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Search for Prognostic Potential of Ubiquitin, SDF1 and CXCR4 in Myocardial Infarction: The Possible New Strategy for Diagnostics

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Despite the high diagnostic value of cTn in MI, increased cTn levels are known in patients with Myocarditis and Chronic kidney failure (CKF). Patients with CKF have increased levels of creatine kinase-myoglobin binding protein (CKMB) and cTn. ECG can be normal or inconclusive in ischemia and MI partially due to electrolyte abnormalities in CKF. Time of diagnosis and treatment determines the outcome of MI. Therefore, it's important to find alternative markers of the diseases. In presented study we used plasma samples from 14 healthy and 18 MI patients. Based on the knowledge of UB, SDF1 and CXCR4 protein functions and nature of their interaction known yet UB, CXCR4 and SDF1 have been chosen for investigation. levels of proteins were measured by ELISA test and further statistical analyses conducted to determine effectiveness of proteins as alternative markers of MI. All three protein levels were elevated in MI patients compared to the healthy patient group with a statistical certainty (P-value < 0.05). A study of the correlation of protein level variability shows a tendency of positive correlation between SDF1 and CXCR4 observed in healthy group samples. Positive correlation tendency between UB and SDF1 is observed in samples of MI patients' group. Although the research was limited by the small number of test subjects and further studies are required to evaluate the value of these proteins as alternative MI markers.

Keywords: cardiovascular diseases, protein markers

INTRODUCTION

Diagnostics of Myocardial infarction

Introduction of coronary artery stenting and CABG has decreased the morbidity and mortality (Grothusen et al. 2017; Arnold, 2018; Shi and Smith, 2018), but MI still causes millions of deaths around the world (Lalloue et al. 2020). The most challenging step in treating MI is the time. Time is essential to prevent or minimize the necrosis of the myocardial cells that in turn will prevent mortality and morbidity (e.g., heart failure, mitral valve regurgitation, myocardial wall rupture etc.) (Faxon and Lenfant, 2001; Hanifi et al. 2021).

As the diagnosis of MI may seem relatively easy considering the specific clinical symptoms associated with it, existence of portable ECG machines which allows paramedics ECG taking on site, Troponin tests that can detect early myocyte damage, and the cardiac US that can show cardiac wall akinesia (Barstow et al. 2017), there are a lot of MI cases that don't follow the "Classic" presentation. Dull crushing sternal pain can often not be felt in patients with long lasting diabetes because of the damage to the sensory nerves (Culic, 2006) or pain can be felt as positional mimicking isolated pericarditis in patients with STEMI complicated with pericarditis (Tingle et al. 2007). Not all MI can be seen on ECG for example posterior wall MI (Van Gorselen et al. 2007) and not always is myocardial damage or death associated with visible akinesia of the ventricular wall on cardiac US (Kjoller, 2002). Finally, the troponin can be elevated in conditions other than MI (McLaurin et al. 1997).

The recurrent MI occurring several hours to several days after the first one renders troponin useless as the troponin levels stay elevated in blood for 7 to 14 days (Hamm et al. 2002). Troponin can also become elevated during myocarditis, after cardiac defibrillation, CABG and coronary stenting, acute heart failure, pulmonary embolism (PE) further complicating the diagnosis of acute MI. Chronically elevated troponin levels occur in patients with severe renal dysfunction and can be especially confusing for cardiologists and ER doctors when a patient presents with moderate and mild MI symptoms as in diabetic patients (McLaurin et al. 1997). The "false" elevation of troponin can lead to late or non-diagnosis of

MI in case of CKF or incorrect diagnosis of MI in case of pulmonary embolism (PE) (Korff, 2006). In any case this leads to increased morbidity and mortality of patients. In some of this cases CK-MB protein testing can be used. CK-MB is rarely used nowadays as Troponin showed superior results in detecting early MI and studies showed that about 10% of the patients with acute MI were sent home from ER during the time when CK-MB was still widely used as a test marker (Collinson, 1998). CK-MB can be falsely elevated in severely ill patients with cancer or rhabdomyolysis, and like troponin is chronically elevated in patients with CKF (Collinson, 1998; Korff, 2006). This small but just as important subset of patients especially CKF patients having MI with minor confusing symptoms Troponin and CK-MB are not very predictive. For this group clearly other markers for early detection of myocyte damage should be looked for to minimize the misdiagnosis, treatment time and of course mortality.

