The recent evolution of vitamin D (vitD) from its long-assumed, exclusive role of calcium homeostasis to majorly influencing living things through involvement with diseases like cancers, cardiovascular disease and auto-immune diseases, has raised considerable scientific curiosity in the past years (1). Identification of vitD receptors (VDR) in virtually all cells as well as the presence of the enzymatic machinery necessary to produce the active form of the vitamin, 1,25-dihydroxyvitamin D (1,25(OH)₂D) (1), has stirred up interest in contemporary vitD research.

On a related note, abundant research investigating the intriguing relationship of vitD with cardiovascular disease (CVD) has spawned limited insight regarding the mechanistic link between them (2).

Previous studies have demonstrated that endothelial cells, besides having VDR, are able to produce 1,25(OH)₂D and hence, investigating the possibility of vitD modulation of endothelial functions is warranted (3, 4).

While several processes have been implicated in the pathophysiology of endothelial dysfunction (5), this study investigates the connection between modulators of the endothelium-derived, potent vasodilator, nitric oxide (NO) and vitD.

Ever since its identification as an endogenous inhibitor of NO synthase (NOS), the enzyme responsible for NO synthesis, in the early 1990s, asymmetric dimethylarginine (ADMA) has gained an unmatchable reputation in the field of cardiovascular research as a novel cardiovascular risk factor (6). Its regioisomer, symmetric dimethylarginine (SDMA), does not share the ability to directly inhibit NOS (7); however, it has been reported to decrease the cellular uptake of the enzyme's substrate, l-arginine, and thus decrease NO production (7).

The inflammation marker high-sensitivity C-reactive protein (hs-CRP) has been identified as a diagnostic marker for patients at high risk for cardiovascular disease (CVD) (8). In view of the association of endothelial dysfunction with arterial inflammation, hs-CRP has also been identified as a marker of endothelial dysfunction (5). Supplementation with vitD₃ has been recently reported to improve systemic inflammatory markers (9), including hs-CRP as well as, in a different study, a negative correlation between 25(OH)D, the agreed upon biomarker of vitD status (1), and hs-CRP (10). Noteworthy is that a recent study denied any association between 25(OH)D and hs-CRP (11).
Assessed by different parameters such as reactive hyperaemia index (12), flow-mediated dilation (13) and circulating endothelial progenitor cell count (14), endothelial dysfunction was previously associated with vitD levels. However, to our knowledge, only one study demonstrated an inverse correlation between 25(OH)D and ADMA levels in an ambulant ageing population (15), making our study the first to investigate such a relationship in patients with coronary artery disease (CAD), with the purpose of examining the hypothesis of vitD’s modulation of the dimethylated arginines and thus affecting endothelial function.

Additionally, the controversial issue of vitD form efficacy is also tackled in this study in terms of association affecting endothelial function.

RESULTS

Study population. Sixty-nine male patients, all between 35 and 50 y of age, with verified single or multi-vessel CAD were recruited from in- and outpatient settings of the National Heart Institute (NHI), Imbaba, Cairo. CAD verification was obtained through coronary catheterization or a history of either myocardial infarction or percutaneous coronary intervention (PCI). Twenty age- and sex-matched controls were also recruited provided that they presented with no diagnostic signs of CAD and that they had a controlled blood pressure of below 140/90 mmHg. CAD patients also had a controlled blood pressure of below 140/90 mmHg since they were all receiving anti-hypertensive medication, which do not interfere with serum 25(OH)D levels.

Exclusion criteria for both groups, besides being out of the age range, included concomitant acute or chronic severe diseases such as renal failure, hepatic insufficiency, severe chronic heart failure and diabetes mellitus.

Female subjects were not recruited since our study was confined to males who developed CAD at a young age. Females are protected from the disease until menopause possibly by sex hormones, namely estrogen (16).

Courteously, all subjects were informed of the nature of the study and thus written consent, which abided by the principles of the Helsinki declaration, was obtained from all of them.

CAD patients were further classified into two groups, acute (n=11) and chronic (n=58), depending on the severity of coronary insufficiency based on coronary catheterization. The former group comprised patients suffering from acute myocardial infarction whereas the latter comprised patients under conservative medical intervention, those directed for PCI and those advised to undergo coronary artery bypass graft surgery.

Biochemical analyses. Blood samples obtained were collected in EDTA-Eppendorf tubes and resulting plasma, after centrifugation at 2,500 rpm for 10 min at 4°C, was preserved in a −80°C freezer until analysis.

