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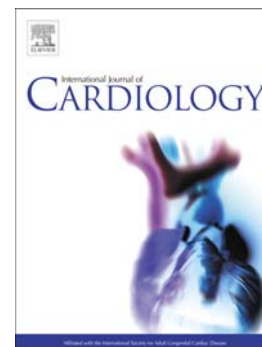
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**Fo ATP synthase C subunit serum levels in patients with ST-segment
Elevation Myocardial Infarction: preliminary findings.**

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CONFLICT OF INTEREST.

None.

STRUCTURED ABSTRACT.

Background: Recent studies in cell cultures hypothesized that the long-sought molecular pore of the mitochondrial permeability transition pore could be the Fo ATP synthase C subunit (Csub). We assessed Csub in patients with ST-segment elevation myocardial infarction (STEMI) and if it is associated with surrogate endpoints of myocardial reperfusion.

Methods: We enrolled 158 first-time acute anterior STEMI treated with successful percutaneous coronary intervention (PCI). Csub was measured, after the procedure, in serum by ELISA. Csub values were related to thrombolysis in myocardial infarction (TIMI) myocardial perfusion grade (TMPG), TIMI frame count (TFC), ST-segment resolution and cardiac marker release. Echocardiography and clinical outcome were recorded at 6-month.

Results: Csub was detectable in serum and it was not normally distributed (6.3% [4-9.3%]). Csub values were higher in patients with poor values of TMPG and TFC ($p=0.002$ and $p=0.001$, respectively). Csub values were higher in patients with absent or partial ST-segment resolution as compared to those with complete ST-segment resolution ($p<0.0001$ and $p=0.003$, respectively). After adjustment for potential confounding factors, Csub emerged as independent determinant of absent ST-segment resolution (HR 1.8, 95%CI 1.5-2.3, $p=0.007$), TMPG 0-1 (HR 1.7, 95%CI 1.3-2.5, $p=0.01$) and TFC above the median value (HR 1.5, 95%CI 1.3-2.1, $p=0.03$). Left ventricle ejection fraction, wall motion score index and cumulative incidence of death and heart failure were worse in patients with elevated Csub.

Conclusions: Our study is the first evidence that Csub is detectable in STEMI patients and that it is significantly related to several surrogate markers of myocardial reperfusion.

KEYWORDS

ST-segment elevation MI; reperfusion injury; mitochondrial permeability transition pore; F₁F₀ ATP synthase C subunit; infarct size; ST-segment resolution.

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INTRODUCTION

Timely reperfusion therapy by primary percutaneous coronary intervention (PCI) has significantly improved the outcome of patients with ST-segment elevation myocardial infarction (STEMI) [1]. However, despite reperfusion therapy, several patients develop large myocardial infarction (MI) and subsequent heart failure (HF) [1-3]. Paradoxically, the process of restoring blood flow to the ischemic myocardium can itself induce and/or accelerate injury [2-3]. This phenomenon, termed reperfusion injury (RI), may account for as much as half of the final infarct size (IS) [2-3]. Mechanistically, the mitochondria, and more specifically the mitochondrial permeability transition pore (mPTP), a nonselective channel of the inner mitochondrial membrane (IMM), have emerged as most critical subcellular signalling elements of RI [2-4]. Its opening within the first few minutes after reperfusion collapses the mitochondrial trans-membrane potential, with a complete arrest of adenosine triphosphate (ATP) synthesis and other mitochondrial activities [2-4]. The molecular identity of mPTP remains elusive [2-5]. Recent studies from our and other groups in cell cultures hypothesized that the long-sought molecular pore of the mPTP could be the Fo ATP synthase C subunit (Csub) [6-9]. Nevertheless, we did not have data regarding presence, function and pattern of Csub in humans and, especially, in the STEMI setting. Thus, the aim of this study was to investigate, for the first time, whether serum levels of Csub were detectable in STEMI patients and whether they were associated with surrogate markers of myocardial reperfusion.

METHODS

Study design

This is a single-centre, investigator-driven, prospective study. The trial was performed according to the Declaration of Helsinki and approved by the local ethics committee (Comitato Etico Unico della Provincia di Ferrara). All subjects gave written informed consent before inclusion. All patients were enrolled from December 2013 to January 2015 in the Cardiology Unit of the Azienda Ospedaliera Universitaria di Ferrara.

