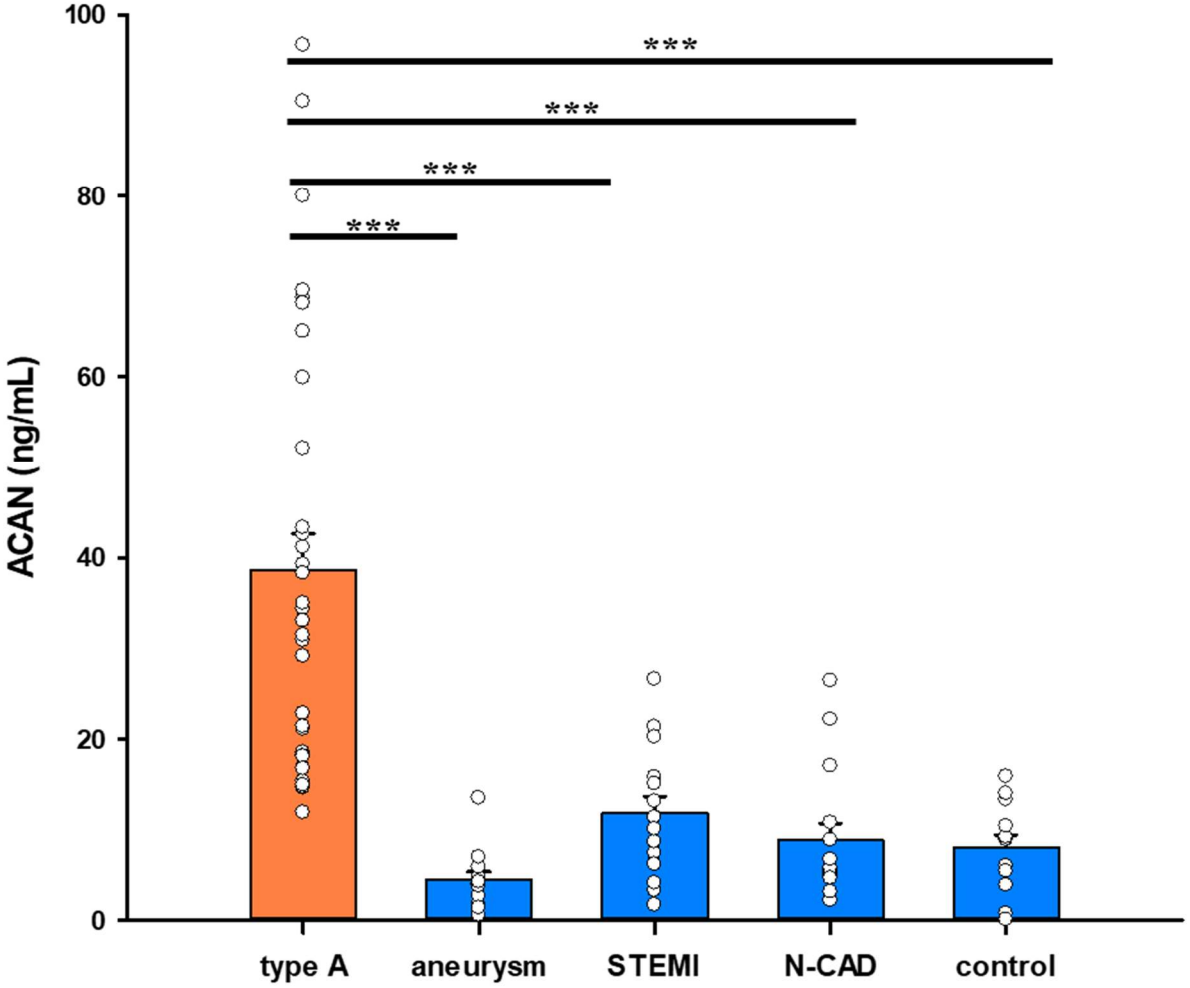

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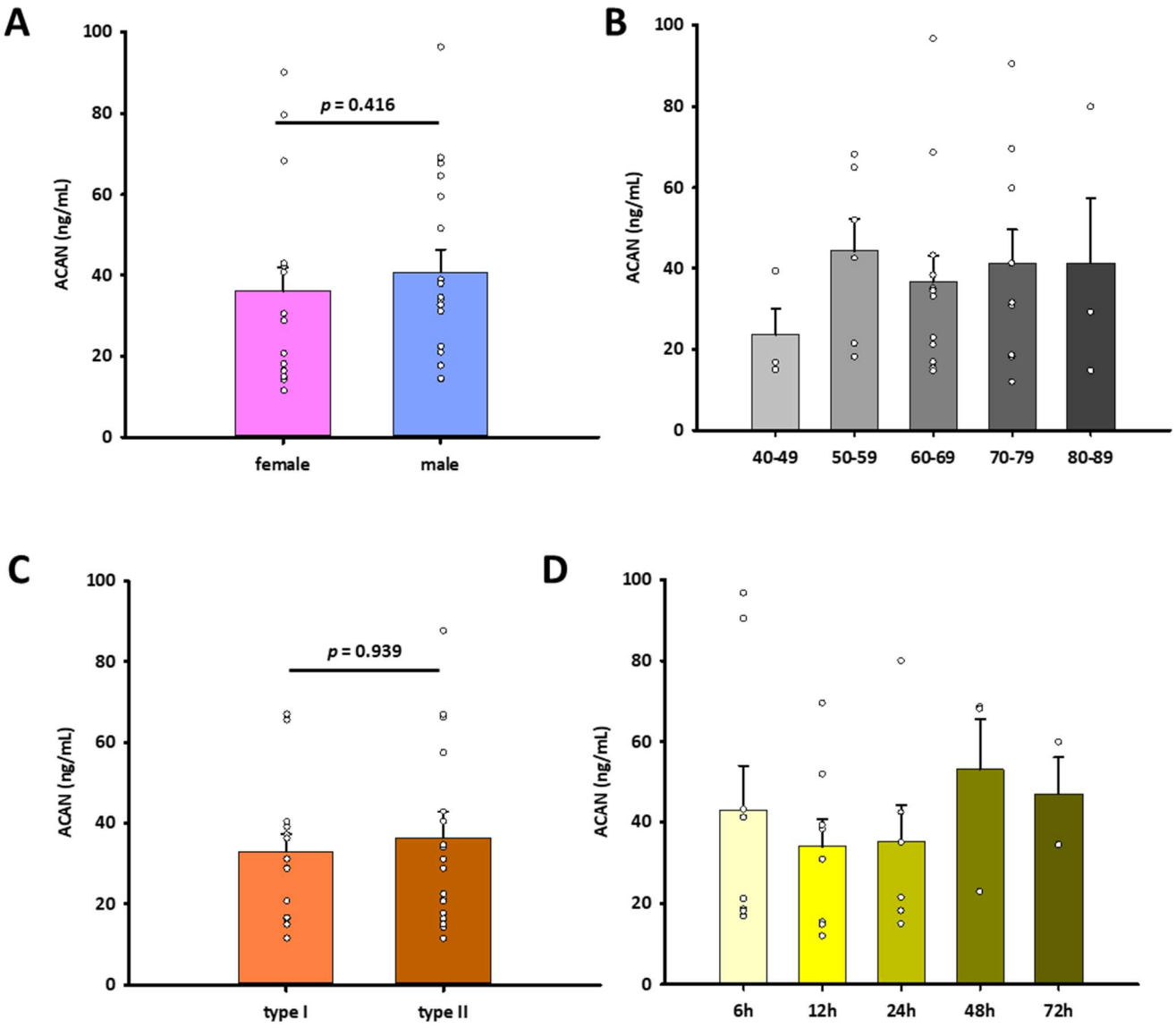

