

Relationship between cardiac contractile performance and mitochondrial respiratory capacity in patients undergoing coronary artery bypass grafting surgery

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**UNIVERSITY OF SPLIT
SCHOOL OF MEDICINE**

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**RELATIONSHIP BETWEEN CARDIAC CONTRACTILE
PERFORMANCE AND MITOCHONDRIAL RESPIRATORY
CAPACITY IN PATIENTS UNDERGOING CORONARY ARTERY
BYPASS GRAFTING SURGERY**

Diploma thesis

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LIST OF ABBREVIATIONS

CVD	-	cardiovascular disease
CAD	-	coronary artery disease
AMI	-	acute myocardial infarction
PCI	-	percutaneous coronary intervention
CABG	-	coronary artery bypass grafting
EF	-	ejection fraction
ATP	-	adenosine triphosphate
CS	-	citrate synthase
FCCP	-	trifluoro carbonyl cyanide phenylhydrazone
OCR	-	oxygen consumption rate
mPTP	-	mitochondrial permeability transition pore
LVEF	-	left ventricular ejection fraction
ROS	-	reactive oxygen species
mtDNA	-	mitochondrial DNA

1. INTRODUCTION

1.1. Coronary artery disease

1.1.1. Epidemiology

Each year cardiovascular disease (CVD) causes 3.9 million deaths in whole Europe (1). Together with cerebrovascular disease, coronary artery disease (CAD) is the most common type of cardiovascular disease, with insufficient blood delivery of oxygen and nutrients to the affected areas of myocardium. CAD accounts for 1.8 million deaths in Europe, corresponding to 19% of all deaths in men and 20% in women respectively. It is the leading single cause of mortality, responsible for 862,000 deaths a year among men and 877,000 deaths among women. Also, about one-third of all deaths in people older than 35 years are due to CAD (1,2,3,4). It is also notable that twice as many fatal cases due to CAD occur in men more than women below the age of 75 (1,4). The economic costs associated with temporary and permanent disability among the working population in Europe amounted to about 106 billion euros in 2009. Therefore, CAD remains an important socioeconomic problem and focusing on treatment options and prevention strategies is of utmost importance (3,4).

Most commonly, CAD is caused by an atherosclerotic obstruction of the coronary arteries with the main clinical presentations being angina pectoris, myocardial infarction and chronic ischemic heart failure. The main risk factors for developing atherosclerosis and thus CAD include age, gender, hypertension, smoking, dyslipidemia/hypercholesterolemia, diabetes mellitus and a family history of CAD (5). In addition, a sedentary lifestyle and lack of exercise are proven to cause atherosclerosis (6).

1.1.2. Pathophysiology

Atherosclerosis remains the predominant underlying mechanism for CAD. More rarely, CAD is caused by the coronary spasm or congenital abnormalities. Atherosclerosis starts as a low-grade inflammation of the inner lining of the arterial wall. With time, this state progresses to formation of a plaque consisting of fatty streaks, with subsequent thickening of the arterial wall and narrowing of the arterial lumen (7). By definition, coronary blood flow limitation starts when the luminal cross-sectional area is reduced by at least 75% (8). Therefore, 75% stenosis is used as a cut-off value for interventional therapy (9). Two main types of atherosclerosis, stenotic and non-stenotic, in the coronary arteries are described in literature (Figure 1). Stenotic

lesions tend to have smaller lipid cores, more fibrosis, calcification, thick fibrous caps and less compensatory luminal enlargement. They cause constant ischemia by narrowing the vessel lumen, but are of low risk for rupture and acute myocardial infarction (AMI). Non-stenotic lesions undergo compensatory luminal enlargement that makes them non-symptomatic in many cases. Due to their thin fibrous cap, they are susceptible to rupture and thrombosis, subsequently leading to AMI. However, most of occlusive lesions lie somewhere between these two extremes and are of a mixed type (10).

Atherosclerosis progresses slowly, however, plaque disruption with subsequent formation of a non-occlusive intraluminal thrombus or a plaque hemorrhage could result in rapid disease advancement. This slow development intermixed with rapid cycles of progression leads to growth of the plaque and further narrowing of the lumen (11). If the narrowing of the arterial lumen reaches a level that the blood flow to the cardiac tissue is impaired, ischemia in the corresponding parts of the myocardium and damage of the cardiac tissue are the consequence (12).

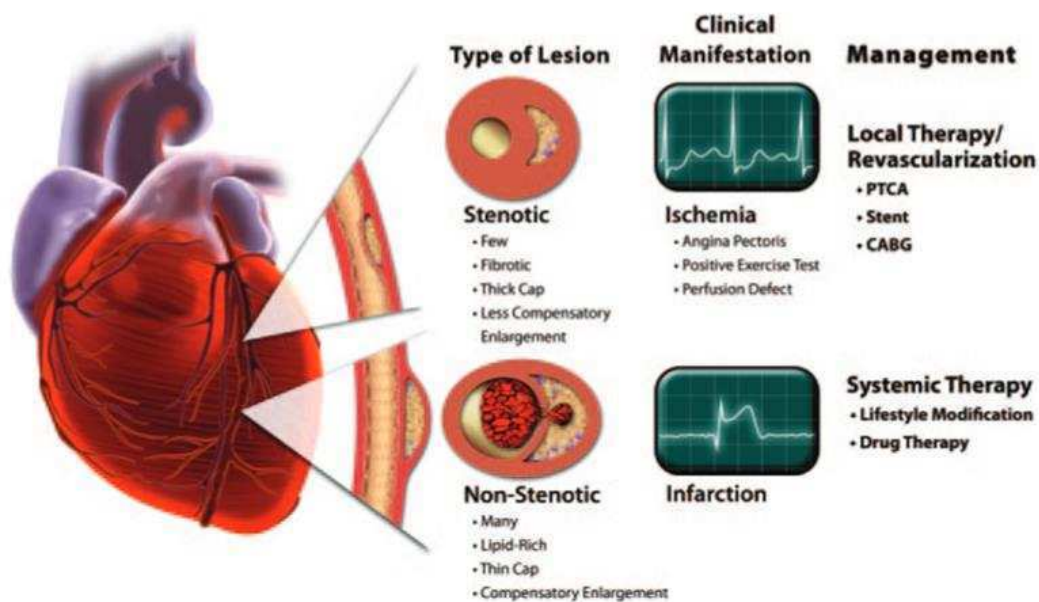


Figure 1. A simplified scheme of coronary artery occlusive lesions due to atherosclerosis. (Image taken from the article by Libby P and Theroux P (10)).

Blood supply of the heart comes directly from the aorta *via* left and right coronary arteries. Left main coronary artery divides into the left anterior descending and the circumflex artery, whereas right coronary artery continues without further major branching. Subsequently, all arteries will subdivide into much smaller arterial branches.

An obstruction of any part of a coronary artery leads to imbalance between the functional requirements of the heart and the capacity of the coronary arteries to supply oxygenated blood. The heart with its tremendously high metabolic requirement consumes up to 7% of the body's oxygen consumption during rest although it accounts for only 0.3% of the full body weight. A reduced oxygen supply therefore will end up in irreversible injury if perfusion is not restored within 40 to 60 minutes (13).

The lack of oxygen supply can lead to clinical signs and symptoms of CAD (12). Typical clinical manifestations of CAD range from not present at all to severe in the individual patient. Angina pectoris is the most common manifestation associated with CAD (13). Angina pectoris is a complex of symptoms presenting with thoracic pain, shortness of breath, diaphoresis, nausea, and vomiting. Typically, the pain radiates into the left arm and the back of the patient. If the pain occurs only during high myocardial oxygen demand such as exercise, it is called stable angina and if the pain occurs at rest also, the term unstable angina is used. If chest pain persists for a prolonged period without interruption unstable angina may have proceeded to AMI (13,14). AMI is defined as an irreversible myocyte damage with the formation of scar tissue in the heart. It is usually caused by the plaque rupture and formation of a fixed thrombus occluding the lumen (13,15). Furthermore, acute or chronic ischemic states caused by CAD can disturb mechanical and electrical cardiac abilities, leading to heart failure and arrhythmias.