Study population

Patients eligible for enrolment were individuals with a first-time acute anterior STEMI following successful PCI and: i) time from onset of symptoms to balloon <6 hours; ii) culprit lesion in the proximal or mid portion of left anterior descending (LAD); iii) baseline thrombolysis in myocardial infarction (TIMI) flow 0-1. Major exclusion criteria included prior myocardial infarction and/or percutaneous or surgical coronary revascularization and/or prior angina and/or evidence of ischemic heart disease, previous HF, cardiac arrest and/or cardiogenic shock, atrial fibrillation, pacemaker, concurrent inflammatory, infectious or malignant disease, liver and/or renal failure, recent significant bleeding and/or major surgery (< 4 weeks), use of oral anticoagulants or contraceptive. PCI procedure, antithrombotic drugs and all other medications were performed according to standard clinical practice, institutional protocols and current guidelines [1,10-11].

Blood Sample Collection

Blood withdrawal was performed 6-18 hours after the end of successful PCI (median 10 [8-13] hours). Blood samples were collected from an antecubital vein using a 21-gauge needle.

The first 2 to 4 mL of blood were discarded. The remaining blood was collected in empty tubes and, after 45 minutes, centrifuged at 1,700g at 4°C for 15 min. The serum obtained was stored at -20°C.

Determination of Fo ATP synthase C subunit

Csub was measured in serum from patients with ELISA, using the human ATP synthase lipid-binding protein, mitochondrial (ATP5G1) ELISA kit (Cusabio Biotech, China). The ELISA was performed according to the manufacturer's instructions using strips of microtiter wells with a capture antibody, specific for Csub coated onto the wells of microplates. Because albumin and globulin are the most abundant proteins in human serum, which masked the expression of low molecular weight (LMW) proteins, we selectively removed them to enrich for proteins of lower abundance (among these, Csub at 8 kDa). So, serum samples were first handled to obtain a concentrated solution of LMW proteins by using Amicon Ultra-4 10K Centrifugal Filter Devices at 3000g for 40 min (+ 4°C) and then diluted 1:5 in phosphate-buffered saline (PBS) as kindly suggested by the supplier. In the details, ELISA assay was performed as follow. One hundred µl samples were placed in the wells. After 2 h of incubation at 37°C, the liquid was removed from each well. The strips were again incubated for 1 h at 37°C after adding 100 µl biotin-antibody 1X to each well. The wells were then washed three times with wash buffer and after the last one 100 µl HRP-avidin 1X was added to each well and incubated for 1 h at 37°C. The wells were washed five times as in the previous step and 90 µl TMB substrate was added. If the Csub protein was present, a blue color developed between 15 to 30 min. The blue color changed to yellow with the addition of 50 µl phosphoric acid, which was used to stop the reaction. The result could be read with the aid of a spectrophotometer and compared with the negative control and the calibration curve supplied with the test kit. A spectrophotometer (450 nm) was used to obtain protein

concentration duplicate readings. A computer software (MARS) capable of generating a four parameter logistic (4-PL) curve-fit was used to obtain concentrations as pg/ml protein. To standardized Csub values, pg/ml concentrations were converted into percentages on the basis of the highest value that the calibration curve is able to read. Intra- and inter-assay CV% were <8% and <10%, respectively. In the appendix online we showed a schematic representation of the assay (supplemental Figure 1).

Endpoints and clinical follow-up

According previous studies [12-14], we selected as surrogate markers of myocardial reperfusion the following endpoints: corrected TIMI frame count (TFC), TIMI myocardial perfusion grade (TMPG), cumulative ST-segment resolution (supplemental Figure 1), release of markers of myocardial necrosis. These endpoints were related to Csub values. TFC and TMPG were measured as previously described by an independent reviewer (SB) blinded to Csub values [13]. ST-segment resolution was expressed as percentage and calculated by an independent reviewer (RP) blinded to Csub values [12]. It was assessed immediately at the end of primary PCI and categorized as complete ($\geq 70\%$), partial (<70% to 30%), and absent (<30%) [12]. The markers of myocardial necrosis (troponin T and CK-MB) were reported as peak and as area under the curve (AUC) (expressed in arbitrary units) [14]. Plasma samples for CK-MB and troponin T were collected on admission and subsequently during the hospitalization every 6-8 hours for 2 days [14]. Cardiac markers were measured in the laboratory of our hospital (high-sensitivity troponin T: upper reference limit 0.014 ng/ml; CK-MB: upper reference limit 5 ng/ml; Cobas, Roche Diagnostics, Mannheim, Germany). Before hospital discharge (median 5 [4-6] days) and after 6 months [median 190 [170-200] days] all patients received transthoracic echocardiography. An independent reviewer (SB) blinded to Csub values analyzed images to measure left ventricle ejection fraction (LVEF) and wall

