Detection of Biological Nano-Particles in Egyptian Patients with Coronary Artery Disease

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Abstract: Cardiovascular diseases accounted for more deaths than any other major cause of death worldwide. Biological nanoparticles (BNP) are novel bacteria with the smallest cellular dimension known on earth (50–300 nm). BNP are recently accused to play the role in the process of endothelial injury and hence arthrosclerosis facilitating coronary artery disease (CAD). **Objective:** The aim of this study was to detect BNP in serum and urine samples of CAD patients to determine their role, incidence, prevalence and correlation with occurrence of coronary artery disease in Egyptian patients. **Method:** The study was conducted on 38 patients and was divided into two groups. Group (1) included 28 patients with confirmed CAD by coronary angiography. Group (2) included 10 patients with excluded CAD by coronary angiography as control group. Both groups were subdivided into 2 subgroups according to sample type; urine and serum. Patients were subjected to clinical examination, chest X ray, EGG recording, echocardiography, coronary angiography and laboratory investigations. BNP was detected in human blood and urine by scanning electron microscope (SEM). **Results:** BNP were detected by SEM in 78.57% of CAD patients, in serum and urine 53.57% and in serum only 25%, showing a strong association between BPN detection and CAD ($p< 0.01$). No statistical significance was shown between BNP serum and urine negative and positive groups regarding age, gender, hypertension, diabetes, smoking and lipid profile. Coronary angiography results in BNP serum and urine positive patients emphasized same significant LAD lesions in 19 (86.36%) and 17 (100%) of patients ($p < 0.01$), followed by LCX in 16 (72.73%) and 13 (76.47%) of patients ($p < 0.05$) then RCA in 16 (72.73%) and 12 (70.59%) of patients ($p > 0.05$) and LMCA was the least to be affected showing 3 (13.64%) and 14 (8.35%) of patients with a same significant statistical reverse correlation ($p < 0.01$) respectively. Nine (52.94%) BNP urine positive versus one (9.1%) BNP urine negative patients showed significant statistical finding between BPN detection in urine and RWMA as a component of estimating the cardiac muscle condition. **Conclusion**: BNP was detected by SEM in (78.57%) of the total patients with known CAD with variable grades and sites of coronary lesions. These data may help to understand the critical medical importance of already demonstrated effects of BNP on atherosclerosis and pathologic calcification in the human body especially coronary arteries. Screening of body fluids for BNP on a large scale could be necessary for the assessment of co-infections with BNP especially in susceptible persons with risk of developing CAD.

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1.Introduction:

Atherosclerosis is the development of plaques within the intimal layer of large vessels. It underlies coronary artery disease (CAD) and cerebrovascular disease and it is considered the most common form of life threatening cardiovascular disorder **(Doherty *et al.*, 2004).** The process of atherosclerosis and hence vascular calcification is associated with significant mortality and morbidity of cardiovascular disease **(Wayhs *et al.*, 2002)**. Indeed clinically, vascular calcification is accepted now as a valuable predictor of coronary heart disease including myocardial infarction and stroke **(Greenland *et al.*, 2007).**

Biological Nanoparticles (BNP) were first discovered by Olavi Kajander since 1988 **(Kajander *et al.*, 1998).** They are novel cell-walled self-replicating bacteria with the smallest cellular dimension known on earth, about 100-fold smaller than standard bacteria and many viruses, with dimensions of 20-300 nm in length and hundreds of a cubic micron in volume **(Sommer *et al.*, 2003).** BNPs isolated from blood characterized by being slowly growing, pleomorphic, and apatite mineral-forming agents, with spherical or ovoid architecture. Furthermore they have widespread abundance in human and animal blood and tissues (**Kajander, 2006).** BNPs are mysterious particles that have spurred

one of the biggest controversies in modern microbiology (**Drancourt *et al*., 2003).**

BNP was listed as a possible etiologic infectious agent because of its unique ability to cause infection, inflammation and calcium deposition. BNP can facilitate the precipitation and growth of calcium phosphate in pathological conditions and hence they have been involved in a long list of diseases **(Ciftcioglu and McKay, 2010).** BNP were isolated from kidney stones and urine of patients with renal lithiasis **(Badawi *et al.,* 2013a),** renal fluid taken from patients with polycystic kidneys **(Hjelle *et al.,* 2000)** and biliary tract in patients with cholecystitis **(Badawi *et al.,* 2013b),** inclusions of psammoma in ovarian cancers **(Hudelist *et al.,* 2004),** peripheral blood from healthy subjects and atheromatous plaques **(Bratos-Perez *et al.,* 2008)**. BNP are thought to play an important role in extra skeletal calcifying diseases including, periodontal disease **(Ciftcioglu *et al.,* 2003**), rheumatoid arthritis **(Cassell, 1998)** and prostatitis **(*Geramoutsos et al.,* 2004).**

***Vita and Loscalzo* (2002)** presented an overview of the relationships among infection, atherosclerosis, and consequent endothelial dysfunction. They concluded that chronic infection may be one of the important factors inducing endothelial dysfunction. Microbes, their toxins or their shed components may contribute to pathological calcifications in several ways **(Garcia *et al.,* 2000).** BNP or its fragments may be nidi for the formation of pathological calcifications and may also contribute directly to the primary pathogenesis of disease by acting as a system for the delivery of microbial and other toxins to tissues **(Garcia *et al.,* 2000; Kajander *et al.,* 2001**). BNP play a crucial role in calcification involving CAD by decreasing vascular lumen and hence leading to acute coronary syndrome and cardiac muscle affection, aortic sclerosis and stenosis. Besides they may play a role in rheumatic heart disease **(Ciftcioglu *et al.,* 2010**). One Mayo Clinic study cites evidence showing the presence of BNP near plaque-filled arteries. The study suggests that BNP potentially represent a previously unrecognized factor in the development of arteriosclerosis and calcific arterial disease. BNP was isolated and propagated from human arteriosclerotic aneurysms and kidney stones. Thus they may result into arterial injury, possibly leading to triggering, propagation of atherosclerosis and arterial calcification **(Nkomo *et al.,* 2006).**