Symptomatic CAD is usually treated pharmacologically, as well as by percutaneous coronary interventions (PCI) and coronary artery bypass grafting (CABG) (16). Various drugs can be used for pharmacological treatment; the following are the key pharmacological approaches. Reducing cardiac metabolic demand is crucial and achieved by the use of beta-blockers, calcium channel blockers, angiotensin-converting enzyme blockers or angiotensin II receptor blockers. These agents are used either alone or in combination to decrease blood pressure and reduce the heart rate, reducing overall cardiac workload. Hyperlipidemia reduction is achieved with cholesterol-modifying medications, which reduces the amount of low-density lipoprotein in the body. Commonly used are statins, niacin and others. Aspirin and clopidogrel are other recommended drugs to prevent thrombosis through their platelet aggregation inhibitory function. Oral anticoagulants are used as well but their use remains controversial due to their higher bleeding risk (15,16).

1.2. Cardiac contractile performance in CAD

Normal myocardial contractile performance is of utmost importance for the blood perfusion of all parts of the body. Myocardial contractile strength depends on three major causal factors: myocardial health, preload and afterload. Ischemic events caused by CAD intervene with myocardial health and therefore with the function of myocardial relaxation and contraction.

A widely used measure to describe cardiac contractile performance is ejection fraction (EF). It describes the "fraction of left ventricular end-diastolic volume ejected per beat" (Folse *et al.* (17)), calculated by stroke volume divided by end-diastolic volume. In a healthy 70-kilogram male individual the EF is around 58% (stroke volume 70mL divided by end-diastolic volume 120mL equals 0.58) (18). Healthy normal range of EF is between 50% and 65%, different sources in literature, however, give different reference values (18).

Scarring of the myocardium resulting from ischemic events in CAD can lead to abnormal contraction patterns or localized absence of movement in the heart (ventricular dyssynergy (19)), which in turn causes a decrease of EF. Cardiac contractile performance is therefore often diminished in patients with CAD (19). An EF below 40% is considered severe and indicates heart failure (20).

EF is also used to classify as well as to describe the severity of heart failure (21). Revascularization procedures such as CABG are used to eliminate further drop of EF in patients with heart failure due to CAD or AMI (20).

1.3. Mitochondria in the heart

1.3.1. Mitochondria in the healthy heart

Mitochondria are recognized as cellular "power plants" providing the energy needed for cellular metabolism. As the most metabolically active organ in the body, the heart must continuously produce the main energy currency - adenosine triphosphate (ATP), to keep its level of performance. About 80-90% of cellular ATP is produced in mitochondria by the process of oxidative phosphorylation (22). Therefore, as the heart relies primarily on mitochondrial oxidative metabolism to provide most of its energy, cardiomyocytes contain the

highest concentration of mitochondria in the body (22). To understand the present study, one must grasp the general principals of this energy producing process.

Mitochondrial respiration or oxidative phosphorylation is the process by which the mitochondrion produces ATP out of macronutrients stored in the cell. The "high-energy" molecule ATP serves as a universal energy donor required for cell metabolism (23). Mitochondria consist of a double membrane, with a smooth outer membrane and an inner membrane forming many folds – the mitochondrial cristae (24). The inner membrane is the location of the so-called *electron transport chain* that consists of a set of membrane protein complexes. Four electron transport chain complexes are known (Complex I – IV), with a fifth one being an ATP synthase enzyme. The first two complexes: complex I and II receive the electrons from the high-energy electron-donors (e.g. NADH) formed by the upstream biochemical processes: glycolysis, beta-oxidation and citric acid cycle. Each complex consists of a specific number of subunits, and the energy released by the transfer of high-energy electrons will be ultimately used for the generation of ATP (25,26).

The main substrates for ATP production in the heart are free fatty acids and glucose. In order to be utilized, the fatty acids are shuttled from the cytosol into mitochondria *via* the acyl-carnitine shuttle and then broken down to acetyl coenzyme A (acetyl-CoA) in the process of beta-oxidation (27). Cytosolic glucose is converted into two molecules of pyruvate by the process of glycolysis. As a "by-product", the molecules ATP and NADH are also formed. Pyruvate will be used to create acetyl-CoA and proceed to enter the citric acid cycle. Glycolytically created cytosolic NADH will transfer its electrons *via* malate-aspartate shuttle to the mitochondrial NADH, an electron donor for the mitochondrial respiratory chain. However, most of high-energy electron donor molecules are formed by the citric acid cycle (Krebs cycle) (28).

The citric acid cycle consists of a series of chemical reactions used for oxidation of major fuel molecules – fatty acids, carbohydrates and amino acids. The entry point for most of the energy substrates is acetyl-CoA, which, through a series of oxidation reactions will be oxidized to form carbon dioxide. In this oxidation process, the high energy electrons will be generated and will be shuttled to the electron transport chain *via* electron carrying molecules of NADH and FADH₂ (28).

The biochemical purpose of oxidative phosphorylation is to convert energy of the high-energy electrons into the ATP. *Complex I (NADH-Q oxidoreductase)* receives electrons by oxidating NADH into NAD⁺ and transfers them *via* flavin mononucleotide cofactor towards ubiquinone, also called coenzyme Q. Upon accepting the two electrons, ubiquinone is reduced

into ubiquinol (QH₂). The energy released by the electron flow is used for pumping of the protons from the mitochondrial matrix into the intermembrane space. The main sources of NADH is the citric acid cycle, while other sources include beta oxidation and glycolysis.

Complex II (succinate-Q reductase complex) contains one of the citric acid cycle enzymes, the succinate dehydrogenase, which breaks down succinate from the citric acid cycle into fumarate and transfers its high-energy electrons to form ubiquinol *via* FADH₂. Ubiquinol transfers its electrons further to complex III. Unlike complex I, the complex II does not pump protons across the inner mitochondrial membrane.

Electrons from NADH and FADH₂ that were transferred to ubiquinol by complex I and II, will be passed to cytochrome c by the *complex III (Q-cytochrome c oxidoreductase)* with concomitant pumping of protons to the mitochondrial intermembrane space.

Finally, electrons from the reduced cytochrome c are transferred to the final electron acceptor - molecular oxygen (O₂) by the *complex IV (cytochrome c oxidase)* in mitochondrial matrix. Each oxygen molecule needs four electrons to completely reduce it to H₂O. The availability of oxygen, therefore, enables perpetuation the electron transfer, and is often the limiting factor of oxidative phosphorylation. Complex IV also transfers protons across the inner mitochondrial membrane. Finally, the protonmotive force created by complex I, III and IV by moving H⁺ into the intermembrane space links the electron transport chain to complex V or ATP synthase. An increased concentration of protons in the intermembrane space causes the electrochemical gradient, pulling the protons back into the matrix. *Complex V (ATP synthase)* allows protons to enter back to the mitochondrial matrix and uses this protonmotive force to phosphorylate ADP into ATP on the matrix side. The generated ATP will be used by the cell as energy (25,26) (Figure 2).