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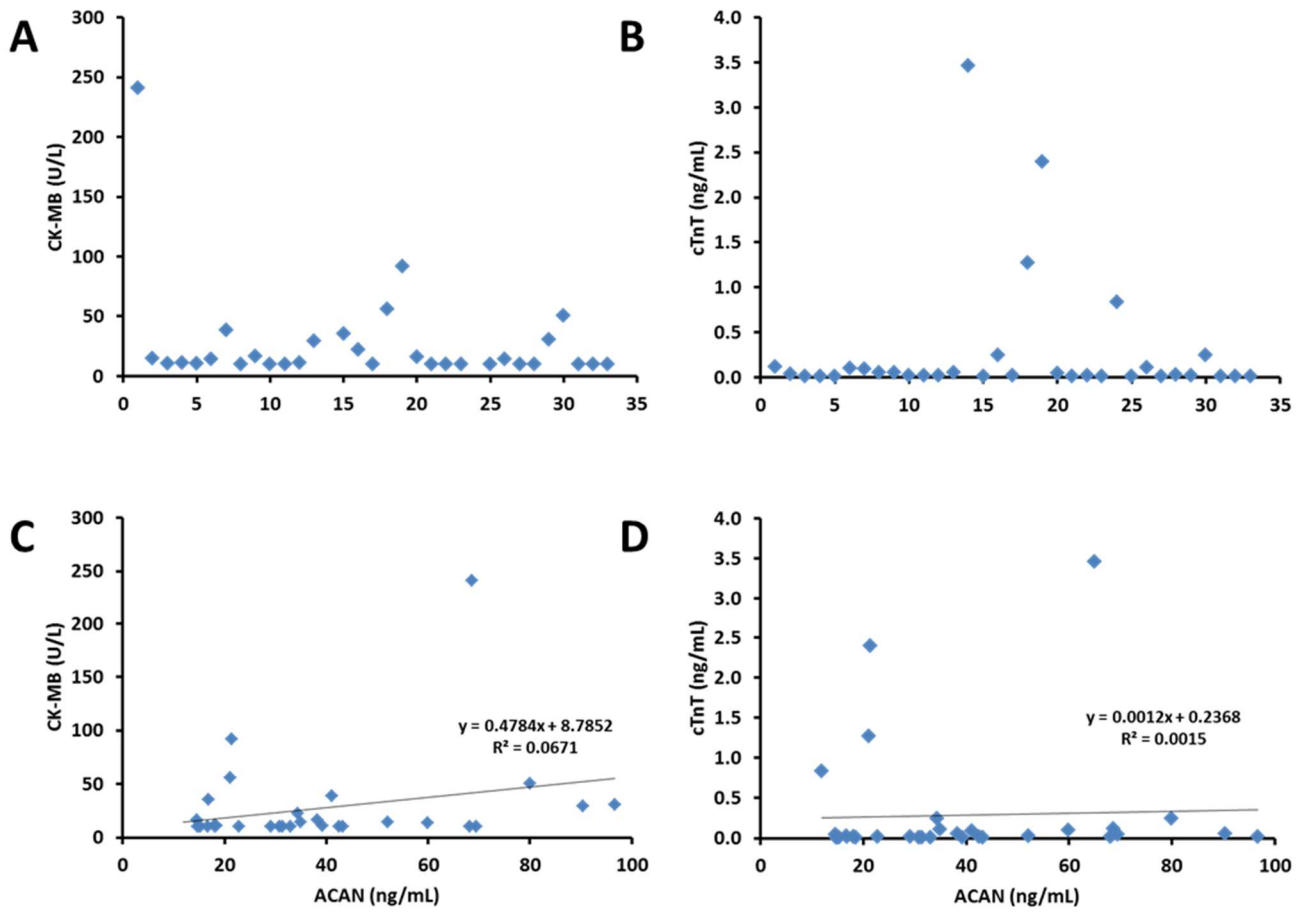

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