Methods of detection of BNP in cells, tissues, blood, and urine include electron microscopy, culture techniques, PCR and immuno-detection with BNP specific monoclonal antibodies (**Miller-Hjelle *et al.,* 2003).** High-resolution SEM imaging is used as an application to the study of BNP and nano structured to illustrate the critical role of the various SEM techniques in nanotechnology and nanoscience research (**Jingyue**, **2005).**

The aim of this study was to detect BNP in serum and urine samples of CAD patients by the aid of Scanning Electron Microscopy as a step to determine their role, incidence, prevalence and correlation with occurrence of CAD in Egyptian patients.

2. Patients and Methods

1- Patients

This study was designed to investigate the prevalence of CNP in Egyptian patients with known and documented coronary artery disease (CAD) as compared to those without as a control. All enrolled subjects, whom were previously known to have or suspected to have CAD, 38 underwent Coronary angiography, after which they were classified into 2 groups: Group 1 (patient group) included 28 subjects with confirmed CAD by coronary angiography. Group 2 (control group) included 10 patients with excluded CAD by coronary angiography. All patients were then investigated to detect CNP in blood and urine samples by the aid of the Scanning Electron Microscopy. The two groups were subdivided according to the sample type into: Group (1-A) included serum samples of Group (I) patients, Group (1-B) included Urine samples of Group (I) patients, Group (2-A) included serum samples of Group (2) controls and Group (2-B) included Urine samples of Group (2) controls. The study was carried out in the period between October 2011 and January 2013. Coronary angiography procedures were carried out in Benha University Hospital in addition to Assalam International Hospital. Blood and urine samples were analysed and processed in Microbiology and Electron Microscopy Departments, Theoder Bilharz Research Institute (TBRI).

Inclusion criteria included were: patients with known and documented coronary artery disease as diagnosed by coronary angiography; patients presented with acute coronary syndrome and suspected to have CAD and underwent coronary angiography; patients with known risk factors for CAD such as diabetes mellitus, hypertension, dyslipidemia, smokers, obesity and positive family history for CAD. Exclusion criteria included were: Patients who were not amenable for coronary angiography procedure such as patient with renal impairment or failure whom are not yet on haemodialysis, patient with known hypersensitivity to contrast media, patients with acute chest and pulmonary conditions i.e. pulmonary embolism, bronchial asthma and severe chest infection, patients in severe infections and sepsis, patient with different bleeding disorders and lastly patients with debilitating illness i.e. tumours or other severe medical or surgical illness.

Approval for undergoing coronary angiography in addition to the use of human samples in this study was obtained from the Institutional Review Board of TBRI (Giza, Egypt). Written informed consents were signed by the individuals who participated in this study.

2- Clinical Examination and Laboratory Investigations.

Every patient was subjected to the following: history of present illness, past history, course of the disease, clinical examination, chest X ray, EGG recording, echocardiography and coronary angiography. Routine laboratory investigations were performed including: kidney functions (urea and creatinine), liver function (albumin and bilirubin), CBC, full coagulation profile (prothrombin time, concentration and INR), creatine kinas, creatine kinas-MB fraction, and/or troponin values for patients with acute coronary syndrome, fasting, post prandial blood sugar level and HbA1c, lipid profile including triglycerides, total cholesterol and LDL.

3- Detection of CNP in Human Blood and Urine by Scanning Electron Microscope (SEM).

Serum and urine samples were collected from CAD patients and control groups. The blood samples (10 ml) were aseptically drawn into sterile Vacutainer tubes without anticoagulant (Becton Dickinson). Whole blood was centrifuged at 1, 5006 g for a period of 15 min at room temperature. The supernatant corresponding to human serum was retrieved and placed into another tube. Collected serum and urine samples were frozen at-85^{0}C till used.

Both serum and urine samples were diluted 1:10 with ultra pure water (passed through a filter of pore size 0.22 µm). A drop from each of them (10 µl) is put in closed polystyrene Petri dish sealed with a permeable tape and placed together with two other dishes containing water into a larger Petri dish which was closed to extend the time of evaporation to be examined by SEM after an evaporation time of 24, 48 and 72 hours at 20^{o}C or 24 and 48 hours at 30^{o}C **(Tsurumoto *et al.*, 2008).** The samples were visualized with Philips EM208 scanning electron microscope (Theodor Bilharz Research Institute). Methods to identify BNP in liquids in general, and in body fluids in particular, has been introduced and validated by the use of 200 nm nanospheres, and successfully applied for detection of BNP in blood and urine **(Wang *et al.*, 2006).**

4- Statistical analysis

Results were expressed as median (minimum-maximum) or number (%). Comparison between different parameters in the two studied groups was performed using Mann-Whitney U test. Comparison between categorical data was performed using Chi square test. The data were considered significant if p value was equal to or less than 0.05 and highly significant if p value < 0.01. Statistical analysis was performed with the aid of the SPSS computer program (version 12 windows).

