



## Protective Antibodies to Bovine Milk Fat Globule and to Xanthine Oxidase Levels in Jordanian Patients with Acute Myocardial Infarction

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### ABSTRACT

**Background:** The previously reported circulating human antibodies against the Bovine Milk Fat Globule Membrane (BMFGM) were found to primarily target xanthine oxidase (XO) enzyme that produces uric acid and reactive oxygen species. It is suggested that XO could potentially be implicated in the pathogenesis of acute myocardial infarction.

**Methods:** In this study, anti-BMFGM and anti-XO IgG, IgM and IgA antibodies were assayed in the sera of acute myocardial infarction patients and healthy control from the Jordanian population using the highly sensitive Enzyme-linked immunosorbent assay (ELISA). **Results:** Serum high in antibodies against xanthine oxidase was used as a reference serum to standardize the assay. The levels of anti-BMFGM IgM antibodies were found to be higher in male controls than myocardial infarction male patients in contrast to female group, but no significant differences were observed in the levels of IgG and IgA antibodies. The levels of anti-xanthine oxidase IgM and IgG antibodies were significantly higher in myocardial infarction patients when compared with their corresponding controls. Conflicting results were obtained when different personnel measured the IgM antibody titres, likely due to infarction factors of IgM aggregation within the assay. Results from this study demonstrate significant differences in the levels of anti-BMFGM and anti-XO IgM antibodies between myocardial infarction patients and controls. **Conclusion:** Collectively, the data suggest that XO may be a risk factor in myocardial infarction patients and the presence of antibodies may act as a protective factor.

**Keywords:** Milk fat globule membrane (MFGM), Xanthine oxidase (XO), Myocardial infarction (MI), Immunoglobulins (Ig)

### INTRODUCTION

Acute myocardial infarction is a life-threatening manifestation of coronary heart disease characterized by the gross reduction, or total cessation, of coronary artery blood flow leading to myocardial tissue necrosis [1]. It causes more than 2.4 million deaths in the USA and 4 million deaths in Europe and northern Asia annually [2,3]. Most acute myocardial infarctions occur due to the rupture of vulnerable coronary atherosclerotic plaques leading to superimposed thrombosis. The superimposed thrombosis reduces or stops blood flow to the myocardium causing myocardial tissue necrosis, clinically detected by elevated serum cardiac biomarkers like Troponin T and CK-MB. Due to the severity and acuteness of the disease, there has been a global interest in finding risk factors that could potentially predict its risk [4].

In the late 1960s and early 1970s, Davies, et al. [5] demonstrated that circulating antibodies to whole dried cow's milk specifically target the bovine milk fat globule membrane (BMFGM) in patients with acute myocardial infarction [6]. This was supported by researchers such as Oster, et al. [7] but contradicted by others [8]. Nonetheless, all studies agreed that antibodies against BMFGM primarily target the enzyme xanthine oxidase (XO) [9]. This was important as it provided a potentially new target that could be used to estimate the risk of myocardial infarction, allowing precautionary measures to be taken in high risk individuals.

Xanthine oxidoreductase (XOR) is a widely-distributed enzyme found in milk, liver, gut, synovium [10] and capillary endothelial cells belonging to the molybdenum family, with a net molecular mass of ~290 kD [11]. Its primary function is the catabolism of purine nucleic acids, catalyzing the oxidation of hypoxanthine to xanthine then to uric acid [9]. The function of this enzyme is vital in the human body as purines are continuously being degraded and renewed, but over-activity could potentially be harmful. XO also exists in another form known as xanthine dehydrogenase (XDH) which is interconvertible with XO and catalyzes the same reactions. XDH reduces NADH while XO reduces oxygen into superoxide anion and eventually forms hydrogen peroxide (a reactive oxygen species (ROS), which in excess, is toxic to mammalian cells. ROSs, especially XO, have been shown to be linked to several disorders such as hypertension and rheumatoid arthritis, sparking interest in researching their pathogenic involvement in ischemia-reperfusion disorders [12], such as myocardial infarction [12]. ROSs have also been recently shown to act as intermediates in normal signal transduction mechanisms [10].