In conclusion, energy released by the transport of electrons *via* membrane complexes, is used to move protons from mitochondrial matrix into the intermembrane space. The proton electrochemical gradient leads to reflux of protons through the ATP synthase, producing ATP from ADP. The above-described process of mitochondrial respiration is oxygen dependent, since molecular oxygen is the final oxygen acceptor. As the heart produces ATP mainly through the oxidative phosphorylation, satisfying the metabolic needs of the heart is tightly coupled to the availability oxygen *via* coronary blood flow, making the heart very dependent to its blood supply (13).

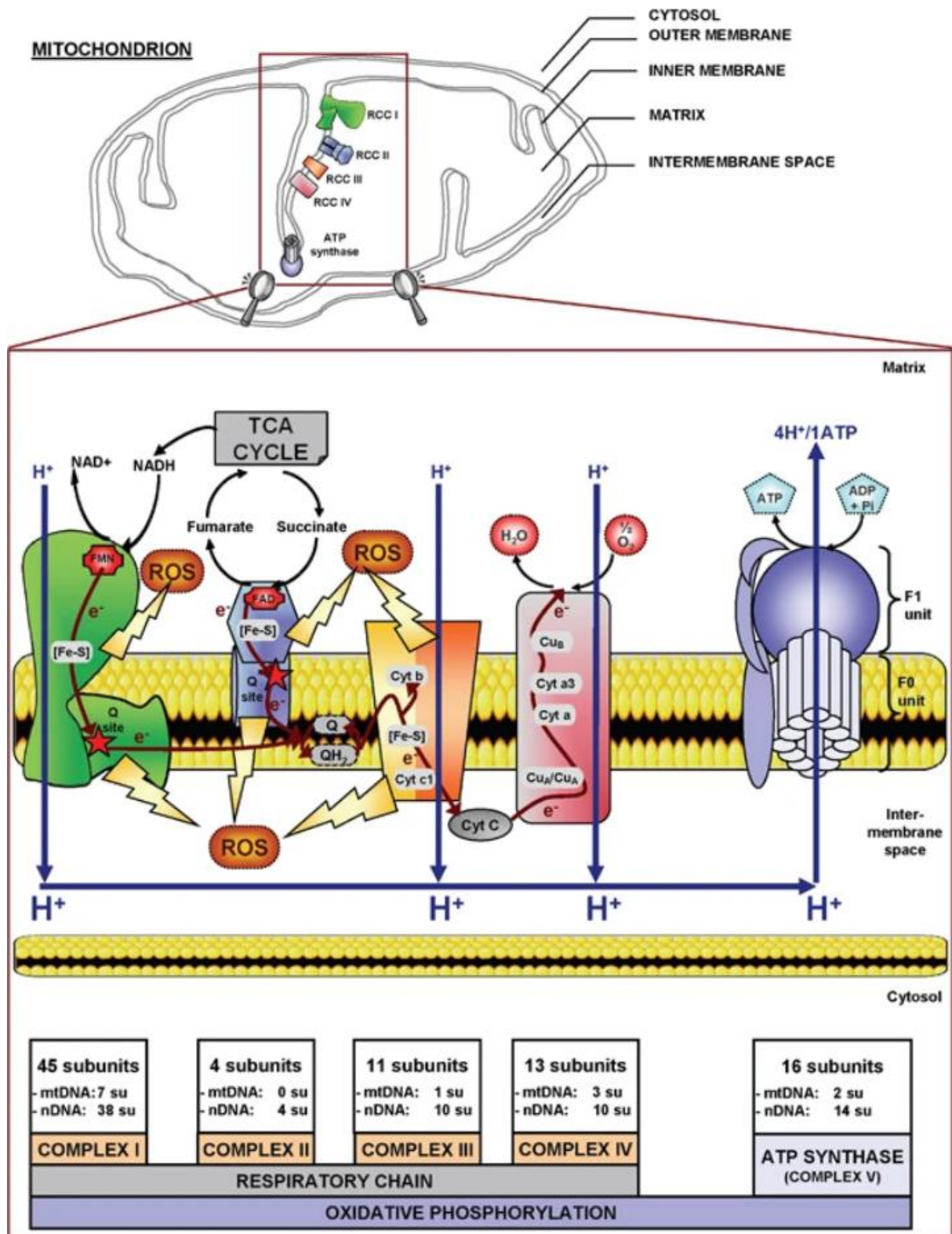


Figure 2. Oxidative phosphorylation. (Image taken from the article by Lemarie A and Grimm S (25)).

The mitochondrial content in a cell can be measured by different biomarkers. Most commonly used is the activity of citrate synthase (CS). Citrate synthase is the initial enzyme for the citric acid cycle that catalyzes condensation from oxaloacetate with acetyl CoA to form

citrate. CS is exclusively localized within the mitochondria, hence the specificity for the mitochondrial content in a cell. High CS activity reflects an abundance of CS and therefore a high mitochondrial content (29,30).

1.3.2. Mitochondria in the ischemic heart

As mentioned above, the main function of mitochondria in the healthy heart is to maintain the ATP supply to the cardiac cells even under stress or increased workload. Conversely, many cardiovascular diseases are associated with mitochondrial dysfunction.

In hearts with CAD, evidence shows an overall decline in mitochondrial function and ATP production (22,31). Damage to the mitochondria or any kind of mitochondrial dysfunction result in cardiomyocyte dysfunction and ultimately lead to cell death. For example, an ischemic event induces sudden biochemical alterations in the mitochondria including mitochondrial membrane depolarization and decline in ATP synthesis. Since ATP is mainly produced *via* the oxygen dependent oxidative phosphorylation, cell metabolism in ischemic heart needs to compensate the reduced mitochondrial respiratory capacity with a metabolic shift towards the anaerobic energy production pathway of glycolysis (32). This induces the production of lactate and leads to a decrease of pH. To get rid of the excess H⁺ intracellularly, the Na⁺-H⁺ exchanger increases its workload, therefore causing an increased entry of Na⁺. Furthermore, the reduced production of ATP causes the Na⁺/K⁺ATP-ase to fail, leading to an even greater accumulation of Na⁺. Consequently, the Na⁺/Ca²⁺ exchanger reverses its physiologically normal direction and starts extruding the accumulated intracellular Na⁺ in exchange for Ca⁺ ions, leading to a Ca⁺ overload. With depleted ATP, the Ca²⁺ ATP-ase cannot perform its function, and consequently the Ca²⁺ overload cannot be reversed (31). The pathological conditions of Ca²⁺ overload and decline of ATP levels can induce assembly of a protein complex called mitochondrial permeability transition pore (mPTP) in the inner membrane of the mitochondria. mPTP increases the permeability of the mitochondrial membrane, allowing the passage of any molecule below 1500 Daltons molecular weight and resulting in mitochondrial membrane potential dissipation. The influx of molecules through mPTP causes mitochondrial swelling and mediates necrotic and apoptotic cell death (22,32,33).

Furthermore, evidence indicates a decreased mitochondrial integrity in clinically diagnosed CAD. Under the influence of DNA deletions and damage, the mitochondrial genome itself mediates a metabolic defect in the hearts of CAD patients (31). However, this needs

further studies to understand the role of mitochondrial DNA in CAD patients. In the future, this might lead to a possibility of mitochondrial DNA-targeted therapies for preventing cardiovascular diseases (31).

1.4. Coronary artery bypass grafting

Coronary artery bypass grafting (CABG) is one of the treatment options for CAD. The procedure consists of by-passing the occluded part of the coronary artery with an autologous graft (arterial or venous) to restore the blood flow to the ischemic area in the heart. By doing so, CABG surgery reduces the clinical symptoms of CAD, decreases the risk of heart attack and improves survival. Several different types of the procedure exist: a conventional CABG surgery, an "off-pump" CABG and a minimal invasive CABG surgery (34,35).