3. Results

Thirty eight (38) patients were included in this study. All patients meeting the inclusion criteria underwent coronary angiography for diagnostic purposes and they were divided into two groups accordingly: Group (1) included (28) patients with confirmed and documented CAD. They were considered as the patient group, 22 (78.5%) patients were males with ages ranging from 38 to 77 years and 6 (21.4%) were females with ages ranging from 57 to 75 years. which then subdivided into 2 subgroups according to sample types; serum samples referred to as group (1-A) and urine samples; referred to as group (1-B). Group (2) included 10 patients with excluded CAD. They were considered as the control group, 7 (70%) patients were males with ages ranging from 39 to 56 years and 6 (30%) were females with ages ranging from 41 to 56 years which then subdivided into 2 subgroups according to sample types: Group (2-A) serum samples and Group (2-B) urine samples.

BNP were detected by scanning electron microscopy in (78.57%) of the total patients with known CAD. There was a strong association between BPN detection in serum and urine with CAD (p< 0.01). It was observed that patients with CAD have the highest BNP detection rate in serum and urine positive cases (53.57%), followed by serum only positive cases (25%). On the other hand (7.14%) of cases with serum negative and urine positive in addition to (14.28%) of cases with serum negative and urine negative were detected. Regarding the control group)Group 2), BNP were not detected in all 10 (100%) patents whom are excluded to have CAD by coronary angiography, neither in serum nor in urine samples, affirming that BNP were been detected exclusively in patient with CAD and not in patient with normal coronaries (Table 1).

Coronary angiography in patients with detected BNP in serum samples showed LAD to be the most significantly affected vessel, followed equally by LCX and RCA, besides LMCA was found to be the least affected vessel. Significant LAD lesions were found in 19 (86.36%) patients versus 3 (13.64%) patients with non-significant lesions, highlighting then a highly significant statistical correlation between detection of BNP in serum and CAD specially LAD (P< 0.01). LCX and RCA significant lesions were equally detected in 16 (72.73%) patients versus 6 (27.27%) with non-significant lesions, with a significant statistical correlation (P< 0.05). LMCA was the least to be affected showing 19 (86.36%) patients with non-

significant lesions versus 3 (13.64%) patients with a significant statistical reverse correlation ($P< 0.01$). There was no significant statistical correlation between different grades of coronary occlusion and detection of BNP in serum ($P> 0.05$).

Coronary angiography in patients with positive urine samples for BNP showed LAD to be the most significantly affected vessel, followed by LCX then RCA, while LMCA was the least to be affected. Significant LAD lesions were found in 17 (100%) patients versus 0 (0%) patients with non-significant lesions, hence focusing on a highly significant statistical correlation between detection of BNP in urine and CAD specially LAD ($P < 0.01$). LCX significant lesions were detected in 13 (76.47%) patients versus 4 (23.53%) with non-significant lesions, showing a significant statistical correlation ($P< 0.05$). RCA significant lesions were detected in 12 (70.59%) patients versus 5 (29.41 %) with non-significant lesions, with a border line non-significant statistical correlation ($P > 0.05$). LMCA was the least to be affected showing 14 (8.35%) patients with non-significant lesions versus 3 (17.65%) patients with a significant statistical reverse correlation ($P < 0.01$). On the other hand, a border line relation in LAD lesions was detected with BNP urine positive patients; otherwise there was no significant statistical correlation between different grades of coronary occlusion and detection of BNP in urine. It is worth to mention that out of the 28 patient group, 4 serum and urine BNP negative patients (14.28%) were recorded. One of those patients has three vessels disease, two of them have two vessels disease and one patient has one vessel disease (Table 2).

In addition estimating the cardiac muscle condition by means of cardiac enzymes, 12 lead surface resting ECG and Echocardiography, it was found that 9 (52.94%) BNP urine positive patients versus 1 (9.09%) BNP urine negative patient showed significant statistical finding between BPN detection in urine and RWMA as a component of estimating the cardiac muscle condition, otherwise there was no statistical significance detected in neither serum positive nor serum negative patients (Table 3).

Table 1: Detection of BNP in Serum and Urine In Patients and Control Groups.

Sample	Control (n= 10) No %	Patients (n= 28) No %	*P* value
BNP Serum Results			
Negative	10 (100%)	6 (21.43%)	0.001**
Positive	0 (0%)	22 (78.57%)	
BNP Urine Results			
Negative	10 (100%)	11 (39.29%)	0.001**
Positive	0 (0%)	17 (60.71%)	

**$P < 0.01$= highly significant.

Table 2: Coronary Angiography Results of BNP Serum Positive (Group 1-A) and Urine Positive Patients (Group 1-B).

Coronary angiography	BNP serum positive patients (Group 1-A)			BNP urine positive patients (Group 1-B)		
	Non sig. lesion	Sig. lesion	*P* value	Non sig. lesion	Sig. lesion	*P* value
LMCA	19 (86.36%)	3 (13.64%)	0.001**	14 (82.35%)	3 (17.65%)	0.008**
LAD	3 (13.64%)	19 (86.36%)	0.001**	0 (0%)	17 (100%)	0.001**
LCX	6 (27.27%)	16 (72.73%)	0.033*	4 (23.53%)	13 (76.47%)	0.029*
RCA	6 (27.27%)	16 (72.73%)	0.033*	5 (29.41%)	12 (70.59%)	0.090 (NS)

NS= $P > 0.05$= not significant. *$P < 0.05$= significant. **$P < 0.01$= highly significant.