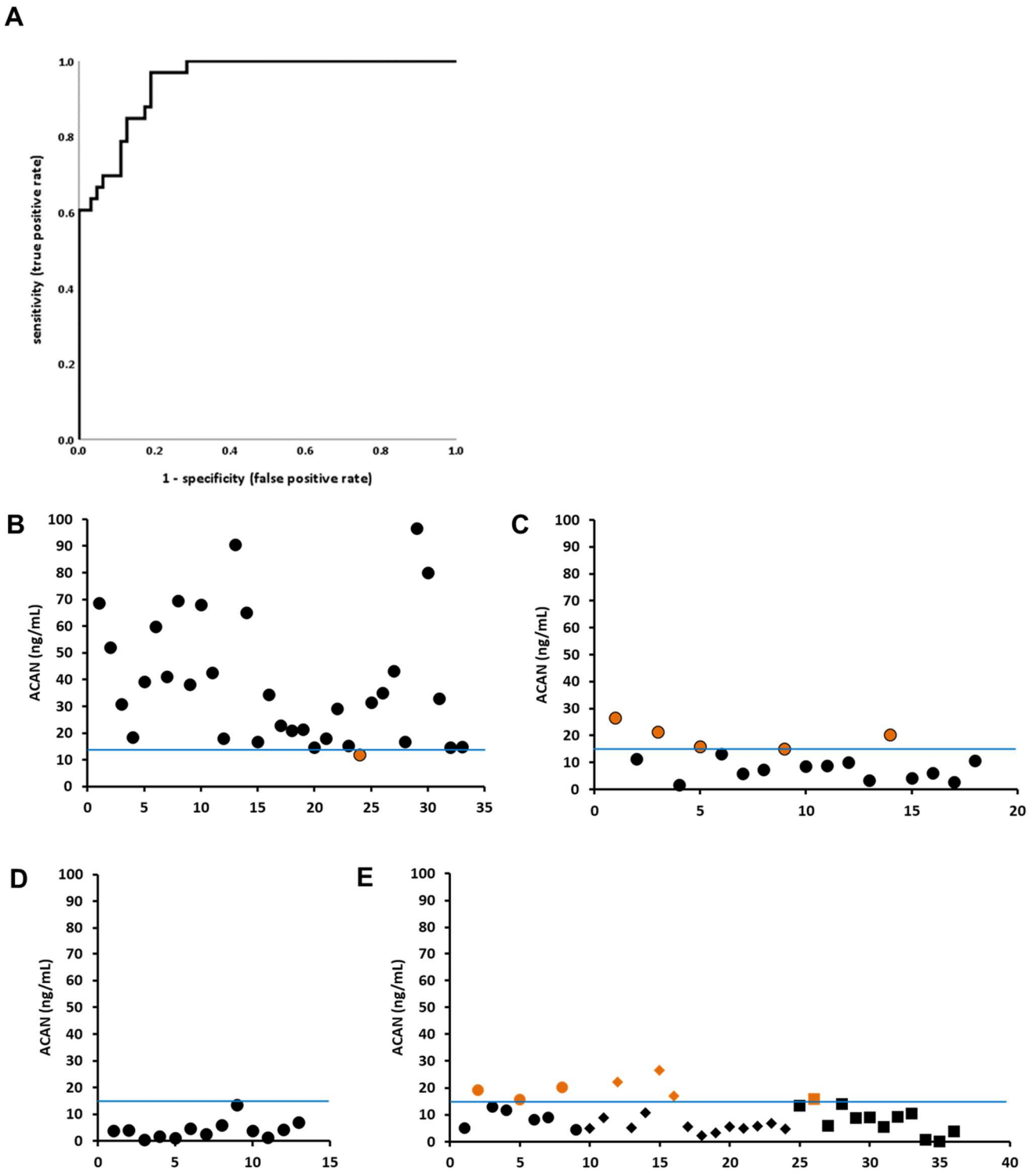

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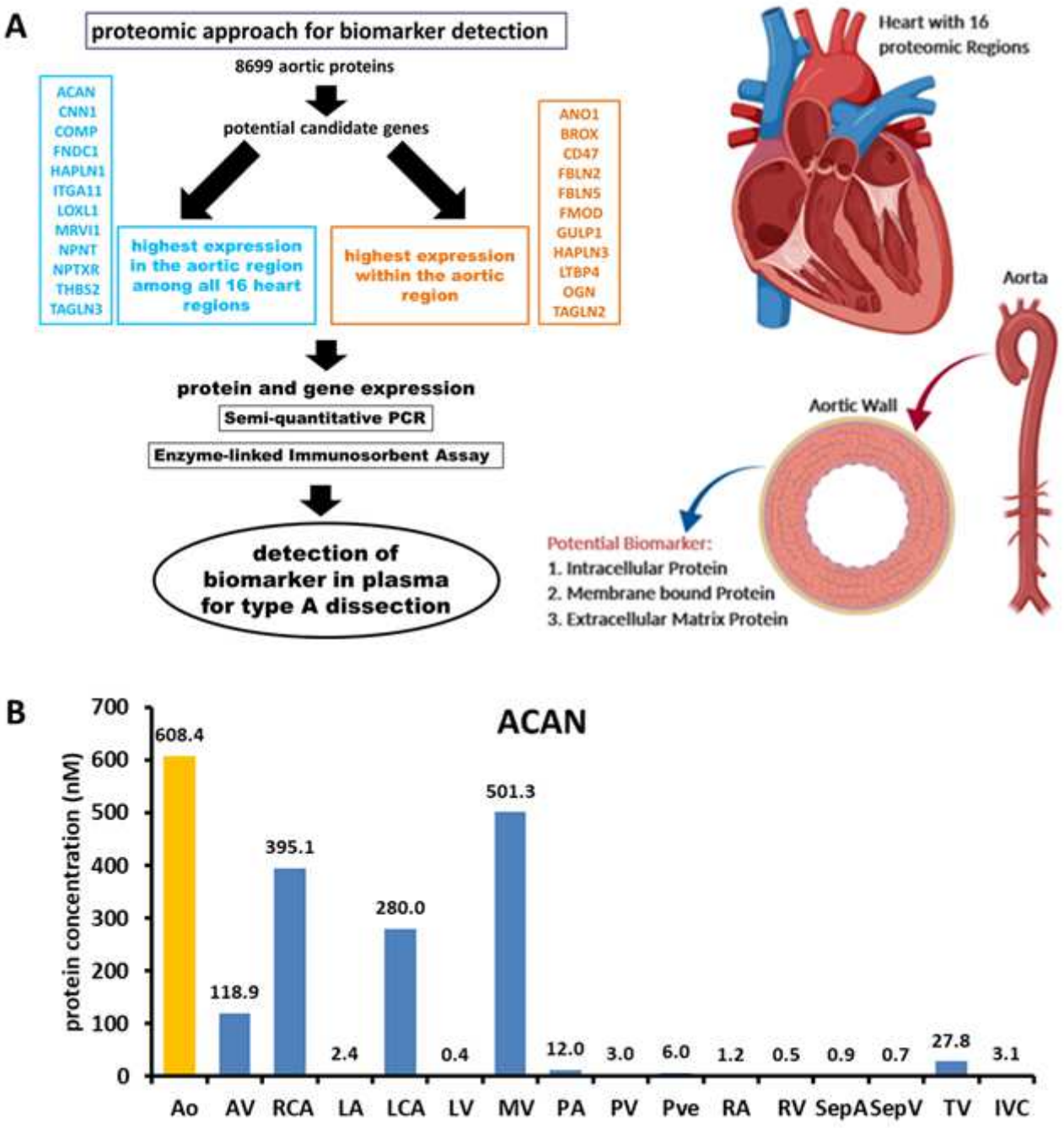
Figures