Measuring the levels of UB, CXCR4 and SDF1 in plasma may prove helpful in detecting myocardial damage in situations when Troponin and CK-MB are not helpful and clinical symptoms are confusing. UB is a small globular protein that plays a role in huge number of cellular and extra-cellular processes including protein degradation, repair of damaged DNA, cell movement, inflammation, and immunity (Kliza and Husnjak, 2020). UB has also demonstrated a role in various cardiac diseases including ischemia and MI (Yu and Kem, 2009).

SDF1 and its membrane receptor CXCR4 have long been studied in human MI and laboratory MI models. SDF1 levels often rise after MI, and it promotes the mobilization of bone marrow stem cells to the damaged myocardium (Askari et al. 2003). In addition, artificial upregulation of SDF1 levels in rats with MI showed better engraftment of bone marrow stem cell in the damaged myocardial tissue and hence better cardiac function (Askari et al. 2003; Ghadge et al. 2011). UB is also the ligand for CXCR4 receptor. UB binding to the CXCR4 promotes similar effects as SDF1 (Chemotaxis, Ca mobilization etc.) (Saini et al. 2011). Considering facts, extracellular UB levels were increased in patients with CAD compared to the control group and the levels of UB positively correlated with the severity of the CAD (Ji et al. 2020) and the fact that SDF1 is rapidly expressed in myocardial cells already within minutes to 1 hour after ischemia (Penn, 2009; Doring et al. 2014) gives us a hope UB, CXCR4 and SDF1 could serve as a potential marker for detection of myocardial injury when this is complicated by other traditional means.

MATERIALS AND METHODS

Selection and division of patient groups

We used plasma samples from 14 healthy and 18 MI patients. The patients were admitted to the hospital with suspicion of acute coronary syndrome (ACS), all had their troponin levels measured (Troponin I TnI), ECG, cardiac ultrasound taken, and coronarography done. The UB, SDF1 and CXCR4 levels were measured from the same plasma sample as TnI so they show the levels of all these four proteins at the same time.

Research involving human participants performed in accordance with the requirements of the Council of Europe Convention on Human Rights and Biomedicine, Biomedical Research, as well as the UNESCO Declaration of Bioethics and Human Rights and regulations established by the New Vision University Hospital ethic committee (Protocol N07/05.06.2017). We have obtained consent letters from volunteers to use their biological material for research.

Immunosorbent assay

ELISA kits for UB, CXCR4 and SDF1 quantitative measurement were purchased from the MyBioSource company (Catalog numbers: MBS726848, MBS264286, MBS2885086) and tests were performed in accordance with the instructions given.

Blood serum preparation

The sample types were all plasma taken from venous blood. The serum samples were prepared in the serum separator kit and allowed to clot for two hours at room temperature, centrifuged at 1000g (or 3000rpm) for 15 minutes and frozen at -20C degrees for storage.

Statistical methods

The concentrations of proteins tested did not follow a normal distribution in the preliminary statistical analysis, thus nonparametric statistical analyses were performed. Data are expressed as means +/- SD. Data were compared by Shapiro–Wilk test, Wilcoxson t-tests, and test comparisons were analyzed by linear regression analysis. These statistical analyses were performed with RStudio software and packages ggpmisc, ggplot2, tidyverse, car, tidyr, cowplot, MASS, rstatix, ggpubr and xtable were employed (Bruce and Bruce, 2022; Kassambara, 2020; Venables et al. 2022).

RESULTS AND DISCUSSION

For the presented study, we selected healthy volunteers and patients with a diagnosis of MI without other complications to determine the prognostic potential of ubiquitin (UB), SDF1 and CXCR4 proteins in blood plasma. If they prove to be comparable markers to troponin in patients with MI, UB, CXCR4 and SDF1 would serve as alternative markers in patients with other complications like diabetes or CKF when both Troponin and CKMB are elevated making the diagnosis of MI obscure.

To establish the concentration difference ranges between three proteins in groups of healthy and MI diagnosed patients, we conducted an ELISA test of blood serum provided by the clinic. The results were studied in detail by statistical methods. Statistical evaluation of the

test results is presented in Table1.