Table 3: Correlation of Cardiac Enzyme, ECG and Echo Findings of Group (1-A) with BNP Serum Detection Patterns and Group (1-B) with BNP Urine Detection Patterns.

	Group (1-A)			Group (1-B)		
	BNP Negative cases (n= 6) No %	BNP Positive cases (n= 22) No %	*P* value	BNP Negative cases (n= 11) No %	BNP Positive cases (n=17) No %	*P* value
Card Enzyme (+ve)	1 (16.67%)	7 (31.82%)	0.466 (NS)	3 (27.27%)	5 (29.41%)	0.903 (NS)
ECG Findings						
ECG STEMI	1 (16.67%)	2 (9.09%)	0.595 (NS)	2 (18.18%)	1 (5.88%)	0.304 (NS)
ECG NSTEMI	0 (0%)	5 (22.73%)	0.198 (NS)	1 (9.09%)	4 (23.53%)	0.330 (NS)
Echo Findings						
Ejection fraction						
35%	0 (0%)	1 (4.55%)	0.646 (NS)	0 (0%)	1 (5.88%)	0.268 (NS)
40%	0 (0%)	2 (9.09%)		0 (0%)	2 (11.76%)	
50%	0 (0%)	2 (9.09%)		0 (0%)	2 (11.76%)	
> 55%	6 (100%)	17 (77.27%)				
Echo RWMA (+ve)	2 (33.33%)	8 (36.36%)	0.891 (NS)	1 (9.09%)	9 (52.94%)	0.018 *

NS= $P > 0.05$= not significant. *$P < 0.05$= significant.

Table 4: Risk Factors for CAD and CNP in Serum (Group 1-A) and Urine Patients (Group 1-B).

Risk factors	Group 1-A			Group 1-B		
	Negative cases (n= 6) No %	Positive cases (n= 22) No %	*P* value	Negative cases (n= 11) No %	Positive cases (n= 17) No %	*P* value
Age (mean &rang)	56.5 (44-75)	57.5 (38-77)	0.892(NS)	52 (41-75)	61 (38-77)	0.225 (NS)
Gender						
Female	3 (50%)	3 (13.64%)	0.054(NS)	2 (18.18%)	4 (23.53%)	0.736 (NS)
Male	3 (50%)	19 (86.36%)		9 (81.82%)	13 (76.47%)	
HTN (+ve)	6 (100%)	18 (81.82%)	0.259(NS)	11 (100%)	13 (76.47%)	0.082 (NS)
DM (+ve)	2 (33.33%)	10 (45.45%)	0.595(NS)	4 (36.36%)	8 (47.06%)	0.576 (NS)
Smoking (+ve)	3 (50%)	6 (27.27%)	0.291(NS)	4 (36.36%)	5 (29.41%)	0.700 (NS)
Cholesterol mg/dl	196.5 (171-238)	221.5(163-308)	0.460(NS)	211 (167-280)	220 (163-308)	0.781 (NS)
TG mg/dl	145.5 (118-250)	165 (64-300)	0.978(NS)	170 (110-268)	145 (64-300)	0.487 (NS)
LDL mg/dl	112.5(100-167)	134 (90-175)	0.494(NS)	128 (90-167)	115 (96-175)	0.963 (NS)

NS= $P > 0.05$= not significant. *$P < 0.05$= significant.

Regarding the different risk factors for CAD including age, gender, hypertension, diabetes, smoking and lipid profile, no statistical significance was shown between BNP serum and urine negative and positive groups (Table4).

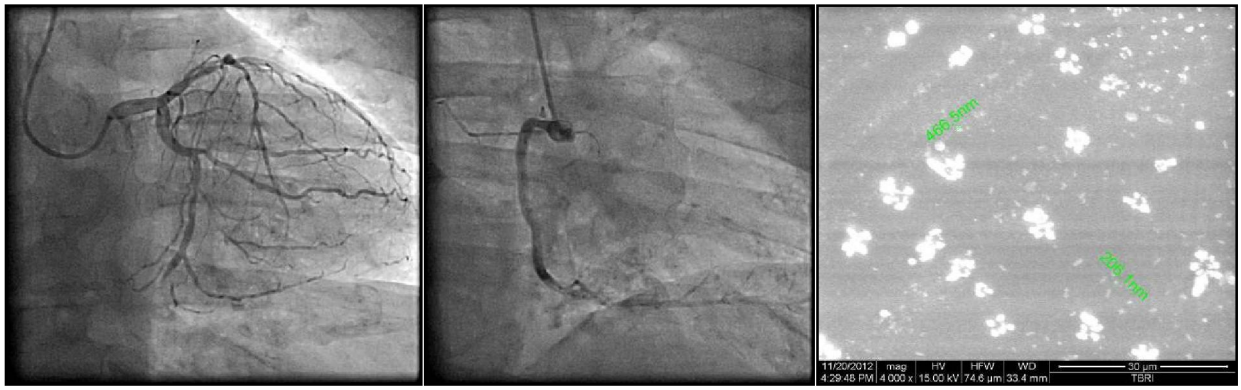

Fig. 1: Coronary angiogram for case number (9), showing multi vessel disease**; (A)** LAD is atherosclerotic, calcific proximal 90% lesion, 1st diagonal branch with 90% biforcational stenosis, LCX is atherosclerotic vessel with 1st obtuse marginal with 90% stenosis**; (B)** RCA showing 70% PDA stenosis; **(C)** SEM image of serum ring periphery showing a multitude of dots in the size range 100-300 nm (BNPs).