The suggested pathogenic mechanism of XO primarily depends on the proteolytic conversion of XDH to XO secondary to ischemia, and is based on the established properties of rat liver and bovine milk enzymes [9,13]. In addition, ischemia causes the breakdown of ATP and ADP to hypoxanthine which upon reperfusion, is used as a reducing substrate for newly formed XO. This generates ROSs through the consumption of molecular oxygen. Given that ischemia-reperfusion is a major pathophysiological process in myocardial infarction, investigating the role of XO in disease pathogenesis is paramount. It is important to note that XO activity levels in healthy subjects are usually very low [12], however, it has been demonstrated that XO levels are significantly higher in rheumatic disease [14], liver disease [10] and ischaemia-reperfusion diseases [12]. Higher than normal levels may suggest an underlying pathological process and can be used to predict and prevent disease. More importantly, the presence of anti-XO antibodies in the serum highlights the pathogenic nature of this enzyme and its potential dangers.

Human antibodies, or immunoglobulins, are proteins produced mainly by plasma cells and occur in different isotypes including IgM, IgG and IgA that differ in molecular structure, distribution and target specificity and are determined routinely in clinical practice because they give key information on the humoral immune status [15] and play an important role in immunity by helping fight and neutralize pathogens. IgG is the predominant isotype circulating in the human serum while IgA is predominantly found in human milk and the colostrum [16]. In this study, the levels of circulating immunoglobulins against BMFGM and XO were measured in Jordanian patients with acute myocardial infarction and healthy control. The aim was to identify the relationship between myocardial infarction and anti-XO and anti-BMFGM, and identify new risk factors for the disease in the Jordanian population. In addition, we evaluate the presence of antibodies as protective factors in myocardial infarction.

## MATERIALS AND METHODS

### Patients and Controls

605 acute myocardial infarction patients (290 females and 315 males) aged 40-50 years-old were recruited into the study from the Arabic Centre Hospital, Amman, Jordan. Acute myocardial infarction was diagnosed by clinical history of central crushing chest pain lasting at least 30 minutes, positive electrocardiogram (ECG) and elevated cardiac enzymes including Troponin T, aspartate transaminase (AST), alanine transaminase (ALT), creatine kinase (CK), iso-enzyme for creatine kinase (CK-MB) and lactate dehydrogenase (LDH). Only those who were in a stable clinical and metabolic rate were recruited. 580 healthy controls (280 females and 300 males) were also recruited into the study from different out-patient clinics. Patients and controls were comparable for age, height, body mass index (BMI) and family history of myocardial infarction. Inclusion criteria: all participants must have a sedentary life-style, are non-smokers, have no significant past medical history and are not on any medications.

### Serum Samples and Measurement of Cardiac Enzymes

The BMI of all participants was calculated as weight in kg divided by the square of height in meters. Blood samples were collected and allowed to coagulate. The serum was extracted and stored at -70° for later use. The levels of AST, CK, CK-MB and LDH were measured using Cobas 800 analyzer, Roch Diagnostics GmbH, Germany as per manufacturer's protocol. The results were expressed in international unit/l of enzyme activity. The level of Troponin T was measured using a commercially available sandwich ELISA kit (Roch Diagnostics GmbH, Germany) as per manufacturer's protocol and results were expressed in nanogram/millilitre.

### **C-reactive Protein CRP Concentration Estimation**

CRP levels were determined by latex agglutination test using plates purchased from Behringer, Germany. The test was done as per manufacturer's protocol. The resulting agglutination was measured by spectrophotometer at 580 nm. Positive and negative controls were included. Values were considered as positive if they were higher than 9.4 mg/l for male and 8.55 mg/l for female.

### **Erythrocyte Sedimentation Rate ESR Estimation**

Modified Westergren method was used to determine the rate [16].