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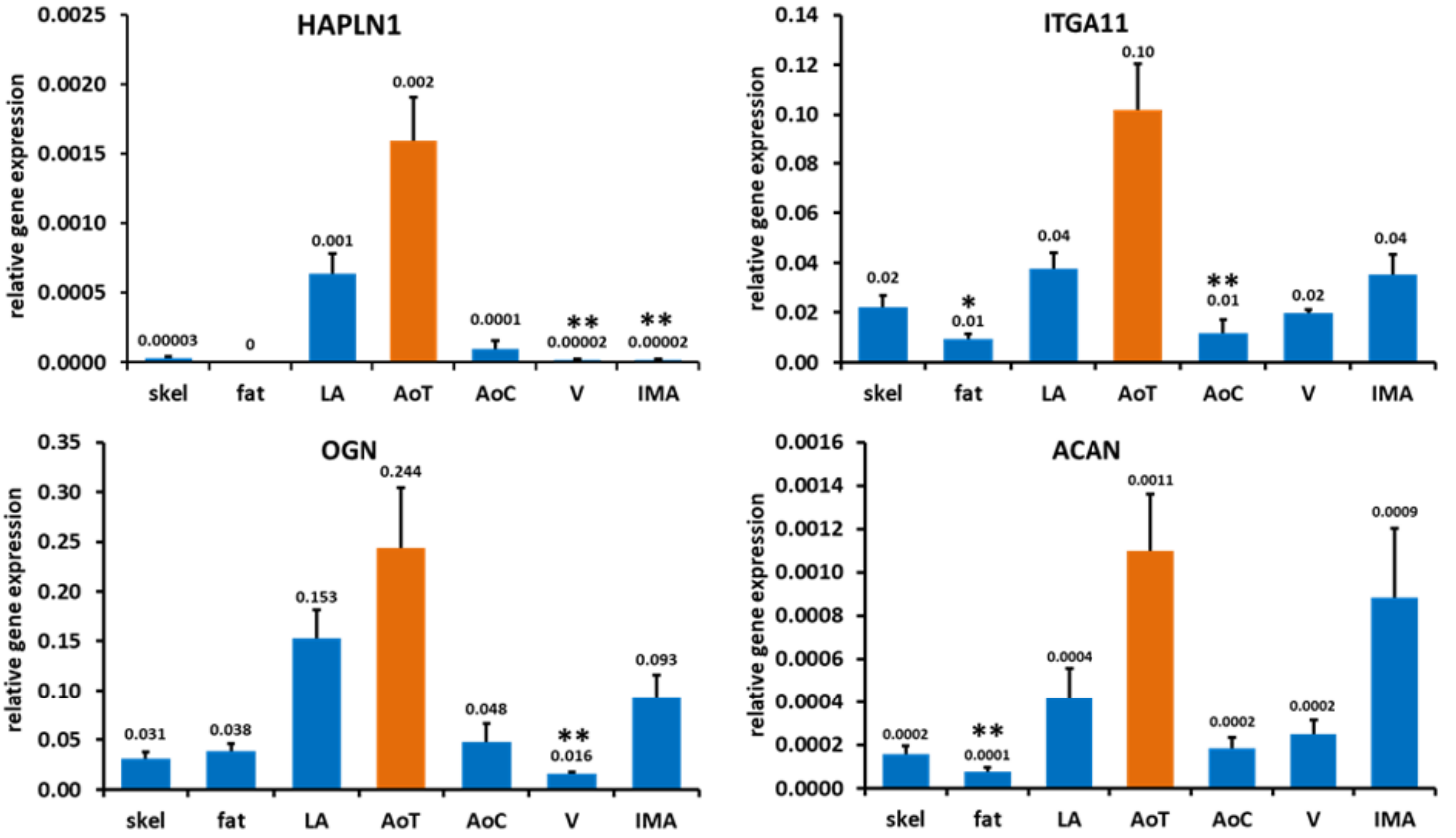

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