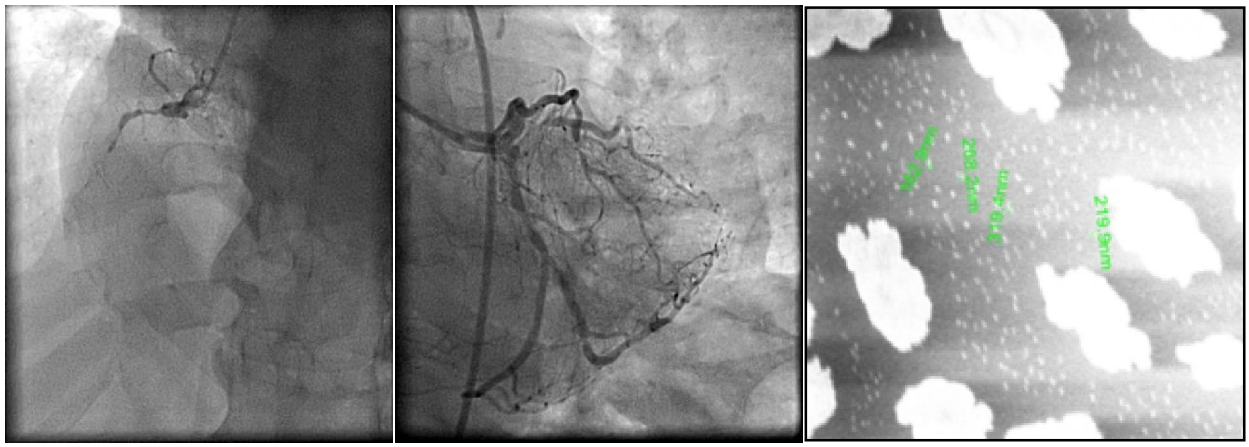

Fig. 2: Coronary angiogram for case number (14), showing multi vessel disease; **(A)** LAD is atherosclerotic, calcific with distal 90% stenosis, LCX is atherosclerotic with 80% mid segment stenosis**; (B)** RCA showing proximal segment total 100% stenosis; **(C)** SEM image of serum ring periphery showing a multitude of dots in the size range 100-300 nm (BNPs).

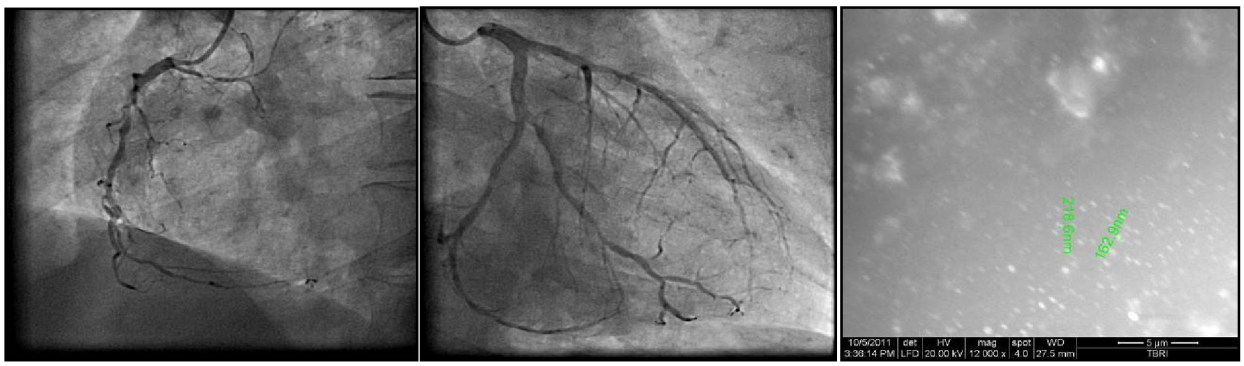

Fig. 3: Coronary angiogram for case number (14), showing multi vessel disease: **(A)** LAD is atherosclerotic, with proximal and mid segment 80% stenosis, LCX is atherosclerotic vessel with 2nd obtuse marginal 95% stenosis; **(B)** RCA is diffusely diseased, showing proximal to mid segment 80% stenosis with distal 100 % total occlusion; **(C)** SEM image of urine ring periphery showing a multitude of dots in the size range 100-300 nm (BNPs).