### **Measurement of Biochemical Parameters**

Total cholesterol (TC) was measured using an Enzymatic (CHOD-PAP) colorimetric method [17], total triglyceride (TTG) was measured using an Enzymatic (GPO-PAP) method [18]. High and low density lipoprotein Cholesterol (HDL-C, LDL-C) were estimated using a Precipitant method [19] and Friedewalds formula respectively [20]. Friedewalds formula:  $LDL-C = TC - HDL-C - (TG/5)$ . Glucose was measured by using standard techniques [21].

### **Ethical Approval**

The study was conducted in accordance to the standards of ethics outlined in the Declaration of Helsinki and general informed consent was obtained from all participants regarding the use of their specimens. The study protocol was also approved by the hospital and university ethics committees.

### **Milk and Reagents**

Fresh bovine milk was prepared according to the methods outlined in Benboubetra, *et al.* [22] and Sanders, *et al.* [23]. Purified XO enzyme was purchased from Biozyme Laboratories, Blaenavon, Wales, UK and all reagents were purchased from Sigma, Poole, UK unless otherwise stated.

### **Preparation of BMFGM**

12L of fresh uncooled milk were centrifuged at 37° (172 g, 15 min) to separate the cream. Equal volumes of distilled water at 37° containing 0.15 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM ethylenediaminetetraacetic acid, disodium salt (EDTA) and 2 mM benzamidine were used to wash fat globules before centrifugation at 37° (250 g, 10 min). Cream was washed for at least 3-4 times until a clear wash was obtained and the final wash was performed at 4°. The cream was re-suspended (5:1 v/v) in 50 mM Tris/ HCL, pH 7.5 and chilled to 4° in an ice bath before mixing to butter in a warring blender for 5-10 minutes. To release the BMFGM, the butter was collected and liquefied at 37° then centrifuged at 100,000 g for one hour at 20°. BMFGM was collected and stored at -80°. Yields varied from 2-6 wet weight of BMFGM, depending upon the time of year.

### **Determination of Anti-XO Antibodies and Anti-BMFGM Antibodies by Enzyme Linked Immunosorbent Assay (ELISA)**

Antibodies used were as described by Harrison, *et al.*, Benboubetra, *et al.* and Ng, *et al.* [8,22,24]. ELISA was performed to determine the level of different classes of antibodies in acute myocardial infarction patients and controls using purified XO enzyme as antigen. To measure the antibodies against BMFGM, the procedure was identical to that of XO except that BMFGM was homogenized in 50 mM sodium carbonate buffer pH 9.6 at a concentration of 10 Mg BMFGM protein/mL carbonate buffer. Standard protocols were followed.

### **Statistical Analysis**

Statistical significance was determined using an unpaired Student's t-test calculated by Statistical Packages for Social Science Software (SPSS). The results are expressed as mean ± SD standard deviation for triplicate, and p-value <0.05 was considered statistically significant.

## **RESULTS**

Demographics and the biochemical parameters of MI patients and control are demonstrated in Table 1.

**Table 1 Demographics of acute myocardial infarction (MI) patients and control group**

Parameter (Unit)	Males		Females	
	Controls	MI Patients	Controls	MI Patients
	n=300	n=315	n=280	n=290
Age (years)	42.4 ± 6.6	40.8 ± 8.8	41.8 ± 7.5	41.7 ± 5.8
BMI (Kg/m <sup>2</sup> )	29.2 ± 7.6	28.9 ± 9.1	28.9 ± 8.9	29.6 ± 8.8
ESR (mm/h)	11.2 ± 5.6	55.6 ± 35.9 <sup>a,b</sup>	10.6 ± 6.2	61.8 ± 33.6 <sup>a,b</sup>
CRP (mg/l)	2.91 ± 1.4	13.16 ± 4.8 <sup>a,c</sup>	3.21 ± 2.6	16.8 ± 5.7 <sup>a,c</sup>
Hypertension risk factor	-	All	-	All
Physical activity	Active	Moderate	Active	Moderate