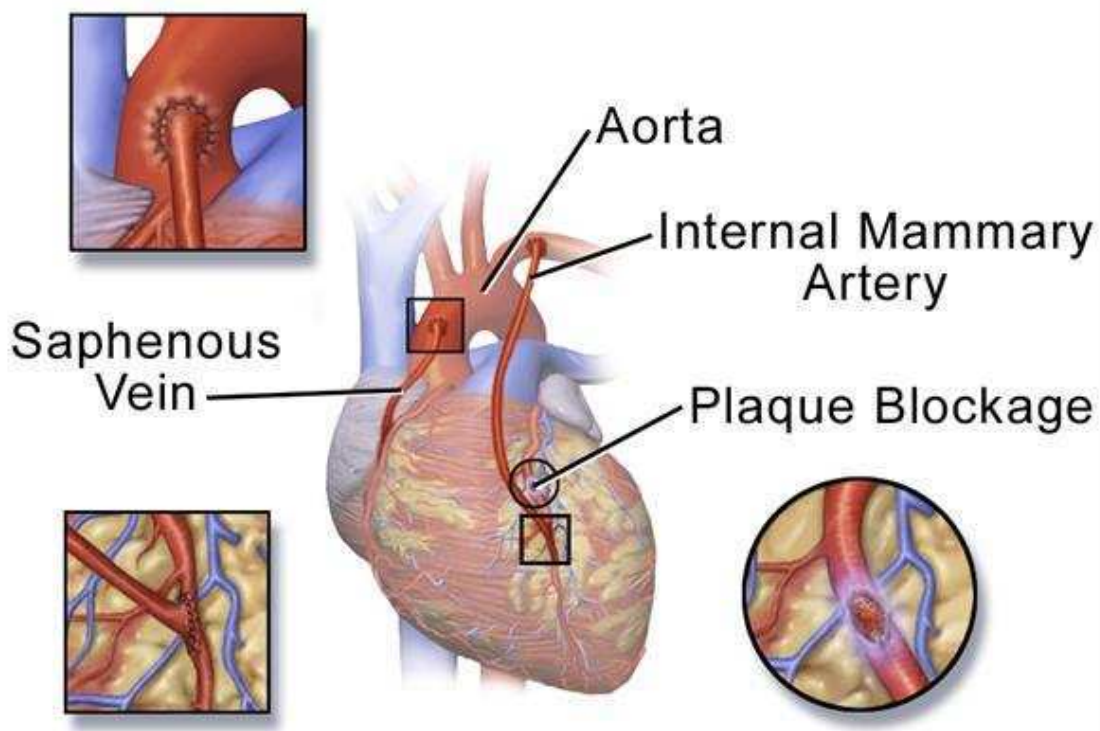


Figure 3. Schematic of CABG procedure using either the great saphenous vein of the leg or the internal mammary artery as a graft (image taken from the Medical gallery of Blausen (36)).

Conventional CABG is an open-heart surgery in which a median sternotomy is necessary to access the heart. The use of a cardiopulmonary bypass machine maintains the oxygen-rich circulation to the rest of the body, while the heart is kept inactive during the surgery

(37). During the procedure, the heart and body is cooled down to reduce metabolic activity and energy expenditure of the tissue. This reduces the need of blood perfusion of the body during surgery. An *off-pump CABG* uses a similar surgical approach, but without the cardiopulmonary bypass. Also, the stopping of the heart (cardioplegia) is not performed and the whole procedure is done on the beating heart (37). The minimal invasive approach is a relatively new technique of CABG surgery. The surgeon approaches the heart through several small incisions without the need of a sternotomy (37).

In all three techniques mostly the left internal thoracic artery, the radial artery or the great saphenous vein from the leg are used as grafts to bypass the coronary occlusion (Figure 3). Major complications of CABG surgery include re-exploration for bleeding, stroke, AMI and death. Yet the outcomes of CABG surgery are excellent with a low mortality rate of coronary artery surgery at around 1-3%. Long term prognosis is very good with reduced risk for AMI as well as overall improvement of survival (8,35,38). Especially for patients with an EF below 40% CABG showed a survival benefit over PCI (20).

To calculate the risk of fatal outcome after a heart operation the EuroSCORE II (European System for Cardiac Operative Risk Evaluation) model is used, in which the patients' individual information, the state of the heart and the proposed surgery are given into the EuroSCORE calculator, available for free online. The result is given in percentage of risk of mortality (39).

2. OBJECTIVES

2.1. Aims

A relationship between cardiac mitochondrial dysfunction and decreased cardiac contractile performance has been demonstrated in both animal models and humans with chronic heart failure (40,41,42). However, no studies exist that investigate the relationship of mitochondrial respiratory function and cardiac contractile function in patients with preserved systolic performance. Therefore, the main purpose of this study was to investigate whether there is a relationship between mitochondrial respiratory capacity and cardiac contractile function in patients undergoing CABG surgery.

If our results show a connection between the contractile performance of the heart and impaired mitochondrial metabolism associated with respiratory chain dysfunction, further research can be done on developing treatment options modulating or targeting mitochondrial function to improve heart diseases.

2.2. Hypothesis

There is a positive correlation between mitochondrial respiratory capacity and cardiac contractile performance in patients undergoing coronary artery bypass grafting surgery.

3. MATERIALS AND METHODS

3.1. Study design

Forty-one hemodynamically stable patients undergoing CABG surgery were included in the present study. Emergency patients, patients with EF below 40%, patients with concomitant valve replacement and patients who had several renal, hepatic or pulmonary diseases were excluded. All clinical data was collected immediately before CABG surgery. This cross-sectional study was performed at Department of Physiology (University of Split School of Medicine) in association with the Department of Cardiac Surgery of the University Hospital Split.

Pre-, intra- and post-surgical procedures were performed according to the standard clinical routines of the Department of Cardiac Surgery. All chemicals used for this study were purchased from Sigma-Aldrich (St Louis, MO, USA), unless stated otherwise. The experimental protocol was approved by the Ethics Committee of the University Hospital Split (Klasa: 500-03/12/01/33; Ur. broj: 2181-147-01/M.J.-12-2). All performed procedures were in compliance with the ethical standards of the institutional research committee and the 1964 Helsinki declaration. All patients signed an informed consent form prior to their inclusion into the study.

3.2. Biopsy acquisition and tissue sample preparation

At the end of the CABG surgery, which was performed in the above described "off-pump" mode, two to three cylinder-shaped biopsies were taken from the anteroseptal part of the left ventricle. The biopsy tissue was immediately immersed in cold mitochondria-preserving storage solution (Solution S, in mmol/L: 2.77 CaK₂EGTA, 7.23 K₂EGTA, 6.56 MgCl₂, 5.7 Na₂ATP, 15 phosphocreatine, 20 imidazole, 20 taurine, 0.5 DTT and 50 K methanesulfonate, pH 7.1 at 0°C), kept on ice and transferred to the laboratory within 15 min. Upon arrival, a portion of the biopsy tissue was snap-frozen for later investigation, while the remaining amount of tissue was used immediately to obtain the mitochondrial respiration measurements.

Still on ice and in solution S, the remaining biopsy tissue was first cleaned under a microscope from excess non-myocardial tissue such as fibrous tissue or fat. It followed a fine dissection of the tissue in order to separate the myocardial fibers. Next, the tissue was permeabilized by mild agitation at 4°C with the addition of saponin (50µg/ml). Saponin is a substance used for chemical skinning. It acts mainly on cholesterol which it dissolves, thereby

making holes in the cell membrane. As mitochondrial membranes contain only negligible amounts of cholesterol, mitochondrial membranes remain intact. These so-called *skinned cardiac fibers* have, therefore, permeabilized sarcolemma and mitochondria that are directly accessible to the substrates (43).