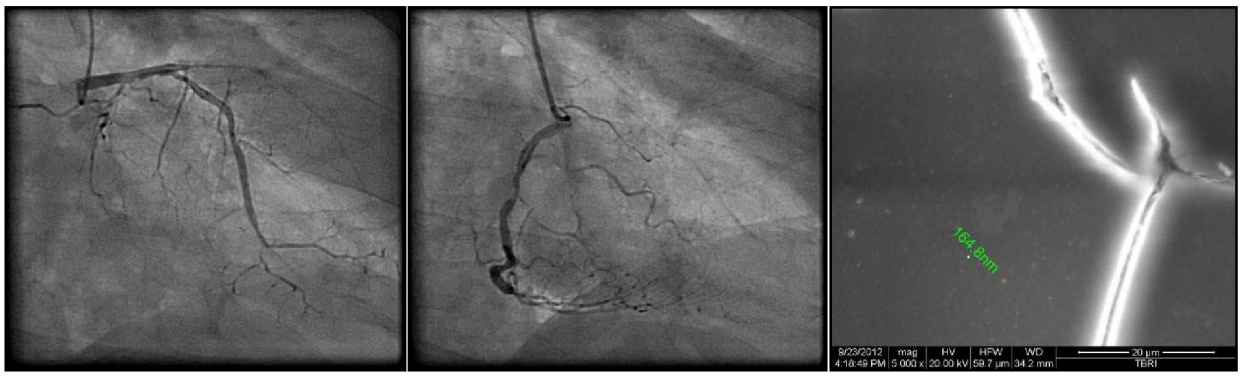

Fig. 4: Coronary angiogram for case number (20), showing multi vessel disease; **(A)** LAD is atherosclerotic, calcific with proximal to mid segment total 100% stenosis, LCX is atherosclerotic with ostial 90% stenosis; **(B)** RCA is atherosclerotic, heavily calcific with mid segment 80% stenosis; **(C)** SEM image of serum ring periphery showing a multitude of dots in the size range 100-300 nm (BNPs).

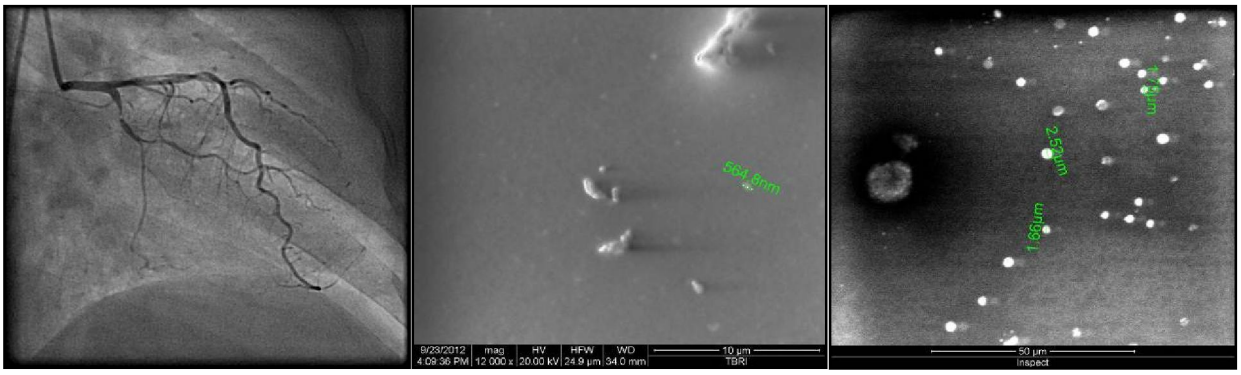

Fig. 5: Coronary angiogram for case number (7), showing tow vessel disease; **(A)** LAD is atherosclerotic, with mid segment 70% stenosis. LCX is atherosclerotic with proximal subtotal 99 occlusion; **(B)** SEM image of serum ring periphery; **(C)** SEM image of urine ring periphery showing a multitude of dots in the size range > 500-600 nm i.e. serum and urine Negative samples.

4. Discussion

BNP are putative living entities characterized by pleomorphic nature, forming amorphous mineral complexes containing calcium as well as other ions like carbonate, which then rapidly acquire phosphate, resulting in accumulation and formation of hydroxyapatite **(Young *et al.*, 2009). Takemura *et al.* (2010)** presented an overview of the relationships among infection, atherosclerosis, and consequent endothelial dysfunction. It was concluded that

"chronic infection may be one of the important factors inducing endothelial dysfunction". Multiple causative organisms including BNP were mentioned. In addition medical researchers reported to the American Heart Association's Scientific Sessions 2004 that a test for BNP is an accurate predictor of heart disease risk **(*Douglas* and Hansen, 2005)**.

Human atherogenesis appears to be of multifactorial etiology, and no single entity can fully explain the pathogenesis. Risk factors such as genetic predisposition, hypercholesterolemia, hypertension, smoking, and diabetes mellitus are major predisposing conditions for atherosclerosis. There is substantial evidence those infectious agents are associated with atherosclerosis, but their exact role in the pathogenesis of atherosclerosis is unknown. The most compelling evidence to date is the presence of infectious agents in the arterial wall, particularly in diseased vessels or within atherosclerotic plaques **(Chiu *et al.,* 1997)**.

In our study correlation between BNP detection in serum and urine with different risk factors for CAD, including age, gender, diabetes miletus, hypertension, dyslipidemia and smoking showed no statistical significance. These data complies with **Wang *et al*. (2004),** who stated that age and sex did not seem to be related to the infectious risk of BNP in the serum of Chinese healthy people. However, the infectious rate was lower in those below 30-year-old than that of people over 60-year-old (P< 0.05)**.**

Fallah *et al*. (2006) stated that infection with BNP is claimed to be a new risk factor for CAD. **Tulunay *et al*. (2011)** showed that antibodies to BNP are still correlated significantly with the existence of BNP, especially in the group having Coronary Artery Calcium score (CAC) that scores greater than 400 (P<0.0001). Therefore could be considered as an independent risk factor for CAD in addition to conventional risk factors such as age, hypertension, diabetes mellitus, and high levels of LDL. Epidemiologic studies carried out by **Zhu *et al*. (2004)** have implicated antibodies made by the body against BNP to be a strong independent risk factor for coronary artery atherosclerosis and calcification. The importance of this is that coronary artery atherosclerosis and calcification is an excellent predictor of future coronary events and death. A recent meta-analysis has reported that dietary calcium supplementation is associated with a significantly increased risk of myocardial infarction. This missed link can be attributed nowadays to the existence of BNP being involved in atherosclerosis and calcification, in abundance of serum calcium level **(Bolland *et al*., 2010)**. **Candemir *et al*. (2010)** showed that increasing age, systemic hypertension, diabetes, HDL-cholesterol levels and high BNP antibody titers were risk factors that were independently associated with calcification in the mitral annuli attributing that BNP might play an important role in the pathogenesis of mitral annular calcification **(Huber *et al*., 2002).** It was noted that almost all the values of CAD risk factors in association with BNP serum or urine positive are exceeding those of BNP serum or urine negative despite the statistical insignificance. This might be attributed to the small number of patients involved in this study.