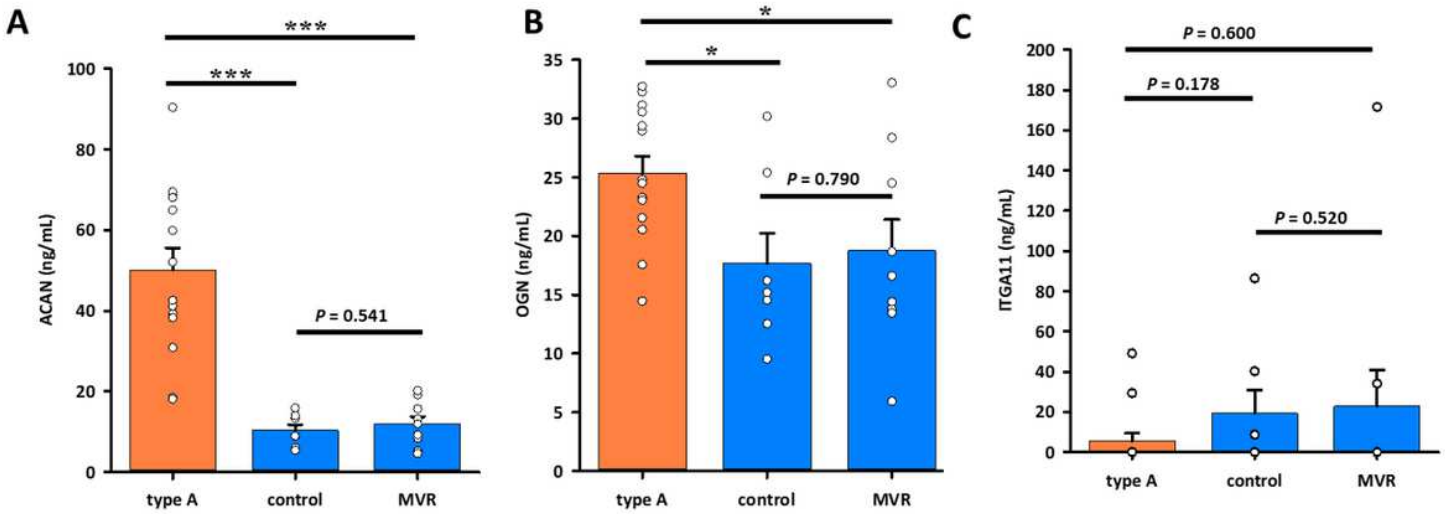

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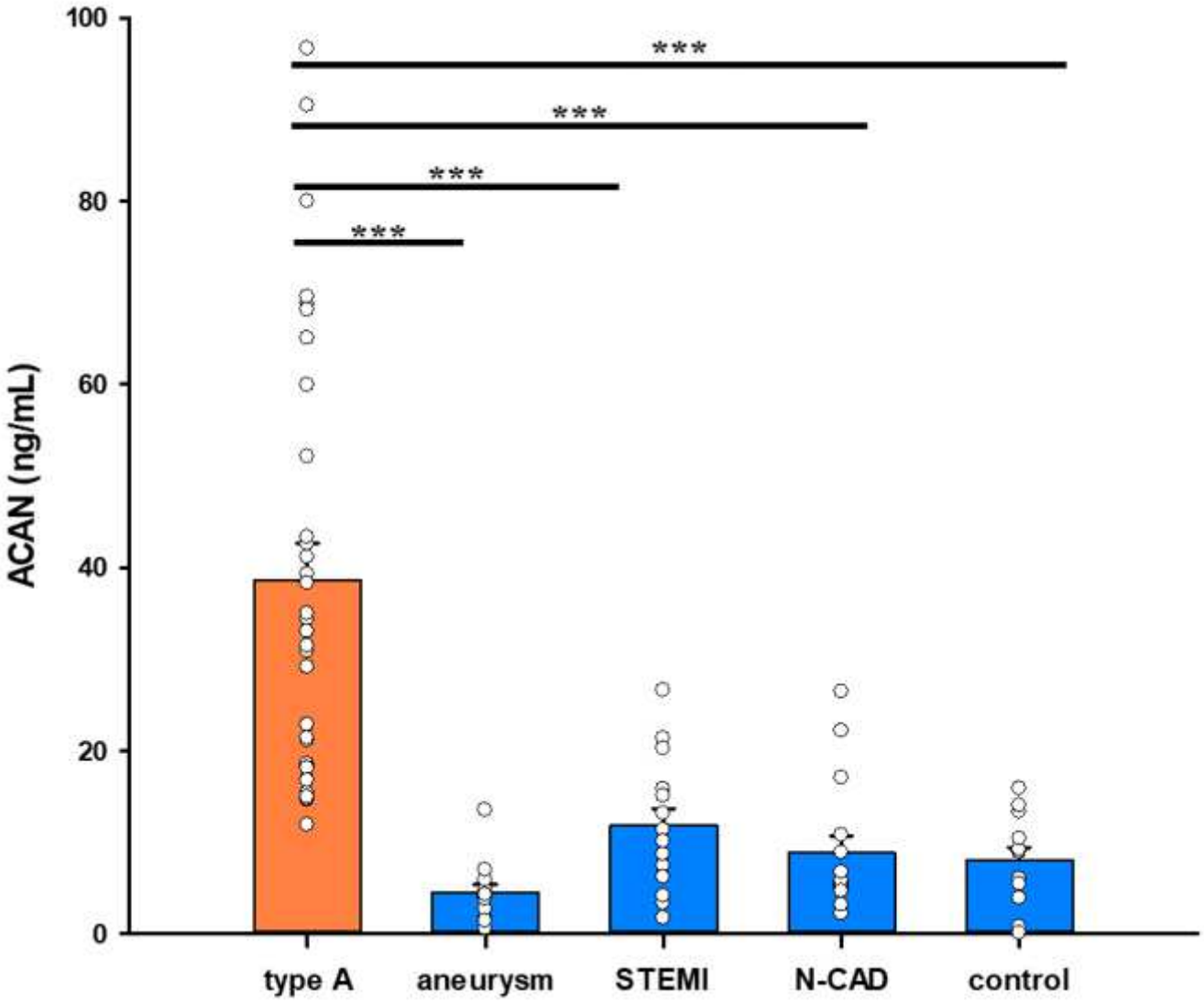