Before respiration parameters were determined, storage solution S was washed off by agitation for 10 min at 4°C with a respiration medium (in mmol/L: 2.77 CaK₂EGTA, 7.23 K₂EGTA, 1.38 MgCl₂, 3 K₂HPO₄, 20 imidazole, 20 taurine, 0.5 DTT, 90 K methanesulfonate, 10 Na methanesulfonate and 0.2% BSA, pH 7.1; 100 nmol/L free Ca²⁺, 1 mmol/L free Mg²⁺). The tissue sample was transferred into a 2ml respiratory chamber filled with the same respiration buffer.

3.3. Mitochondrial respiration measurement

Mitochondrial respiration was evaluated using an oxygen Clark-type electrode (Oxygraph, Hansatech Instruments, Norfolk, UK) at 30°C and under constant stirring. The electrode was connected to a computer software measuring the oxygen concentration. The availability of oxygen in the chamber was always maintained above 210 µmol/l.

Maximal tissue oxygen consumption (mitochondrial respiratory capacity) was assessed at conditions of maximal stimulation of both carbohydrate (by adding pyruvate and malate, 10 mmol/L and 5mmol/l, respectively) and fatty acid (palmitoyl carnitine, 40 µmol/l) utilization in the presence of saturating amounts of ADP (2,5 mmol/l), as well as after addition of trifluoro carbonyl cyanide phenylhydrazone (FCCP, 1 µmol/l), an uncoupler of oxygen consumption from ATP production (44). This way, any potential oxidation limitation of the phosphorylation apparatus was avoided. Oxygen consumption rate (OCR) was then calculated and expressed in pmol O₂/s/m of wet tissue weight.

3.4. Citrate synthase activity assessment

Using a 0.2 mL tissue grinder, the portion of previously frozen biopsy sample was homogenized in the presence of a protease inhibitor cocktail. The extraction of CS was performed using 15% lauryl maltoside detergent solution (ab109858; Abcam, Cambridge, UK). CS concentration was determined with a detergent compatible protein assay (Bio-Rad,

Hercules, CA, USA). The activity of CS in the reaction mixture containing 15 µg of tissue protein was assessed at 30°C using a kit (CS0720; Sigma-Aldrich). Enzyme activity was initiated by adding oxaloacetic acid (10 mmol/L), and absorbance was measured at 412 nm using a spectrophotometer (DU 800; Beckman Instruments, Fullerton, CA, USA). Upon subtraction of background absorbance, the enzyme activity was calculated using the 13.6 (mmol/L)/cm extinction coefficient and expressed in international units of citrate synthase activity per mg tissue protein (U/mg).

3.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software. For metrical parameters normality of distribution was checked using D'Agostino-Pearson test. Correlation between the measured variables of mitochondrial function (maximal respiration rate and citrate synthase activity) and EF was assessed using Pearson's correlation analysis. Data in tables are presented as means ± SD. A two-sided P value < 0.05 was considered as significant.

4. RESULTS

4.1. General observations

Patient clinical characteristics and demographics are shown in Table 1. All CABG procedures went well and no complications in any of the patients related to the biopsy acquisition were evident.

Table 1. Clinical characteristics and demographics of patients enrolled in the study.

	n=41
Female gender, n (%)	9 (21.95)
Age (years)	66.5±7.4
LVEF (%)	61.35±0.1
EuroScore II (%)	2.48±1.7
<i>Clinical characteristics</i>	
Hypertension, n (%)	26 (63.4)
BMI (kg/m ²)	29.2±4.1
HBA1c (%)	7.63±0.1
HDL (mmol/l)	1.06±0.3
LDL (mmol/l)	2.01±0.7
TG (mmol/l)	1.76±0.9
<i>Medications, n (%)</i>	
acetylsalicylic acid	37 (90.3)
clopidogrel	25 (61.0)
beta blocker	33 (80.5)
ACE inhibitor/ARB	26 (63.4)
statin	34 (82.9)
nitrate	10 (24.4)
diuretic	16 (39.0)
Ca-channel blocker	6 (14.6)
amiodarone	3 (7.3)
insulin	4 (9.8)
oral hypoglycemic agent	7 (17.1)
antithrombotic agent	11 (26.8)

Values are mean±SD

LVEF = left ventricular ejection fraction; BMI = body mass index; HbA1c = glycosylated hemoglobin; HDL = high density lipoprotein; LDL = low density lipoprotein; TG = triglycerides; ACE = angiotensin converting enzyme; ARB = angiotensin II receptor blocker.

4.2. Relationship between oxygen consumption rate and ejection fraction

Our analyzed data revealed positive correlation between mitochondrial oxygen consumption and cardiac ejection fraction ($r=0.35$; $P=0.026$). The higher the mitochondrial respiratory capacity in a patient is, the better the cardiac contractility (Figure 4). To point out, we cannot tell from our data whether the EF is changing depending on the OCR or the OCR depending on the EF. However, the enzymatic activity of CS was not correlated with EF ($r=0.13$; $P=0.42$; Figure 5). Furthermore, no association was found between patient age and EF ($r=-0.27$; $P=0.09$; Figure 6), or patient age and mitochondrial respiratory capacity ($r=-0.22$; $P=0.17$, Figure 7).

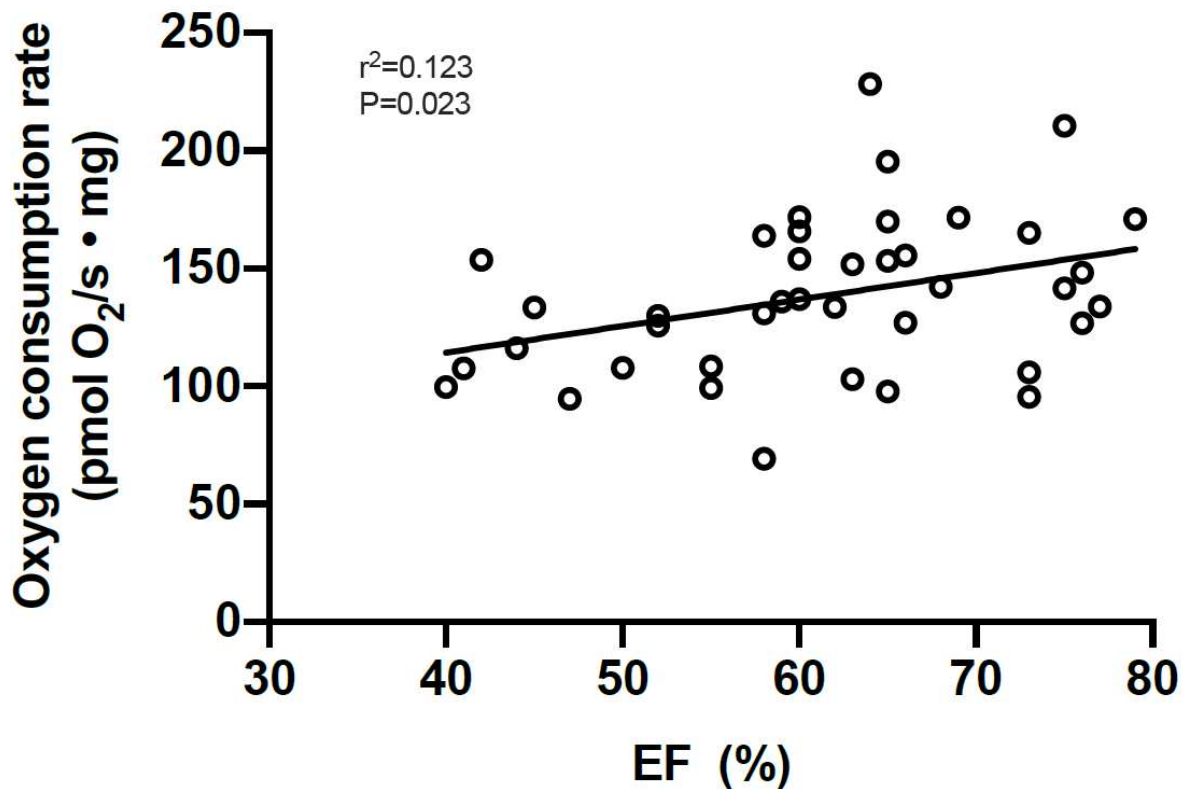


Figure 4: Relationship of maximal oxygen consumption rate (in the presence of all energy substrates and FCCP) and ejection fraction (EF).