BMI: Body Mass Index; ESR: Erythrocyte Sedimentation Rate; CRP: C-Reactive Protein. Values in the table represent the mean ± SD standard deviation, n=3. a p<0.0001: significantly different from corresponding control; b p<0.0001; c p ≤ 0.001: significantly different from opposing gender with MI

CRP levels were determined using latex agglutination and were higher in MI patients compared to controls, indicating the presence of inflammation. The mean age and BMI for both MI male and female patients and control groups were comparable. ESR (1 hr) values were significantly higher in MI patients compared to control. Both male and female MI patients had a positive hypertension risk. MI patients had a sedentary life style when compared to controls.

The cardiac biomarkers serum levels in MI patients and control were shown in Table 2.

**Table 2 Serum levels of cardiac biomarkers in control group and MI patients**

Parameter	Control (n=580)		MI (n=605)	
	Male	Female	Male	Female
	n=300	n=280	n=315	n=290
AST (IU/l)	23 ± 5.2	25 ± 6.5	38 ± 10.4 <sup>*a</sup>	39.5 ± 9.5 <sup>+</sup>
LDH (IU/l)	225-450	225-450	470 ± 39.6 <sup>**b</sup>	500 ± 40.5 <sup>++</sup>
CK (IU/l)	69.2 ± 18.7	66.8 ± 16.5	130.2 ± 21.6 <sup>*b</sup>	126.5 ± 26.5 <sup>++</sup>
CK-MB (IU/l)	12.5 ± 2.8	11.5 ± 1.6	88.6 ± 14.5 <sup>**b</sup>	97.2 ± 8.7 <sup>++</sup>
Troponin T (ng/ml)	0.025 ± 0.007	0.03 ± 0.01	1.19 ± 0.27 <sup>*a</sup>	1.92 ± 0.1 <sup>+</sup>
TC (mg/dl)	<200	<200	246.11 ± 10.41 <sup>**b</sup>	233.81 ± 9.27 <sup>++</sup>
LDL-C (mg/dl)	<100	<100	170 ± 15.12 <sup>**b</sup>	142.81 ± 16.11 <sup>++</sup>
HDL-C (mg/dl)	35-55	35-55	39.81 ± 8.64 <sup>*</sup>	46.91 ± 9.25 <sup>+</sup>
TTG (mg/dl)	<200	<200	95.81 ± 13.22	90.91 ± 12.33
Glucose (mg/dl)	70-100	70-100	93.61 ± 4.21	90.91 ± 5.22

AST: Aspartate Transaminase; LDH: Lactate Dehydrogenase; CK: Creatine Kinase; CK\_MB: iso-enzyme for creatine kinase; TC: total cholesterol; LDL-C: Low Density Lipoprotein-Cholesterol; HDL-C: High Density Lipoprotein-Cholesterol; TTG: Total Triglyceride. Values in the table represent the mean ±SD standard deviation, n=3. \* p<0.01, \*\* p<0.001, \*\*\* p<0.0001 between MI male and female patients. a p<0.001, b p<0.0001 between MI male patients and the male control group. + p<0.001, ++ p<0.0001 between MI female patients and the female control group

As demonstrated in Table 2, all the cardiac biomarkers including AST, LDH, CK, CK-MB, and Troponin T were elevated in patients with MI compared to control. The lipid profile in the control group was normal, while in MI patients, the TC, LDL-C and HDL-C were elevated but TTG was not. Blood glucose levels were within normal range.