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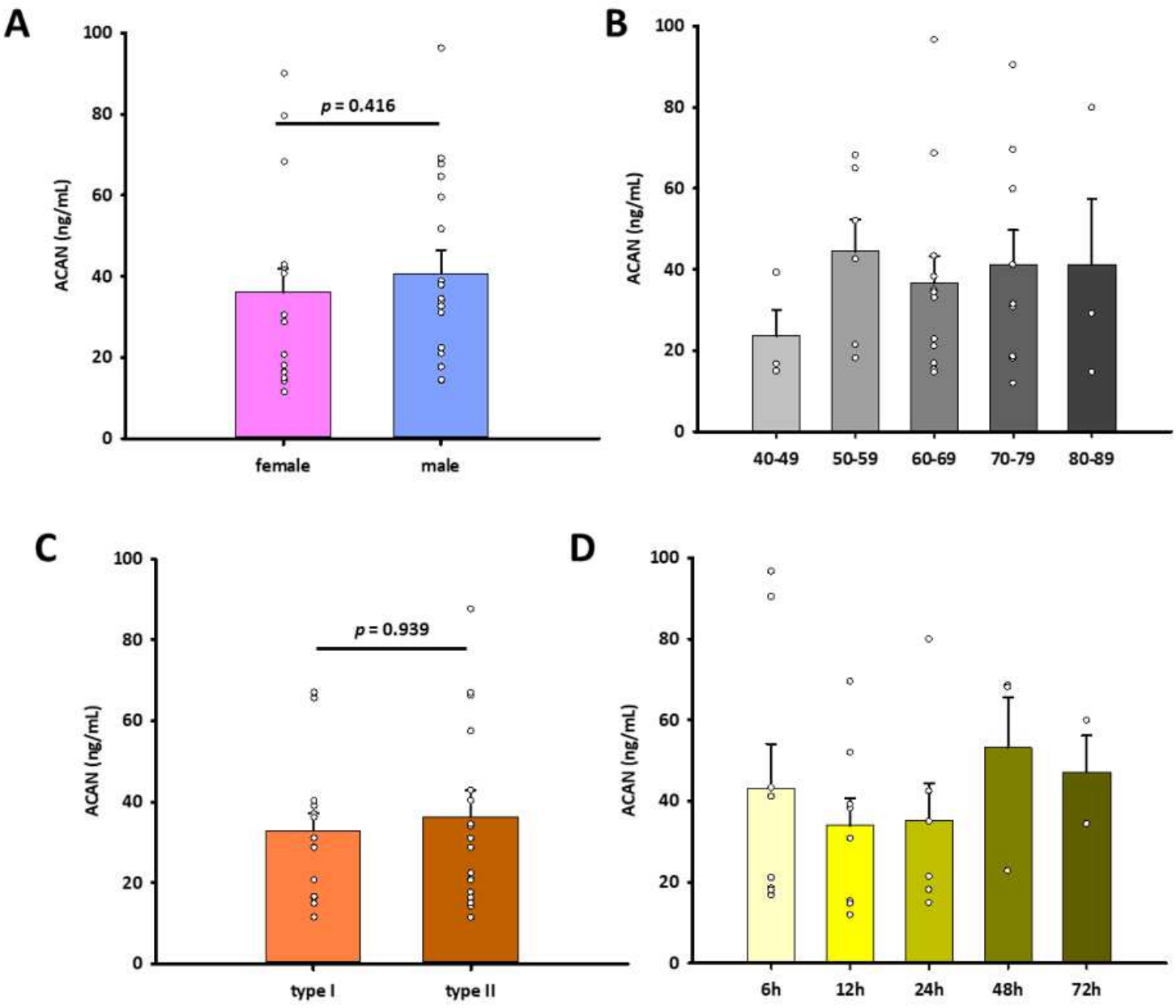

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