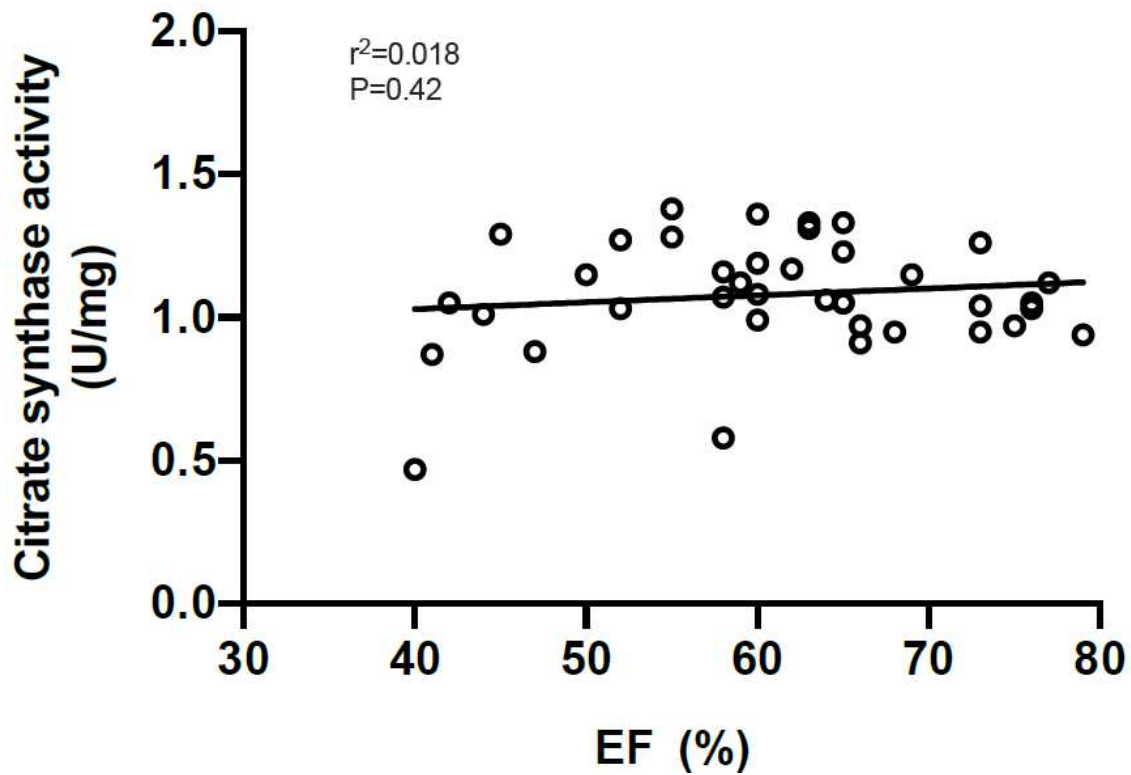


Figure 5: Relationship between citrate synthase activity and ejection fraction (EF).

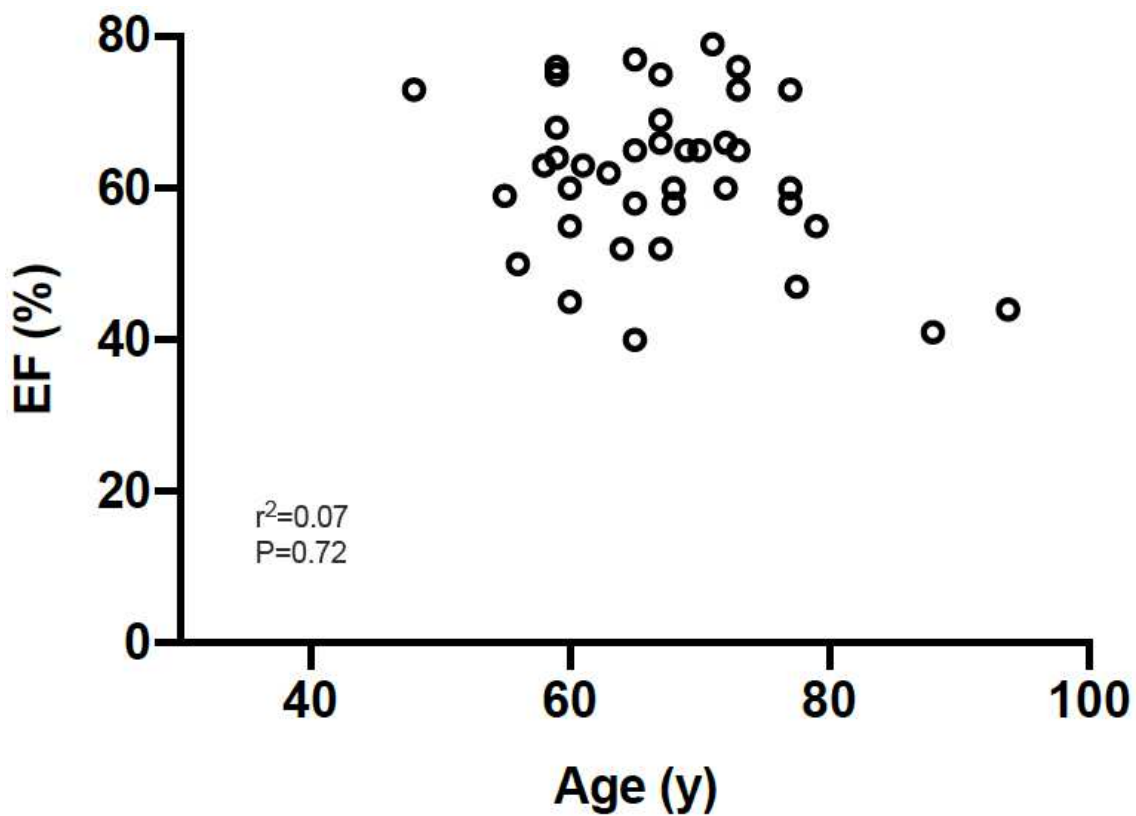


Figure 6. Relationship between patient age and ejection fraction (EF).