motion score index (WMSI). The 6-months occurrence of principal ischemic adverse events (death, hospital admission for recurrence of acute coronary syndrome [ACS] and/or HF) was recorded.

Statistical Analysis

Being our study explorative and hypothesis-generating and considering the lack of prior data of Csub values in humans and STEMI patients, a formal sample size was not possible. In line with recommendations for pilot studies, an arbitrary sample size of 150 patients was chosen [19]. Categorical variables are reported as counts and percentages. Differences between percentages were assessed by chi-square or Fisher exact tests. Continuous data were tested for normal distribution with the Kolmogorov-Smirnov test. Normally distributed values were presented as mean \pm SD; otherwise median value [interquartile range] was used. Student t tests and analysis of variance were used for normally distributed continuous variables. Appropriate nonparametric tests (Mann-Whitney, Kruskal-Wallis, and Spearman rank correlation tests) were used for all other variables. To better define the relationship between Csub and endpoints, we described it as continuous marker and as above vs. below the median value. The effects between all variables listed in Table 1, Csub and endpoints were assessed by logistic regression models. Variables with a probability value <0.1 at univariate analysis (symptom-balloon time, first medical contact-balloon time, Csub serum level, age) and several potential confounding factors (sex, glycoprotein IIb/IIIa administration, manual thrombectomy, proximal LAD) were then entered into a multivariable analysis to identify the independent predictors for endpoints. All tests were 2-sided and the statistical significance was defined as $p<0.05$. All analyses were performed with *STATISTICA 8* (Statsoft Inc, Tulsa, Okla, USA).

RESULTS

From December 2013 to January 2015, we enrolled 158 patients respecting all criteria out of the 447 (35%) admitted to our center for STEMI (Table 1). Csub was detectable in serum and it was not normally distributed (median 6.3% [4-9.3%]; range 1.1-47.5%). Out of all variables reported in Table 1, there was only a weak inverse correlation between age and Csub serum levels ($R=-0.16$, $p=0.04$). Baseline characteristics did not differ between groups after stratification of study population according Csub median value (Table 1).

Csub values and angiographic markers of myocardial reperfusion

TMPG and TFC were not measurable in 3 (2%) and 13 (8%) patients, respectively. TMPG was 2-3 in 70 (44%) patients. TFC median value was 55 [40-80] frames. Csub values were higher in patients with poor values of TMPG and TFC (Figure 1). At multivariable analysis, Csub emerged as independent determinant of TMPG 0-1 (HR 1.7, 95%CI 1.3-2.5, $p=0.01$) and TFC above the median value (HR 1.5, 95%CI 1.3-2.1, $p=0.03$).

Csub values and ST-segment resolution

ST-segment resolution was classified as absent, partial, and complete in 47 (30%), 54 (34%) and 57 (36%) patients, respectively. Csub values significantly differed between patients stratified according ST-segment resolution ($p<0.0001$, Figure 2) (supplemental Figure 1). They were higher in patients with absent and partial ST-segment resolution as compared to those with complete ST-segment resolution ($p<0.0001$ and $p=0.003$, respectively) (Figure 2). At multivariable analysis, Csub emerged as independent determinant of absent ST-segment resolution (HR 1.8, 95%CI 1.5-2.3, $p=0.007$).