Differences between protein concentrations were detected between groups. It is evident that the levels of all proteins in healthy group is lower than in MI group. To obtain the information about statistical importance of this fact we used the unpaired Wilcoxon rank sum test which is used to compare two independent groups of samples if data are not normally distributed (Table 2, Figure 1).

Table 1 Total descriptive summary of proteins.

Protein	Diagnosis	n	mean	SD	min	Q1	median	Q3	max
Ubiquitin ng/ml	**Healthy**	14	29.893	9.024	18	25	28	35.5	51
	MI	18	62.817	25.749	38	40	55.5	78.3	114
CXCR4 ng/ml	**Healthy**	14	0.418	0.289	0.1	0.225	0.325	0.5	1.1
	MI	18	0.741	0.317	0.2	0.435	0.8	0.975	1.2
SDF1 ng/ml	**Healthy**	14	0.619	0.114	0.49	0.502	0.62	0.685	0.81
	MI	18	0.712	0.191	0.3	0.65	0.74	0.8	1

Table 2 Wilcoxon rank sum test summary statistics.

Protein ng/ml	group1	group2	n1	n2	statistics	*P. value*	*P. value significance*	Effect size	magnitude
UB	Healthy	MI	14	18	11.5	0.0001	****	0.77	large
CXCR4	Healthy	MI	14	18	60	0.01	*	0.44	moderate
SDF1	Healthy	MI	14	18	70.5	0.035	*	0.375	moderate

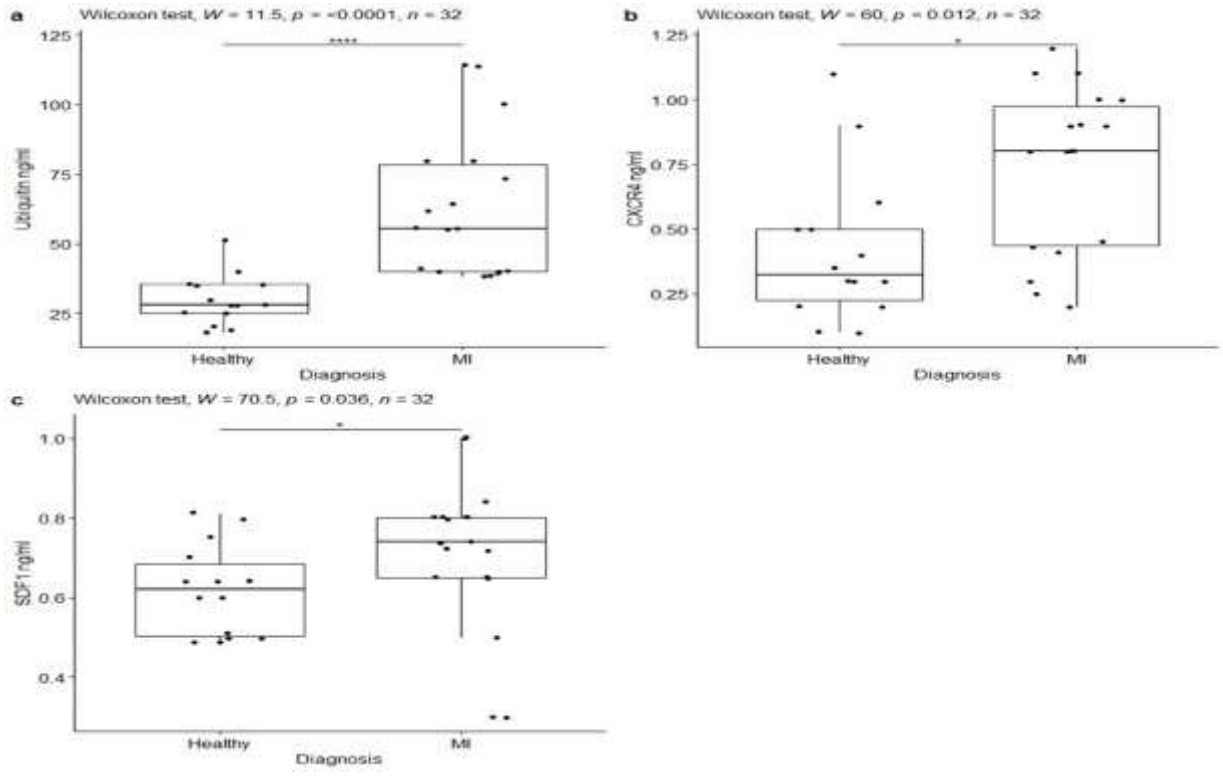

Figure 1: Wilcoxon rank sum test to compare significance of differences of proteins amount according to diagnosis: a. Ubiquitin, b, CXCR4, c. SDF1