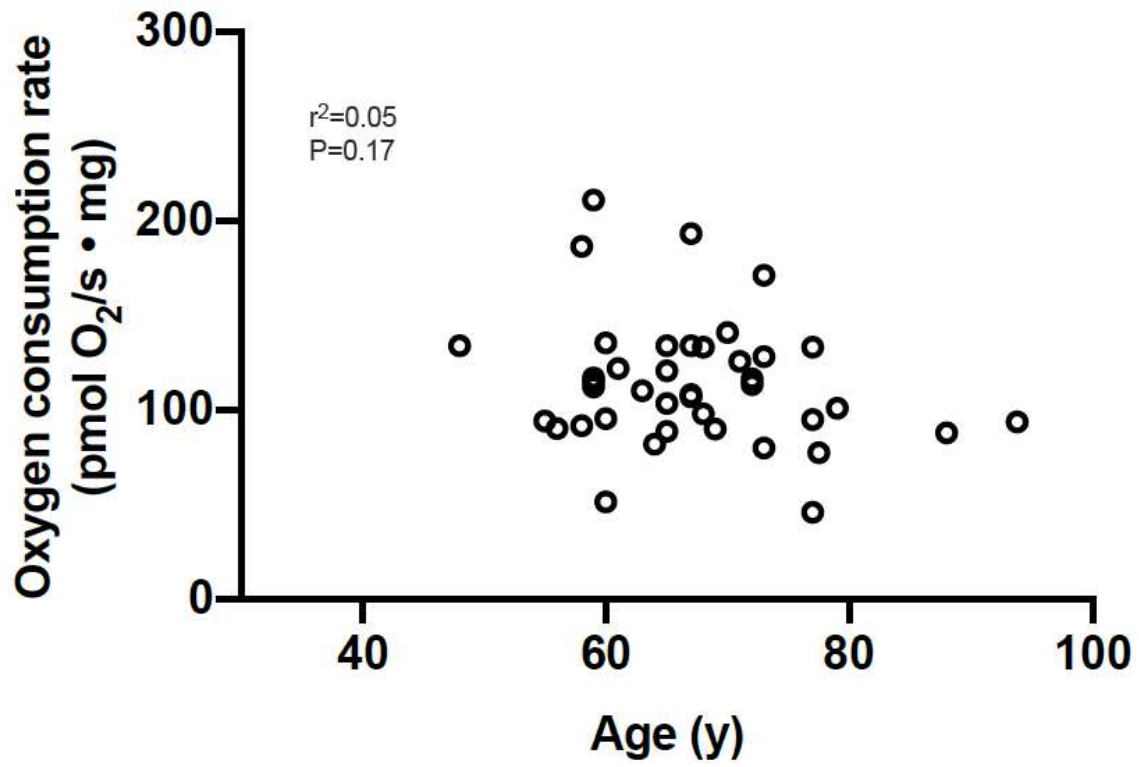


Figure 7. Relationship between patient age and maximal oxygen consumption rate (in the presence of all energy substrates and FCCP).

5. DISCUSSION

Our data demonstrate a positive correlation between left ventricular ejection fraction (LVEF) and mitochondrial oxygen consumption rate in patients with preserved systolic LV function. There was no correlation between LVEF and activity of citrate synthase suggesting that the association between mitochondrial respiratory capacity and LV contractile function is not due to altered mitochondrial mass, but rather qualitative mitochondrial changes.

Contractile cells of the heart are characterized by the high rate of energy consumption and strong dependence on adequate mitochondrial function to meet their energy needs. There is accumulating evidence that mitochondrial defects in energy production positively correlate with development and progression of heart disease (40,41,42). However, most studies of mitochondrial (dys)function in human heart are mostly limited to end-stage chronic heart failure (45), and only little is known about the role of impaired oxidative phosphorylation during still preserved contractile function, while the disease is still in progression.

The imbalance of energy supply and demand is central to pathological remodeling leading to heart failure. Due to extensive research on this topic in the last decades, the main events of underlying the pathological remodeling have been identified. However, their cause-and-effect relationships are still largely debated (41). For example, pathological cardiac remodeling such as left ventricular hypertrophy and chamber dilation disturb the cardiac geometry and lead to increased ATP demand. Attempting to meet this demand, mitochondrial function is altered, and metabolic remodeling ensues – for example altered utilization in energy substrates. The failing heart attempts to regain energy homeostasis with metabolic remodeling causing mitochondrial stress, leading to a decrease of ATP production. Decreased ATP production aggravates the unsatisfied metabolic demands of the heart even more (41). This vicious circle leads ultimately to cell dysfunction and cell death initiated by the mitochondria (45). The hypothesis that a failing heart is energy deprived is supported by clinical evidence of beneficial effects of beta-blockers and vasodilators (both decreasing cardiac energy demand) in treating heart failure. Decreasing heart rate, preload and afterload all contribute to reduction of the overall cardiac workload (46). On the other hand, positive inotropic agents, by increasing energy need of the heart, worsen the clinical outcome (47). Possible future therapeutic interventions for heart failure, therefore, should target the decreased ATP supply in diseased hearts through the development of medication that improve the mitochondrial function (41).

Recent studies point towards more complex multifactorial connection between heart failure and mitochondrial function than just the "energy-starvation" hypothesis. Several mitochondria-mediated mechanisms have been identified such as mitochondrial protein modification, mitochondrial Ca^{2+} dysregulation, mitochondrial aging and increased reactive

oxygen species (ROS) production, which drive the heart ultimately towards ATP depletion (41,48).

The relationship between reduced mitochondrial function and heart failure in humans had mostly been investigated by studying the mitochondrial respiration of the end-stage failing hearts (explanted hearts during the cardiac transplant surgery). In these hearts, high-resolution respirometry (the method also used in our study) showed a pronounced limitation of oxidative phosphorylation in end-stage failing hearts as compared to the non-failing (45). On the other hand, isolated mitochondria from LV wall of patients with heart failure and reduced systolic function undergoing heart transplant or LV assist device placement, showed preserved respiratory capacity as compared to mitochondria from non-failing left ventricle (49). However, the non-failing LV wall tissue was obtained from explanted hearts of patients with pulmonary hypertension undergoing heart-lung transplant, which might have influenced the final results. Finally, a recent study in transcatheter endomyocardial biopsies of the LV wall showed a significant reduction of mitochondrial oxidative capacity in failing hearts as compared to non-failing controls (50). In our study, we demonstrate that in patients with preserved systolic LV function ($EF > 40\%$), there is still a positive relationship between contractile function and mitochondrial oxidative capacity. This suggests that not only in end-stage heart failure, but also in CAD without significantly impaired contractile abilities, the capacity of mitochondrial respiration is still related to the contractile function. As citrate synthase activity did not correlate with EF, we conclude that the changed mitochondrial respiratory capacity is not the consequence of a change in mitochondrial content inside the cell, but rather due to qualitative changes in mitochondria related to contractile ability.

Recent research on qualitative mitochondrial abnormalities in heart failure found various qualitative mitochondrial impairments. For example, mitochondrial structural abnormalities were found in animal models as well as human diseased hearts, and include mitochondrial hyperplasia, reduced size, fragmentation, and ultrastructural changes such as loss of electron-dense matrix and disruption of inner and outer membranes (48). These morphological changes in mitochondria were shown to result in abnormal mitochondrial function and therefore leading to a decrease in respiratory capacity.

As abnormalities in mitochondrial dynamics a link was shown by several recent studies between many mitochondrial DNA (mtDNA) mutations and cardiovascular disorders (51,52,53). In particular, seventeen mtDNA mutations were found to be associated with ischemic heart disease in humans due to mitochondrial dysfunction involvement in atherosclerotic development and its risk factors (48). Since mtDNA is inherited through the

mother, evidence suggests that mothers carrying such mutations can transmit increased atherosclerosis risk, and therefore increase risk for CAD, to their children (51,52).

Mitochondrial functional abnormalities can result in increased production of ROS and oxidative stress. Healthy heart mitochondria also produce ROS mostly in the form of superoxide anion, but it is quickly converted to the less damaging hydrogen peroxide by superoxide dismutase, followed by its further catalase-driven conversion to O₂ and water. However, excess ROS production mostly by the mitochondria *via* electron leakage from complex I and III is considered as one of the central events leading to cardiac damage and a major pathophysiological mechanism in development of chronic heart failure. We speculate that mitochondrial ROS might play a similar significant role in pathological remodeling of hearts with CAD and preserved EF and lead reduction in mitochondrial respiratory capacity (41). Furthermore, oxidative stress is also considered as a major initiator of development of atherosclerosis and associated CVD (53).