25(OH)D levels were analyzed by an in-house developed and validated high performance liquid chromatography with ultraviolet detection method after solid phase extraction (SPE) of the metabolite from plasma samples, described elsewhere (17). After SPE, the samples were injected into the HPLC with the following chromatographic conditions: a 150×4.6 mm C18 HPLC column set at 57°C was adopted; 0.02 M phosphate buffer of pH 2.6 was used as mobile phase A and a mixture of methanol and acetonitrile (1:1 v/v) was mobile phase B. A linear gradient of 78 to 95% of mobile phase B in a gradient time of 25 min was utilized. A flow rate of 1 mL/min was also used and ultraviolet detection was set at 256 nm. The assay is capable of detecting both forms of the metabolite, 25(OH)D2 and 25(OH)D3, giving an individualized result for each form, with excellent resolution and a limit of detection of 2 ng/mL for each metabolite. Subjects’ vitD status was classified into normal, having 25(OH)D concentrations greater than or equal to 30 ng/mL, insufficient, with concentrations between 20 and 30 ng/mL and finally, deficient, with concentrations less than 20 ng/mL, which complied with reported reference values (1). VitD insufficient and deficient subjects were collectively referred to as exhibiting suboptimal vitD levels.

NO was indirectly determined as nitrate/nitrite (NOx) using the Griess reaction, described elsewhere (18). Briefly, plasma samples (200 μL) were deproteinated by the addition of 20 μL 30% zinc sulfate followed by a 15 min centrifugation period at 14,000 rpm at 4°C. Three-hundred microliters of vanadium (III) chloride (8 mg/mL) was then added to 100 μL of the supernatant to reduce nitrate to nitrite. Three-hundred microliters Griess reagent, containing 1:1 (v/v) 2% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, was then added and followed by a 30 min incubation period at 37°C in the dark. Absorbance was determined at 540 nm and unknown concentrations were determined using the linear calibration curve constructed from serial dilutions of sodium nitrite standards (0–100 μM).

L-Arginine, ADMA and SDMA were analyzed by liquid chromatography-mass spectrometry using a previously described method (19, 20). A commercially available enzyme-linked immunosorbent assay (ELISA) kit (DRG Instruments GmbH, Marburg, Germany) was obtained for analysis of hs-CRP.

Statistical analyses. Analyses were performed using GraphPad Prism statistics software (GraphPad Software, Inc.). Correlations between two measured parameters were made using linear regression analysis as well as comparison of their means using the t-test. Statistical significance was defined as obtaining a p-value of less than 0.05. All results, unless stated otherwise, are presented as means±standard error of the means (SE). Means of all investigated parameters are presented along with their corresponding 95% confidence interval (95% CI). Cohen’s d was used as a measure of effect size for all significant and insignificant values obtained.

RESULTS

Vitamin D and CAD

With regards to the previously defined reference val-
ues for vitD status, 20 CAD subjects exhibited normal 25(OH)D levels whereas 24 were insufficient and 25 were deficient. Seventy-one percent of the investigated CAD population was thus found to exhibit suboptimal 25(OH)D levels while only 29% presented with normal levels. On the other hand, only 30% of the control subjects exhibited suboptimal 25(OH)D levels. Statistical comparison of their means yielded a significant result (Table 1). Comparisons of the means of the different forms of the metabolite between the investigated groups and among the same group are presented in Table 1.

Vitamin D’s association with the dimethylated arginines, nitric oxide and inflammation

Results of the investigation of the relationship of vitD with NO and its modulators, as well as with hs-CRP in CAD subjects exhibiting normal and suboptimal vitD levels are presented in Table 2 and Fig. 1. An overview of the biochemical investigations of acute and chronic CAD subjects are presented in Table 3.

### DISCUSSION

The first part of our results demonstrate a significant difference in 25(OH)D concentration between CAD subjects and controls adding to the already existing, accumulating evidence of association of vitD deficiency with CVD (Table 1). No significant results were reached from the comparison of 25(OH)D concentrations of patients with acute and chronic CAD (Table 1).

A recently conducted study by Williams et al. (21),

#### Table 1. Total vitD status and different 25(OH)D form comparison between the different classes of subjects (vertical) and within the same class (horizontal).

<table>
<thead>
<tr>
<th>Total 25(OH)D</th>
<th>25(OH)D₁</th>
<th>25(OH)D₂</th>
<th>p-value</th>
<th>Cohen’s d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34.5±2.3</td>
<td>21.9±2.2</td>
<td>12.6±1.5</td>
<td>0.001</td>
</tr>
<tr>
<td>CAD subjects</td>
<td>24.0±1.3</td>
<td>9.9±0.9</td>
<td>14.1±1.1</td>
<td>0.003</td>
</tr>
<tr>
<td>p-value</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
<td>0.497</td>
<td>0.0002</td>
</tr>
<tr>
<td>Cohen’s d</td>
<td>1.00</td>
<td>1.38</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>25.0±4.5</td>
<td>13.8±2.6</td>
<td>11.2±3.7</td>
<td>0.572</td>
</tr>
<tr>
<td>(16.2–33.8)</td>
<td>(8.6–19)</td>
<td>(3.9–18.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic</td>
<td>23.8±1.3</td>
<td>9.2±0.9</td>
<td>14.6±1.1</td>
<td>0.0002</td>
</tr>
<tr>
<td>(22.5–25.1)</td>
<td>(8.5–9.9)</td>
<td>(13.5–15.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.739</td>
<td>0.054</td>
<td>0.258</td>
<td></td>
</tr>
<tr>
<td>Cohen’s d</td>
<td>0.12</td>
<td>0.67</td>
<td>0.45</td>
<td></td>
</tr>
</tbody>
</table>

Statistical significance is obtained from the comparison of the total 25(OH)D concentration between controls and CAD subjects. Significance is also found in the comparison of the different 25(OH)D forms within controls and CAD subjects exhibiting 25(OH)D₁ and 25(OH)D₂ dominance, respectively. 25(OH)D₁ concentration in controls is significantly higher than that of CAD subjects. Finally, 25(OH)D₂ is significantly higher than its counterpart in chronic CAD subjects. 95% CIs are indicated in parentheses following their corresponding means. Values presented are in ng/mL.