According to the output above, it can be concluded that medians of ubiquitin, SDF1 and CXCR4 concentrations in healthy groups are significantly different from the median of their concentrations in MI groups with *P-value* = 0.000142, effect size r = 0.771 for ubiquitin, *P-value* = 0.035, effect size r = 0.375 for SDF1 and *P-value* = 0.0125, effect size r = 0.445 for CXCR4. But the number of lower outliers for CXCR4 in both groups is significant.

All three proteins share the same important statistical dependency on the health condition. But number of lower outliers for CXCR4 receptor confirms the suspicion that this protein may not be useful for diagnosis if further research has not proven otherwise.

Standard statistical normalization technique, like quadratic and log transformation of the initial data, was used for data normalization (not presented here). Still normalization did not significantly improve the quality of the models, and for better understanding of the results, we decided to rely on primal, not transformed data to describe the possible relationship of proteins.

To better identify the interdependences between the proteins within the groups, we analyzed the data by regression method (**Table 3, Figure 2**).

Table 3 : (a)MOD1, (d)MOD2 – Ubiquitin level in a role of dependent variable, CXCR4 level is a predictor; (b)MOD3, (e)MOD4 – Ubiquitin level is a dependent variable, SDF1 level is a predictor; (c)MOD5, (f)MOD6 –SDF1 level is a dependent variable, CXCR4 level is a predictor; models were built up separately for healthy and MI group

	Dependent variable:					
	Ubiquitin level				**SDF1 level**	
Predictors	**(a)MOD1 (Healthy)**	**(d)MOD2 (MI)**	**(b)MOD3 (Healthy)**	**(e)MOD4 (MI)**	**(c)MOD5 (Healthy)**	**(f)MOD6 (MI)**
CXCR4	-1.009 +/-9	-1.443 +/-20.284			0.25* +/-0.08	-0.036 +/-0.15
SDF1			5.394 +/-22.828	77.036** +/-27.709		
Constant	30.315*** +/-4.521	63.886*** +/-16.282	26.552* +/-14.357	7.993 +/-20.378	0.51*** +/-0.044	0.738*** +/-0.12
Observations	14	18	14	18	14	18
R^2	0.001	0.0003	0.00446	0.326	0.404	0.004
Adjusted R^2	-0.082	-0.062	-0.0783	0.284	0.354	-0.059
Residual Std. Error	9.387 (df = 12)	26.537 (df = 16)	9.371 (df = 12)	21.794 (df = 16)	0.091 (df = 12)	0.196 (df = 16)
F Statistic	0.013 (df = 1; 12)	0.005 (df = 1; 16)	0.0558 (df = 1; 12)	7.730** (df = 1;16)	8,137* (df = 1; 12)	0.057 (df = 1; 16)
Note:	**P<0.1; **P<0.05; ***P<0.01*					