The majority of patients in our study were 65 years of age or older (mean 66.5±7.4). In the general population the average age of patients with CVD is well into the 70s (48). Mitochondria play a major role in the aging process; changes in mitochondrial quality over time contribute to cellular senescence with aging (48). Mitochondria are potent regulators of apoptosis, releasing apoptotic factors and activating the caspase system when the membrane integrity and mitochondrial function is altered (53). Mitochondrial biogenesis and turnover through fusion and fission and mitophagy of mitochondria normally allows for the removal of dysfunctional and damaged organelles and replacement with functional ones. However, with advancing age imperfect turnover can alter the quality control of mitochondria and causes cell damage and apoptosis (48,53). In addition, incidence and frequency of aforementioned mtDNA mutations increase markedly with age (48). The mitochondrial respiratory capacity declines with advancing age and prevalence of CAD increases. In our patient group, however, no associations were found between patient age and ejection fraction, or age and mitochondrial oxidative capacity.

All previously discussed points lead us to conclude that impairment of mitochondrial function takes place at initial stages of heart disease, rather than being a consequence of this pathology. The primary underlying disorder leading to a decrease in cardiac contractile abilities must, affect the mitochondrial function and results in pathological remodeling that further worsens the contractile dysfunction and starts the vicious cycle. Targeting these mitochondrial abnormalities with metabolic therapy may offer a promising approach for future therapeutic

interventions to delay the progression of CVD. However, effective treatment options still remain to be found.

6. CONCLUSIONS

Cardiovascular disease affects many patients worldwide. Not only health care expenditure but also mortality is growing and will continue to grow with raising age of the population. Myocardial cells system are characterized by high-energy consumption and are strongly dependent on preserved mitochondrial function. In this paper, we showed a positive correlation between cardiac contractile abilities and mitochondrial respiratory capacity. If the ejection fraction is changing due to changes of the mitochondrial respiratory capacity or *vice versa* is a topic that exceeds the scope of this study and needs further investigation. In addition, we did not find a change in mitochondrial mass, which means that changes in EF are not due to quantitative changes, but result from qualitative mitochondrial abnormalities. Future studies are needed to investigate how mitochondria exactly affect the cardiomyocytes and how we can intervene with mitochondria-targeting therapies to slow down the progression of cardiovascular diseases.

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8. SUMMARY

Objectives: The purpose of this study was to investigate a relationship between cardiac contractile performance and mitochondrial respiratory capacity in myocardium of patients who underwent coronary artery bypass (CABG) surgery.

Materials and methods: The study included forty-one (thirty-two men and nine women) hemodynamically stable patients with coronary artery disease undergoing CABG surgery. Emergency patients, patients with ejection fraction (EF) below 40%, patients with concomitant valve replacement and patients with several renal, hepatic or pulmonary diseases were excluded. Before the CABG procedure, each patient's EF was determined by transthoracic echocardiography. A needle biopsy was performed, and tissue was obtained from anteroseptal area of the left ventricle for evaluation of the mitochondrial respiratory capacity. Following permeabilization of myocardial fibers, tissue oxygen consumption was recorded in three states: in the absence of any substrate, in the presence of substrate only and additionally with saturating amounts of adenosine diphosphate. Additionally, citrate synthase activity was measured to assess the mitochondrial mass. Correlation analysis of potential relationship between ejection fraction and mitochondrial respiratory capacity, as well as citrate synthase activity was performed using GraphPad Prism software.

Results: Our data revealed a significant positive association between mitochondrial oxygen consumption rate and cardiac ejection fraction ($r=0.35$; $P=0.026$). The higher the mitochondrial respiratory capacity in a patient is, the better the cardiac contractility. The activity of citrate synthase was not associated with ejection fraction, indicating unchanged mitochondrial mass in relation to different EF.

Conclusions: We found a positive correlation between cardiac contractile ability and mitochondrial respiratory capacity in patients with preserved cardiac systolic function. Our results cannot differentiate whether the systolic function is changing due to altered mitochondrial respiratory capacity or *vice versa*, and this topic needs further investigation. In addition, we did not find a change in mitochondrial mass in relation to EF, suggesting that changes in mitochondrial oxidative capacity stem from gradual qualitative mitochondrial alterations that parallel the changes in systolic function of the left ventricle.

9. CROATIAN SUMMARY

Naslov: Povezanost između srčane kontraktilne funkcije i mitohondrijskog respiratornog kapaciteta kod pacijenata podvrgnutih operaciji ugradnje srčane prenosnice.

Ciljevi: Svrha ove studije je bila istražiti postoji li povezanost između srčane kontraktilne funkcije i mitohondrijskog respiratornog kapaciteta u miokardu pacijenata podvrgnutih operaciji ugradnje srčane prenosnice.

Materijali i metode: Studija je obuhvatila četrdeset i jednog bolesnika (trideset dva muškarca i devet žena). Svi bolesnici su bili hemodinamski stabilni s koronarnom arterijskom bolesti te podvrgnuti operaciji ugradnje srčane prenosnice. Hitni bolesnici, oni sa srčanom izbačajnom frakcijom nižom od 40%, bolesnici s istodobnom zamjenom srčanih zalistaka, te koji s teškom bolesti bubrega, jetre ili pluća nisu uključeni u studiju. Prije operacije, svakom bolesniku je transtorakalnom ehokardiografijom utvrđena izbačajna frakcija lijeve klijetke. Za procjenu respiratornog kapaciteta mitohondrija analizirano je tkivo dobiveno iglenom biopsijom iz anteroseptalne regije lijeve klijetke. Nakon permeabilizacije mišićnih vlakana, brzina tkivne potrošnje kisika je zabilježena u tri stanja: u odsutnosti supstrata, u prisutnosti supstrata, te u prisustvu saturacijske razine adenozin-difosfata. U miokardu istih pacijenata je također izmjerena je aktivnost citrat sintaze. Kako bi se istražila potencijalna veza između srčane kontraktilne funkcije te mitohondrijskog oksidativnog kapaciteta i mitohondrijske mase, napravljena je korelacijska analiza između izbačajne frakcije, mitohondrijske respiracije i aktivnosti citrat sintaze koristeći GraphPad Prism softver.

Rezultati: Naši podaci su otkrili pozitivnu korelaciju između mitohondrijske potrošnje kisika i izbačajne frakcije lijeve klijetke ($r=0.35$; $P=0.026$). Što je veći mitohondrijski respiratorni kapacitet pacijenta, to je bolja srčana kontraktilna funkcija. Značajna asocijacija između aktivnost citrat sintaze i izbačajne frakcije nije nađena, što upućuje da se mitohondrijska masa u tkivu miokarda ne mijenja ovisno o kontraktilnoj funkciji.

Zaključci: Nađena je pozitivna korelacija između srčane kontraktilne funkcije i mitohondrijskog respiratornog kapaciteta kod pacijenata s očuvanom sistoličkom funkcijom. Na osnovu naših rezultata, ne možemo zaključiti da li je promjena sistoličke funkcije posljedica promijenjenog mitohondrijskog respiratornog kapaciteta ili obrnuto, te su potrebna dodatna

istraživanja kako bi se otkrila uzročno-posljedična veza. Nadalje, nije nađena povezanost između izbačajne frakcije i ukupne tkivne mitohondrijske mase, što upućuje da su nađene razlike mitohondrijskog respiratornog kapaciteta rezultat kvalitativnih promjena u mitohondrijima koje se događaju paralelno promjenama sistoličke funkcije lijeve klijetke.

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