Table 3 levels of anti-BMFGM in control and MI patients

Parameter	Control Group (n=580)		MI Patients (n=605)	
	Male	Female	Male	Female
	n=300	n=280	n=315	n=290
IgM ( $\mu\text{g/ml}$ )	63.3 $\pm$ 30.27 <sup>x</sup>	56.7 $\pm$ 25.18	40.6 $\pm$ 20.66*	59.5 $\pm$ 23.43
IgG ( $\mu\text{g/ml}$ )	33.4 $\pm$ 11.66	35.4 $\pm$ 16.4	38.2 $\pm$ 17.45	38.8 $\pm$ 14.96
IgA ( $\mu\text{g/ml}$ )	19.86 $\pm$ 5.65	19.8 $\pm$ 6.97	23.3 $\pm$ 8.17	21.7 $\pm$ 6.19

Values represent the mean  $\pm$  SD standard deviation, n=3. \* p<0.001 between MI male and female patients. x p<0.01 between male and female control group

Human antibodies of different isotypes (IgM, IgG, IgA) to BMFGM and XO were determined by different researchers and carried out on human sera (males and females). As shown in Table 3, the level of IgM anti-BMFGM in the male control group was higher than the female group. The level of IgG anti-BMFGM in the male control group was lower than the female group. No differences were found in the levels of IgA between male and female controls.

The levels in MI patients demonstrate that IgM anti-BMFGM in males was lower than in females and that no significant differences are found in the levels of anti-BMFGM IgG and IgA between male and female MI patients.

Table 4 levels of anti-XO in control and MI patients

Parameter	Control Group (n=580)		MI Patients (n=605)	
	Male	Female	Male	Female
	n=300	n=280	n=305	n=300
IgM ( $\mu\text{g/ml}$ )	9.55 $\pm$ 2.62 <sup>x</sup>	14.2 $\pm$ 2.22	129.09 $\pm$ 31.30 <sup>a</sup>	143.09 $\pm$ 26.30 <sup>*+</sup>
IgG ( $\mu\text{g/ml}$ )	13.2 $\pm$ 2.05 <sup>xx</sup>	16.2 $\pm$ 3.31	36.37 $\pm$ 2.35 <sup>b</sup>	46.43 $\pm$ 23.62 <sup>**+</sup>
IgA ( $\mu\text{g/ml}$ )	1.65 $\pm$ 0.76	2.05 $\pm$ 0.95	2.15 $\pm$ 1.78	2.40 $\pm$ 1.61

\* p<0.001, \*\* p<0.01 significant difference between MI male and female patients. a p<0.0001, b p<0.001 between MI male patients and the male control group. + p<0.0001 between MI female patients and the female control group. x p<0.001, xx p<0.01 between male and female control group

As shown in the table above, the level of IgM anti-XO in the male control group was significantly lower than female control group. IgG and IgA anti-XO were lower in the male control group compared to the female control group. In MI patients, IgM and IgG levels were higher in females than in males and the levels were significantly higher than the levels in the control group. No significant differences were found in the levels of IgA between MI patient and controls.

The levels of anti-BMFGM and anti-XO IgG antibodies were shown in Tables 3 and 4 above and the results show that the level of IgM in the male control group was higher than in male MI patients, while the levels in female MI patients were higher than the female control group. No significant differences in the levels of IgG and IgA between MI patients and controls were observed in respect to sex.

## DISCUSSION

In patients with MI, the inflammatory response, in particular that of T and B cells, play an important role in the pathogenic remodelling of the myocardium [25]. There is a fine balance between the protective and pathogenic properties of the immune system, wherein a change in the tight homeostasis may lead to disease such as acute myocardial infarction. In this study, ELISA was used to measure the levels of IgM, IgG and IgA to xanthine oxidase and BMFGM to study the protective properties of the immune system in acute myocardial infarction. Our results demonstrate a great variability in serum immunoglobulin levels between different ages and sexes. The levels of anti-BMFGM IgM antibodies in females with acute myocardial infarction were higher than the female control group but interestingly, IgM levels were lower in males with acute myocardial infarction than male control group. But importantly, the levels of anti-BMFGM IgM antibodies in females with acute myocardial infarction were significantly higher than in their male

counterparts. This could potentially be explained by the hormonal differences between the two sexes; it has been long recognised that oestrogen in women delays the onset and progression of atherosclerosis and thus the manifestation of coronary heart disease [11]. Our results agree with some studies but not others, demonstrating a variability in study methods and the complexity of the topic.