In the present study, BNP were detected in sera of 22 (78.57%) patients with CAD, compared to exclusively no BNP detection in all the 10 control patients whom are excluded to have CAD by coronary angiography with a highly significant statistical value (P < 0.001). BNP were detected in urine of 17 (60.71%) CAD patients and no BNP were detected in all the 10 control patients with a highly significant statistical value (*P*< 0.001). Thus in the current study BNP were detected in association with CAD and not in healthy individuals not proved to have CAD. These data are in consistent with those of **Kajander *et al.* (2003**), who reported identification of BNP in 48 of 74 (64.8%) of the vascular tissue and heart valves, which supports the possibility that BNP are not simply innocent by standers but they are playing an active role in coronary atherosclerosis**.** A group of researchers at Mayo Clinic, led by **Miller *et al. (2004)*** cultured BNP from atherosclerotic calcified coronary arteries and heart valves and showed that these entities contained DNA and antigens of BNP. Moreover, BNP nano-sized particles were cultured from calcified but not from non-calcified aneurysms (**Miller *et al*., 2004**). Nanometer- scale particles similar to those described as BNP isolated from geologic specimens and human kidney stones can be visualized and cultured from calcified human cardiovascular tissue **(Puskas *et al*., 2005). Miller *et al.* (2004)** published a study providing anatomic evidence that atherosclerotic calcified human tissue including coronary arteries, carotid plaque, aortic aneurysm, femoral arterial plaque and cardiac valves contain nanometre-sized particles ranging in size from 30 to 150 nm. In addition BNP were found in mammalian blood, human kidney stones and were observed by transmission electron microscopy in a calcified human mitral valve.

In the current study, no BNP were detected in all control healthy individuals whom are excluded to have CAD by coronary angiography. These results were not comply with what was stated by **Kajander (2006)** that (1-5%) of human serum of healthy individuals were positive (tested by culture or ELISA). **Wang *et al.* (2004)** observed that BNP exist

in serum of Chinese healthy people with infection rates of 27 (8.0%) by ELISA and 19 (5.7%) by immunohistochemistry stain. This contradiction has many aspects to look for. One aspect was illustrated by **Hjelle *et al.* (2000)** research which showed that 10% of the Swedish population screened to be positive for BNP has been proved in a later study to have history of animal contacts and to be alternatively infected with Bartonella because of the potential for serologic cross-reactivity among these two organisms. As Nanobacteria purportedly share similar surface antigens with *Bartonella* spp., there may be serologic cross-reactivity; however, the extent to which cross-reactivity occurs has not been well characterized ***(Breitschwerdt et al., 2001).*** Another aspect was shown through **Maurin *et al.* (1997)** who reported serological cross reactions between *Bartonella* and Chlamydia species for LPS and non-LPS epitopes. Previous studies showed that patients with coronary artery disease are significantly more likely to have serologic evidence of past infection with *C. pneumonia* **(Linnanmki *et al.*, 1993).** The third aspect illustrated when depending on the serological diagnosis of BNP, false positive results may be obtained, as BNP antibodies were found to react with serum albumin on the BNP surface instead of antigens produced by BNP **(Barr *et al.*, 2003*).***

In the current study, coronary angiography in patients with detected BNP in serum samples emphasized significant LAD lesions in 19 (86.36%) patients ($p< 0.01$), followed by LCX and RCA significant lesions were equally detected in 16 (72.73%) patients ($p< 0.05$). LMCA was the least to be affected showing 3 (13.64%) patients with a significant statistical reverse correlation ($p<0.01$). **Miller *et al.* (2004)** published a study providing anatomic evidence that atherosclerotic calcified human tissue including coronary arteries, carotid plaque, aortic aneurysm, femoral arterial plaque and cardiac valves contain BNP. Nanoparticles structures exist in areas of atherosclerotic calcified human tissue including coronary arteries and was found to contain calcium phosphate stained by von Kossa staining and X-ray microanalysis and resemble structures described as "BNP" that were cultured from kidney stones **(Folk, 1993).** Studies showed that 94% of people with atherosclerotic calcified coronary arteries have infection with BNP as measured by Nanobacterial Antibody Assay, furthermore antibody results correlated with coronary calcification scoring **(Stephen *et al.*, 2004).** In addition nanobacterial antigens were identified in 9 of 14 (64.2%) plaque specimens, but none were identified in normal coronaries, carotid or aortic tissue. This independent research corroborates similar findings published by Mayo Clinic in the American Journal of Physiology which showed that BNP can be visualized in and cultured from human calcified arteries and heart valves (**Puskas *et al.*, 2005).**It was shown that, there was very high incidence of BNP existence in disease processes known to be associated with atherosclerosis, calcification or thrombosis, for 97.5% of carotid stenosis was found to embrace BNP (**Bini *et al.*, 1999).** Researchers led by **Kajander *et al.* (2003)** focused exclusively on the presence of BNP and on the analysis of the biological content in their interior. The recently reported identification of BNP in 48 of 74 (64.8%) heart valves supports the possibility that BNP are not simply innocent bystanders but play an active role in heart valve calcification. Data led by **Nadra *et al.* (2005)** showed that BNP were shown to be present in atherosclerotic calcification and in soft plaque as nanoscopic calcific particles. This is consistent with a pathological role for nano-sized calcification in the vasculature. Atherosclerosis and calcification can be the driving force for endothelial lining damage and hence athero-thrombosis.