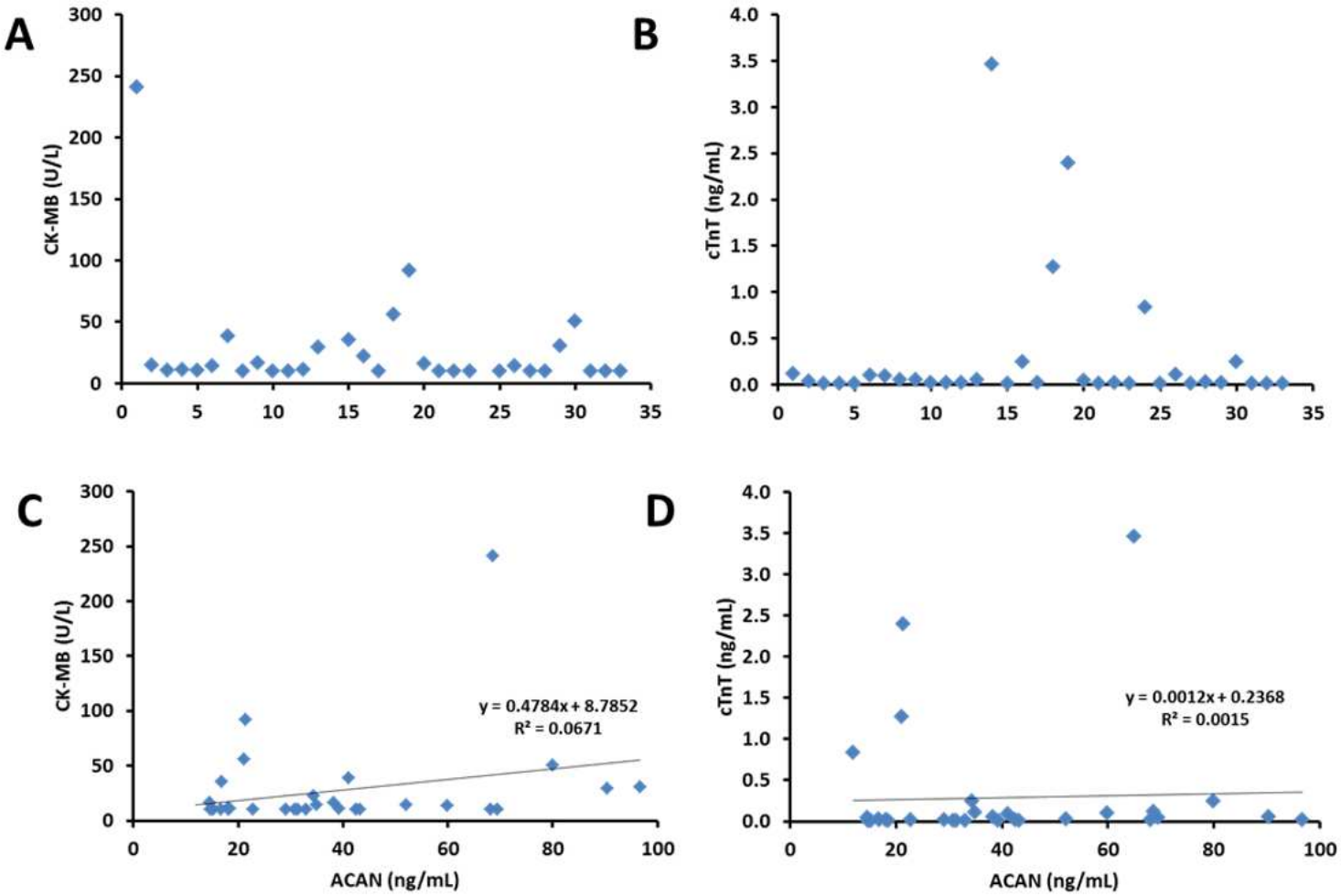

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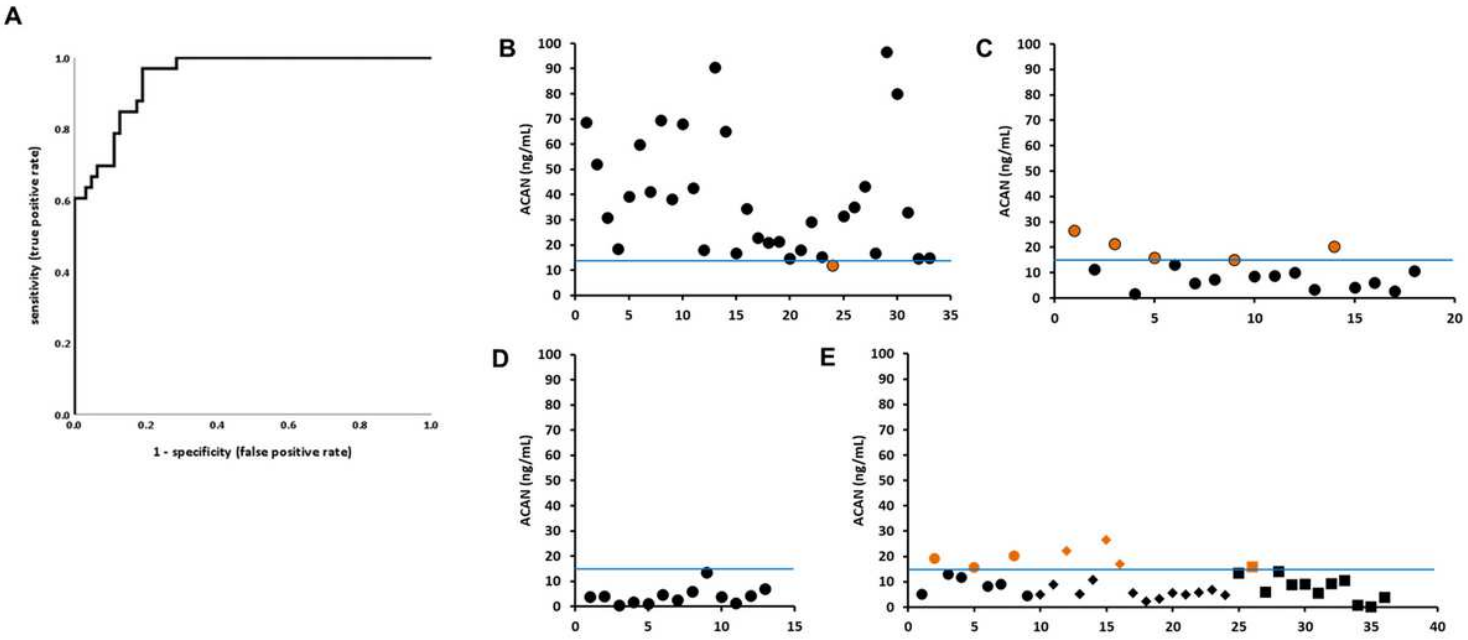

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Supplementary Files

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- Supplement.pdf