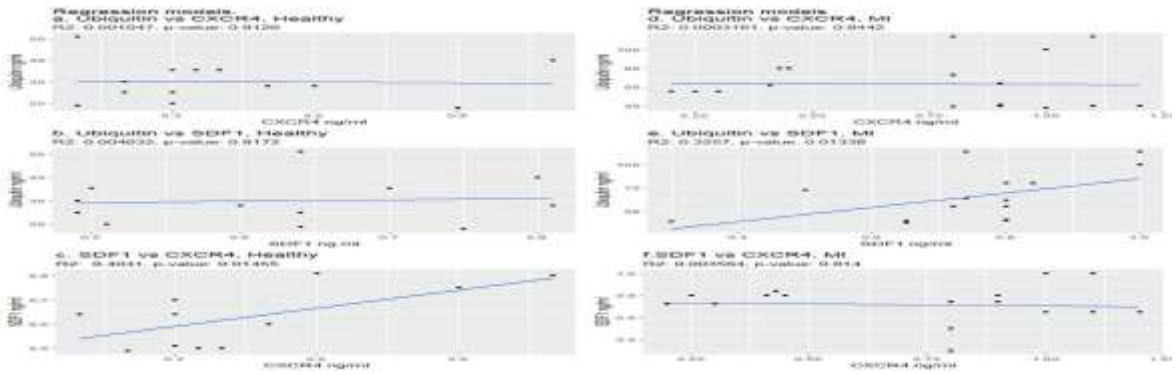

Figure 2 summarized parameters for all simple linear regression models in different groups. Simple Linear regression models: a. (Mod.1) Ubiquitin and CXCR4 in healthy group; b. (Mod.3) Ubiquitin and SDF1 in healthy group; c. (Mod.5) SDF1 and CXCR4 in healthy group; d. (Mod.2) Ubiquitin and CXCR4 in MI group; e. (Mod.4) Ubiquitin and SDF1 in MI group; f. (Mod.6) SDF1 and CXCR4 in MI group.

In this statistical evaluation the simple regression models are created separately for each pair of proteins in different groups (Table 3). The aim was to predict a quantitative outcome of ubiquitin level based on CXCR4 and SDF1 levels used as a predictor and SDF1 level based on CXCR4 level as a predictor (Figure 2).

Even though the investigated proteins' concentrations are elevated in MI group compared to healthy group, relationship between SDF1 and CXCR4 in healthy group and between UB and SDF1 in MI group was observed, though must be noticed that it explains less than a half of the variance.

Coronary artery disease (CAD) which is the part of the cardiovascular diseases (CVD) affects the arteries of the heart and can lead to myocardial infarction (MI). MI is the leading cause of death despite effective treatment methods like coronary artery stenting or coronary artery bypass graft (CABG) being implemented in many hospitals worldwide (Grothusen et al. 2017; Arnold, 2018). The diagnosis of MI is usually based on clinical symptoms, ECG findings and cardiac troponin (cTn) either troponin T (TnT) or troponin I (TnI) measurements (Arnold, 2018; Barstow et al. 2017). Despite the high diagnostic value of cTn in MI, increased cTn levels are known in patients with Myocarditis and Chronic kidney failure (CKF). Patients with CKF have increased levels of creatine kinase-myoglobin binding protein (CKMB) and cTn. ECG can be normal or inconclusive in ischemia and MI partially due to electrolyte abnormalities in CKF (McLaurin et al. 1997; Hamm et al. 2002). So, while some cases of MI in CKF patients can be easily diagnosed even with chronically increased cTns, others are not especially when MI symptoms mimic fatigue or shortness of breath (SOB) etc. instead of "classic" presentation, considering CKF patients often have other conditions that can worsen the outcome of the MI (Culic, 2006). Time of diagnosis and treatment determines the outcome of MI (Faxon and Lenfant, 2001). Therefore, it is important to find alternative markers of the diseases.

CONCLUSION

The level of recognized protein biomarkers of MI is high in other diagnosis as well. Consequently, diagnosis is often missed until the cardiac muscle damage becomes very large and present with visual fields of defects and leads to the irreversible processes or death. Alternative markers should be sought to avoid misinterpretation of MI diagnosis. The results of our study showed that the concentrations of all three proteins are higher in the blood serum of patients diagnosed with MI compared to healthy ones. However, it should be noted that in CXCR4 samples low outlier point number counts about 30%, that raises additional questions. A study of the correlation of protein level variability shows a tendency of positive correlation between SDF1 and CXCR4 observed in healthy group samples. Positive correlation tendency between UB and SDF1 is observed in samples of MI patients' group. That seems to be logical based on the knowledge of UB, SDF1 and CXCR4 protein functions and nature of their interaction known yet. The study clearly shows that further research on at least UB and SDF1 makes sense in search of additional biomarkers for MI diagnostics.

CONFLICT OF INTEREST

No conflict of interest scientific, financial or otherwise is declared by the authors. All authors agree with the content of the manuscript. We certify that the presented article is an original work and is not under consideration at any other journal.

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AUTHOR CONTRIBUTIONS

GJ collected biological material, performed Elisa tests and wrote the manuscript. II provided statistical data analysis. RS supervised the study, designed and monitored experiments, and reviewed the manuscript. All authors read and approved the final version.

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