It was mentioned that, since BNP are detectable just below the endothelium, they can contribute to thrombotic clotting together with the circulating BNP when the endothelium lining is damaged. These results indicate a role for an apatite-mediated clotting system in thrombotic events **(Wen *et al.*, 2005).** Studies carried by **Puskas *et al.* (2005)** confirmed that BNP are present in calcified human atherosclerotic plaques besides they can form a calcium apatite coat. Nanobacterial antigens were identified *in situ* by immuno-transmission electron microscopy in 9 of 14 (64.2%) plaque specimens, but none (0%) of the normal carotid or aortic tissue (5 specimens). Nanobacteria-like particles were propagated from 26 of 42 (61.9%) sclerotic aorta and carotid samples and were confirmed by dot immunoblot, light microscopy and TEM. Studies showed that nanobacteria-like material has been isolated and cultured from calcified cardiac valves with rheumatic heart disease. While 26 of 29 (89.65%) calcified valves stained positive for BNP antibody, all normal valves (100%) stained negative. Transmission electron microscopy analysis indicated that cultured particles from calcified valves ranging in size from 88 to 341 nm had an obvious cell membrane structure similar to that of BNP (**Hu *et al.*, 2010).**

It was stated by **Bratos-Perez (2008)** that calcific aortic valves were obtained from 75 patients undergoing surgical valve replacement. In the microbiology laboratory, valves were screened for BNP using a 4-6 weeks specific culture method. They showed to be positive in 48 of the 75 (64.0%) valves

with aortic stenosis in comparison with zero of 8 (0%) for the control group (P = 0.0005). The observation of cultures by way of scanning electron microscopy highlighted the resemblance in size and morphology of BNP. It was suggested that BNP colonizing the aortic valve, provoking an inflammatory response, are able to infect phagocytosing cells via receptor-mediated internalization and have been shown to exert cytotoxic effects on fibroblasts (**Miguel *et al.*, 2008).** Studies showed that BNP can transmit disease to naive animals. They observed that inoculated BNP localized to areas of arterial injury and invoked a proliferative response that included atherosclerosis, plaque formation and calcification in adult male rabbits pre-treated by endothelial denudation of one carotid in comparison with intravenously inoculation **(Kraemer *et al.*, 2007).** It was stated that samples isolated and cultured from calcified cardiac valves with rheumatic heart disease showed (89.65%) calcified valves stained positive for BNP antibody, on the other hand all normal non calcified valves (100%) stained negative for BNP Antibody (**Hu *et al.*, 2010**). Samples obtained from calcific aortic valves from 75 patients undergoing surgical valve replacement, were screened for BNP using a 4-6 weeks specific culture method. They showed to be positive in 48 of the 75 (64.0%) valves with aortic stenosis in comparison with zero of 8 (0%) for the control group (P= 0.0005). The same observation was confirmed by Scanning Electron microscopy **(Bratos-Perez *et al.*, 2008).**

In our study, coronary angiography in BNP urine positive patients, showed LAD to be the most significantly affected vessel in 17 (100%) patients ($p < 0.01$), followed by LCX, 13 (76.47%) patients ($p < 0.05$) and then RCA, 12 (70.59%) patients ($p> 0.05$). LMCA was the least to be affected showing 14 (8.35%) patients with a significant statistical reverse correlation ($p < 0.01$). So we can conclude that significant lesions causing CAD in those patients are attributed to other traditional risk factors other than BNP. On the other hand, still BNP accused for being involved in the rest of the 24 (85.72%) patients' significant lesions rendering them the title of CAD. Unfortunately no research work in the literature showing the mere relation between existence of BNP in urine and different grades of significant lesions and their distribution in different coronary vessels, since all the research work is focusing on urinary tract stone formation regarding the size and duration required for their formation. In our study, neither BNP detection in serum nor in urine showed significant statistical correlation regarding the cardiac enzymes, 12 lead surface resting ECG and Echocardiography. However 9 (52.94%) patients versus 1 (9.09%) patient showed significant statistical finding between BNP detection in urine and RWMA as a component of estimating the cardiac muscle condition. Unfortunately there were no data in the literature supporting these findings.

In conclusion, BNP was detected by scanning electron microscopy in (78.57%) of the total patients with known CAD, with variable grades and sites of coronary lesions. These data may help to understand the critical medical importance of already demonstrated effects of BNP on atherosclerosis and pathologic calcification in the human body especially coronary arteries and on research, into countermeasures to reverse or eliminate these effects. It is recommended that further epidemiological and experimental data collection are required to determine the critical physicochemical and toxicological properties of BNP in humans, thus facilitating understanding the biological nature of BNP, clinical significance and relative importance of the observed biological responses e.g. vascular dysfunction, atherosclerosis and calcification. Screening of body fluids for BNP on a large scale could be necessary for the assessment of co-infections with BNP especially in susceptible persons with risk of developing CAD.

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