Csub and release of markers of myocardial necrosis

Median values of cardiac markers expressed both at peak and as AUC were shown in Table 1. Csub values were significantly higher in patients with AUC of cardiac markers release above the median value as compared to others (Figure 3). After multivariable analysis, Csub emerged as independent determinant of AUC above the median value both for TnT (HR 1.53, 95%CI 1.26-1.86 as single change unit, $p=0.0005$) and CK-MB (HR 1.65, 95%CI 1.35-2 as single change unit, $p<0.0001$). To further confirm the relationship between Csub values and cardiac markers release, we performed other analyses. After stratification of the study population according Csub median value, we observed that TnT and CKMB AUCs were significantly larger in those with Csub above the median value (Table 1). Supplemental Figure 2 in the appendix online showed TnT release according this stratification.

Csub values, echocardiographic data and clinical outcome

LVEF and WMSI values in overall study population were reported in Table 1. Patients with LVEF under the median value and WMSI above the median values showed higher Csub values (7% [4.5-9.7%] vs. 4.9% [3.8-8%], $p=0.03$ and 7% [4.2-9.5%] vs. 5% [3.6-8.2%], $p=0.04$, respectively). At 6 months, we observed 4 (2.5%) deaths, 4 (2.5%) hospital readmission for ACS and 6 (3.8%) for HF. Death and HF occurred in 8 patients (5%). Patients developing adverse events showed significantly higher Csub values (18% [15-21%] vs. 5.5% [4-8.5%], $p<0.001$).

DISCUSSION

Despite a large number of cardioprotective strategies has been proposed to reduce IS and improve myocardial reperfusion in experimental animals, their translation and application into the clinical setting has been largely disappointing [2-5,16]. Recently, two randomized clinical trials targeting proteins involved in mPTP formation, the first with TRO40303 and the second with cyclosporine A, failed [17-18]. The reasons for this failure are multiple and not the aim of our study. Nevertheless, these and previous trials strongly suggested that newer studies are still needed to clarify and identify all actors and processes involved in the reperfusion injury and in cardioprotection [19-24]. Only a complete knowledge regarding molecular connections affecting myocardial reperfusion and myocardial necrosis will allow the development of effective drugs [4-9, 19-24]. Regarding mPTP structure and generation, it has been suggested that mitochondrial ATP synthase arranges in a super-complex with ANT and PiC to form an ATP synthasome [4-9]. Under favorable circumstances, such as those occurring during myocardial reperfusion, the Csub ring is rearranged and becomes the pore of the mPTP [4-9]. Accordingly, Csub depletion considerably reduced mPTP opening by Ca²⁺ and reactive oxygen species (ROS), whereas Csub over-expression enhanced mPTP opening [6]. Consequently, we may infer that patients with higher Csub values, developing a heightened mPTP activity and mPTP opening at the moment of reperfusion, are those at higher risk of reperfusion injury. This should be translated in poor myocardial reperfusion and larger IS. At present, available data regarding Csub come only from cell cultures. No information about Csub from humans serum and/or patients with STEMI are available. Our study is the first trying to fill this gap and describing Csub in serum from patients admitted to hospital for STEMI and treated with successful primary PCI. Csub was present in serum early after STEMI and we observed a significant inter-patient variability. Consistent with a previous

study in isolated perfused hearts from mice, Csub in humans correlates only with age [25]. To establish a possible link between Csub values and myocardial reperfusion process, we tried to associate this Csub inter-patient variability with validated surrogate markers of myocardial reperfusion. To minimize potential confounding factors, we applied restrictive inclusion and exclusion criteria selecting a highly homogenous study population. We obtained a relatively young study population, at its first event, with medium-large anterior MI receiving a successfully primary PCI in short symptoms-to-balloon and door-to-balloon times. With this background, we found that circulating Csub is an independent predictor of all surrogate endpoints of myocardial reperfusion. Elevated Csub levels were significantly related to poor values of TMPG, TFC and ST-segment resolution. Especially, ST-segment resolution is a well-established marker of reperfusion, it is frequently used as study endpoint and it is significantly related to short- and long-term mortality [26-27]. We found that Csub values are significantly and independently higher in patients with absent and/or partial ST-segment resolution. Consistently, these patients developed a poor 6-month echocardiographic pattern, with lower LVEF and higher WMSI. Finally, we are well aware that our study is not powered for prognostic implication. Nevertheless, we observed a significant relationship with the occurrence of death and HF. Although preliminary, our results are consistent with the rationale of the study and confirm experimental data suggesting that Csub is involved in the myocardial reperfusion process. Our findings support the hypothesis that elevated Csub levels, probably influencing mPTP opening, are determinants of worse myocardial reperfusion, larger IS and poor outcome. Obviously, this study should be considered a proof-of-concept and future investigations are clearly on demand to confirm and extend them.