In contrast, our results demonstrate a non-significant increase in serum anti-BMFGM IgA and IgG antibodies in myocardial infarction patients compared to control. This disagrees with most of the previously published studies [7,8]. The increase in IgA levels may suggest a subclinical event preceding the attacks or a long-term increase in IgG levels in individuals susceptible to myocardial infarction. It is important to note that myocardial infarction is less likely to occur in individuals who generate and maintain higher IgM responses to bovine XO, therefore the increase in IgA and IgG in myocardial infarction supports the suggestion that B-lymphocytes are affected by circulating hormones, giving rise to differences between genders [26]. These differences are important, especially when interpreting the levels of antibodies in diseases with unequal sex distribution as it could provide insight into potential pathogenic differences.

The main antigen of BMFGM has been shown to be XO, which was used to detect anti-XO antibodies in this study. There was a significant increase in anti-XO IgM antibodies in male and female myocardial infarction patients compared to control, which is in agreement with McCord, et al. [12], and the antibody was higher in female MI patients compared to male MI patients which may be due to gender difference as previously reported [26,27], as well as a significant increase in anti-XO IgG antibodies in male and female myocardial infarction patients compared to control which is not in agreement with other studies [12]. This could be explained by the fact that IgG anti-XO antibody titres are considerably higher than those of IgM antibodies, but IgM antibodies are more efficient in immune complex formation. Compared to anti-XO IgM and IgG, IgA levels were higher in male and female myocardial infarction patients compared to control but the increase was not significant, this was also reported by others [12]. The reason for this is not fully understood, but could be due to unidentified genetic variants, differences in quality and type of milk consumed, metabolic differences or even differences in ethnicities of the populations.

What remains unclear is the reason for the increase in human anti-bovine milk (XO) in myocardial infarction patients against the enzyme present in ingested bovine milk. One suggested hypothesis propose that the ingested active enzyme is absorbed from the intestines and deposited in the intima of coronary arteries. The active enzyme then catalyses the oxidation of plasminogen derived fatty aldehydes causing pre-atherosclerotic lesions in the arteries [28]. However, this hypothesis was widely debated and criticized, often on the basis that absorption of enzymatically active bovine XO is certainly continuous [29,30]. Another explanation could be that these antibodies are raised against endogenous XO rather than ingested bovine milk, which is widely available in the small intestine, mammalian liver, and the milk fat globule membrane of several species and localized on endothelial and epithelial cells [31].

There has been an interest in the endogenous form of the enzyme as a source of ROSs since the early 90s which has been shown to be implicated in various diseases. The interest died down after several published studies failed to detect the enzyme in the human heart [31-33]. However, later studies demonstrated anti-XO IgG and IgM antibodies in the human heart with evidence of correlation between IgM antibodies and acute myocardial infarction. These antibodies are directed towards ingested bovine milk X, as well as purified human XO [34,35].

## CONCLUSION

Our results demonstrate a connection between serum anti-BMFGM and anti-XO and myocardial infarction. There were contradictions in our anti-BMFGM results that could be attributed to differences in the antigenic complex mixture (BMFGM) used, or due to anti-BMFGM antibodies binding to platelets in the form of immune complexes. Other reasons include variations in diet especially milk, metabolic differences and differences in ethnicity. This is especially relevant in the Jordanian population as camel, sheep and goat milk are widely consumed whereas bovine milk is the primary type of milk consumed in participants of other studies. Further studies are needed to incur that myocardial infarction is initiated by XO induced endothelial damage leading to atherosclerosis and that bovine XO can be used to determine IgG, IgM and IgA specificities.

## DECLARATIONS

### Authors' Contributions

This research was the result of the collaboration of all authors. All authors participated in the study design, clinical testing and in writing the manuscript. All the authors read and approved the final manuscript.

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### Availability of Data and Materials

Available on request from the authors.

### Ethical Approval

The study protocol was approved by the Hospital and University ethics committees.

### Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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