Study limitations

This is the first attempt to validate in humans the possible relationship between Csub and myocardial reperfusion. It follows that there are several limitations. First, the role of Csub in the mPTP has been previously investigated mainly in cell culture. Further validation in preclinical setting is clearly on demand. Second, we assessed Csub at single time-point. Third, we are unable to explain where the measured Csub was generated. Nevertheless, the differences in the serum values and the finding that Csub levels predict myocardial reperfusion suggest a possible link between acute event (myocardial infarction and reperfusion) in cardiomyocytes and peripheral blood assessment. Finally, we are aware that cardiac magnetic resonance (CMR) is the gold standard to assess IS and reperfusion injury [12]. On the other hand, CMR is expensive, time-consuming and its application on large number of patients may be difficult.

Conclusions

FO ATP synthase C subunit is detectable in serum from STEMI patients. Elevated FO ATP synthase C subunit serum levels in the early phase of MI correlate to several myocardial reperfusion markers (including TMPG and ST-segment resolution) and outcome in terms of death and HF.

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None.

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FIGURE LEGEND.

Figure 1. C subunit values in patients stratified according to TMPG and TFC.

The horizontal line shows the median value. The box shows the interquartile range. The vertical line shows the 10-90th percentile. TMPG: TIMI myocardial perfusion grade. TIMI: thrombolysis in myocardial infarction. TFC: TIMI frame count.

Figure 2. C subunit values in patients stratified according to ST-segment resolution.

The horizontal line shows the median value. The box shows the interquartile range. The vertical line shows the 10-90th percentile.

Figure 3. C subunit values in patients stratified according to median value of TnT and CKMB AUC median value.

The horizontal line shows the median value. The box shows the interquartile range. The vertical line shows the 10-90th percentile. TnT: troponin T. AUC: area under the curve.

Table 1. Main characteristics of study population.

Variables	All (n=158)	C subunit below median value (n=79)	C subunit above median value (n=79)	p
Age, (years)	63±11	65±11	62±10	0.3
Men, no. (%)	119 (75)	61 (77)	58 (74)	0.3
BMI, (kg/m ²)	27±4	27±4	27.5±4	0.7
CrCl, (ml/min)	78±15	77±14	79±13	0.7
<i>Cardiovascular risk factors, no. (%)</i>				
Diabetes,	22 (14)	9 (11)	13 (16)	0.2
Hypertension,	112 (71)	54 (68)	58 (73)	0.5
Current Smoker,	86 (55)	41 (52)	45 (57)	0.6
Hyperlipidemia,	89 (56)	46 (58)	43 (54)	0.7
<i>Angiographic data,</i>				
Multivessel disease, no. (%)	82 (52)	44 (55)	38 (48)	0.1
Proximal LAD, no. (%)	81(51)	41 (52)	40 (50)	0.9
Manual thrombectomy, no. (%)	65 (41)	33 (42)	32 (40)	0.5
DES, no. (%)	129 (81)	64 (81)	65 (82)	0.9
Stent diameter, (mm)	3 [3-3.5]	3 [3-3.5]	3 [3-3.5]	0.9
Stent length, (mm)	24±8	24±8	25±8	0.8
Post-dilatation, no. (%)	34 (21)	18 (23)	16 (20)	0.5
Symptom-balloon time, (min)	128 [93-200]	130 [90-200]	125 [95-190]	0.6
FMC-balloon time, (min)	83±42	85±40	80±42	0.5
<i>Procedural therapy, no. (%)</i>				
Unfractionated heparin,	140 (88)	69 (87)	71 (89)	0.8
Bivalirubin,	18 (12)	10 (13)	8 (11)	0.7
GP IIb/IIIa inhibitors,	31 (19)	15 (19)	16 (20)	0.8
<i>Cardiac markers,(ng/ml)</i>				
CK-MB at peak,	102 [39-255]	48 [21-103]	245 [102-345]	<0.0001
CK-MB AUC,	279 [100-682]	129[58-279]	650 [294-900]	<0.0001
Troponin T at peak,	2.3 [0.9-6.1]	1 [0.5-2.1]	5.2 [2.3-8.4]	<0.0001
Troponin T AUC,	6.4 [2.4-16]	2.7 [1.5-82]	15 [7.4-23]	<0.0001
<i>Therapy at discharge, no. (%)</i>				
Aspirin,	158 (100)	79 (100)	79 (100)	0.9
P2Y12 inhibitors,	158 (100)	79 (100)	79 (100)	0.9
- clopidogrel	33 (21)	16 (20)	17 (21)	0.8
- prasugrel	21 (13)	11 (14)	10 (13)	0.8
- ticagrelor	104 (66)	52 (66)	52 (66)	0.9
ACE inhibitors,	131 (83)	64 (81)	67 (85)	0.6
β-blockers,	137 (87)	66 (83)	71 (89)	0.3
Statins,	155 (98)	78 (99)	77 (97)	0.8
<i>6-month follow-up,</i>				
LVEF, (%)	47±8	53±10	43±6	0.001
WMSI,	1.5 [1.3-1.8]	1.3 [1.2-1.61]	1.7 [1.4-1.9]	0.03
Death, no. (%)	4 (2.5)	1 (1.2)	3 (3.7)	0.3
ACS, no. (%)	4 (2.5)	2 (2.5)	2 (2.5)	0.9
HF, no. (%)	6 (3.7)	1 (1.2)	5 (6.3)	0.1
Death and ACS, no. (%)	6 (3.7)	2 (2.5)	4 (5)	0.3
Death and HF, no. (%)	8 (5)	1 (1.2)	7 (8.8)	0.04

BMI: body mass index. CrCl: creatinine clearance. LAD: left anterior descending. DES: drug eluting stent. FMC: first medical contact. GP: glycoprotein. AUC: area under the curve. ACE: angiotensin-converting enzyme. LVEF: left ventricle ejection fraction. WMSI: wall motion score index. ACS: acute coronary syndrome. HF: heart failure.

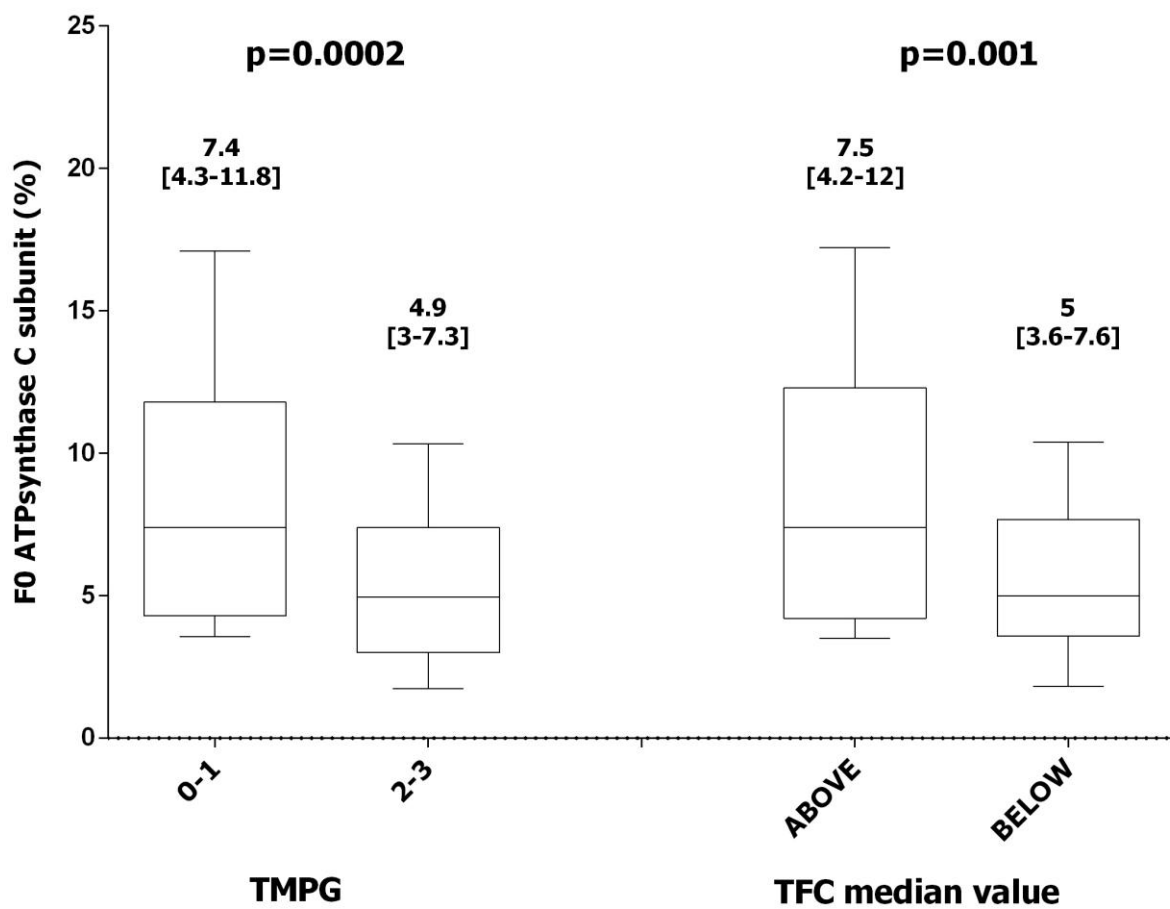


Figure 1

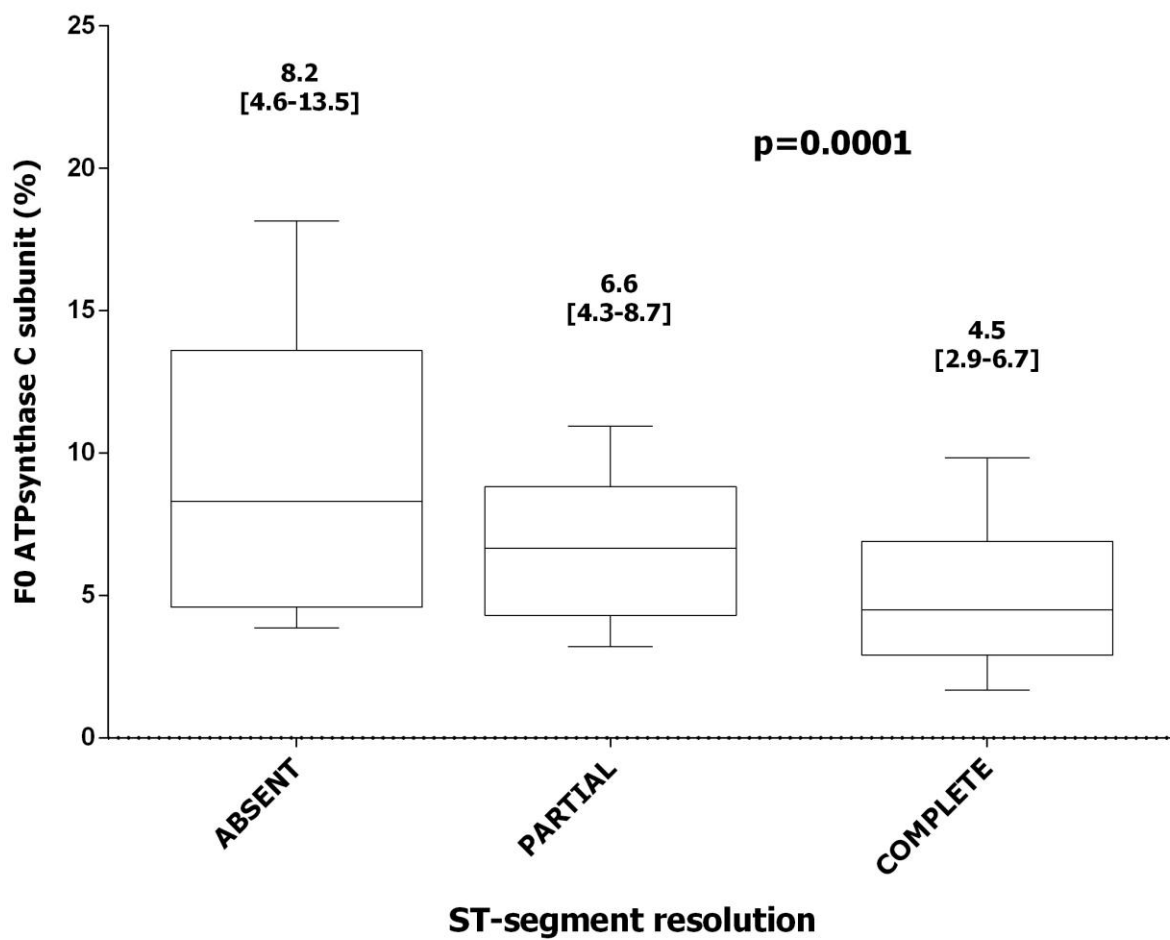


Figure 2

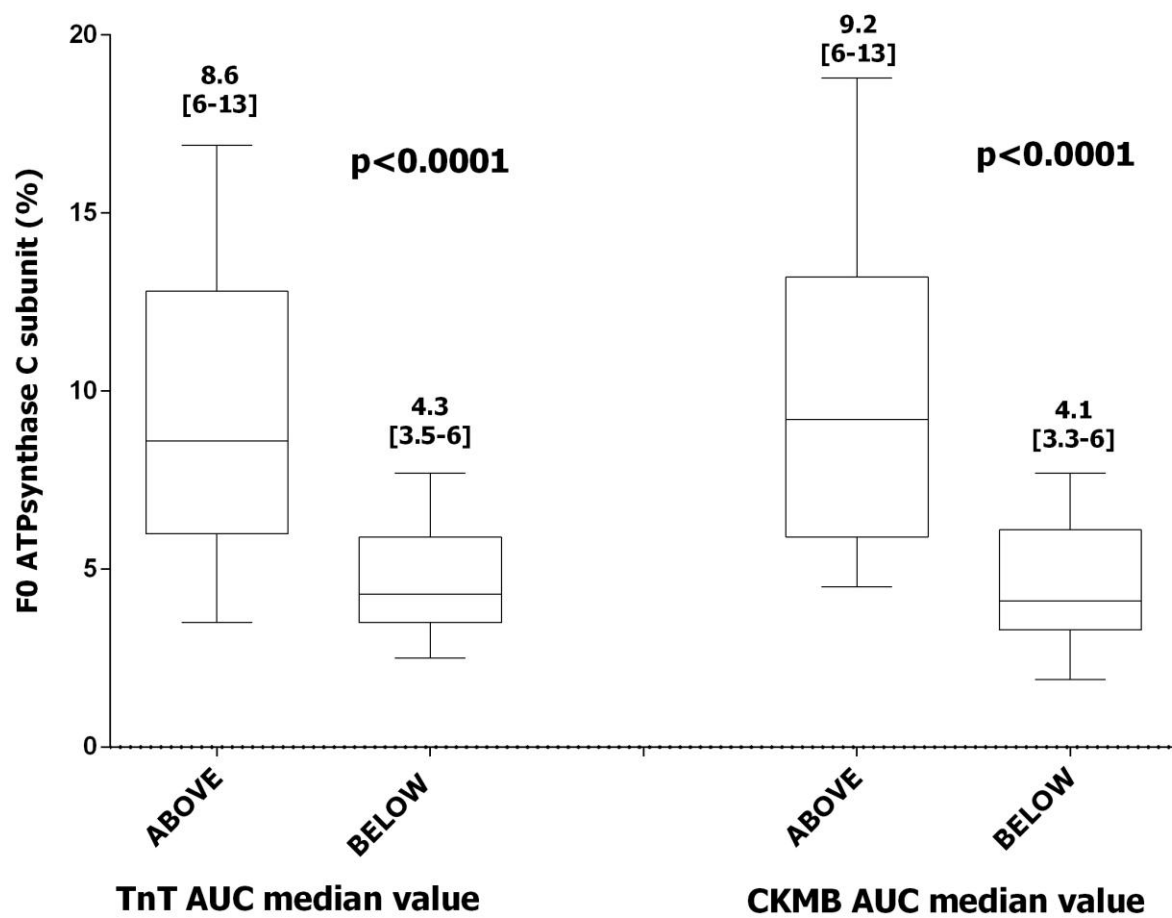


Figure 3