#### Table 2. Overview of the biochemical parameters investigated in CAD subjects exhibiting normal and suboptimal vitD levels.

<table>
<thead>
<tr>
<th>ADMA (μmol/L)</th>
<th>SDMA (μmol/L)</th>
<th>l-Arginine (μmol/L)</th>
<th>NO (μmol/L)</th>
<th>hs-CRP (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6133±0.05234</td>
<td>0.5387±0.04524</td>
<td>84.75±8.625</td>
<td>44.53±5.188</td>
<td>8.277±3.564</td>
</tr>
<tr>
<td>(0.5243–0.7023)</td>
<td>(0.4617–0.6157)</td>
<td>(70.11–99.39)</td>
<td>(37.69–51.37)</td>
<td>(2.647–13.907)</td>
</tr>
<tr>
<td>Suboptimal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5945±0.02127</td>
<td>0.5381±0.03865</td>
<td>94.74±6.238</td>
<td>29.47±3.432</td>
<td>22.89±4.860</td>
</tr>
<tr>
<td>(0.5565–0.6325)</td>
<td>(0.4921–0.6071)</td>
<td>(83.53–105.95)</td>
<td>(24.53–34.41)</td>
<td>(14.92–30.86)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.692</td>
<td>0.998</td>
<td>0.3952</td>
<td>0.03I</td>
</tr>
<tr>
<td></td>
<td>0.003</td>
<td>0.27</td>
<td>0.90</td>
<td>0.035</td>
</tr>
<tr>
<td>Cohen’s d</td>
<td>0.11</td>
<td>0.003</td>
<td>0.27</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Statistical significance is found in the comparison of NO and hs-CRP concentrations between the two groups. 95% CIs are indicated in parentheses following their corresponding means.
Vitamin D and Nitric Oxide Modulatory Abilities

Involving children from the AVON longitudinal study of parents and children, demonstrated the association of 25(OH)D3 with cardio-protective levels of their investigated biomarkers, including high-density lipoprotein cholesterol, apolipoprotein-A1 and adiponectin. Mixed associations of 25(OH)D2 with cardiovascular risk factors such as a positive association with hs-CRP and interleukin-6 and an inverse association with triglycerides and apolipoprotein-A1 was observed. Our study presents interesting results in this regard. 25(OH)D3 is found to be dominant in the control samples whereas 25(OH)D2 is dominant in CAD subjects (Table 1). Additionally, 25(OH)D3 is significantly higher in our controls compared to CAD subjects while 25(OH)D2 is not (Table 1), which raises the question of whether the 25(OH)D2 form possesses a cardio-protective advantage over its counterpart. It also raises the conclusion that 25(OH)D3, rather than 25(OH)D2, deficiency is an inducer of CVD. Another finding presented in this study supporting the aforesaid notion is the dominance of 25(OH)D2 in chronic CAD subjects with the lacking of such a finding in their acute counterparts (Table 1).

The association of vitD deficiency with endothelial dysfunction has been described by quite a few studies, such as Tarcin et al. (13) which not only demonstrated that 25(OH)D deficiency is associated with a lower flow-mediated dilatation compared to their controls, but also presented an improvement in the mentioned endothelial function parameter after supplementation with vitD3.

While the study done by Sugden et al. (22) complements that of Tarcin et al. (13) in terms of improved endothelial function by vitD administration, other studies have provided opposing conclusions (23); thus, randomized controlled trials involving larger cohorts than previously investigated are warranted to determine the efficacy of vitD as a cardio-protective agent.

VitD’s beneficial effects on vascular functions have been recently described, in terms of NO production, by Molinari et al. (3). They described an elaborate experiment that led to the understanding that administration of 1,25(OH)2 D produces a dose-dependent increase in NO production in cultured endothelial cells, with the involvement of VDR, by activation of endothelial NOS. Our results agree with the aforementioned study in terms of association of a statistically significant higher concentration of NO in CAD subjects presenting with normal vitD levels compared to those exhibiting suboptimal levels (Table 2). Such findings are thus immensely valuable in cardiovascular research and provoke further investigations that aim to promote vitD as an anti-atherogenic agent.

The previously conducted study by Ngo et al. (15) was the first to demonstrate a correlation between 25(OH)D

Table 3. Overview of the biochemical parameters investigated in acute and chronic CAD subjects.

<table>
<thead>
<tr>
<th></th>
<th>ADMA (µmol/L)</th>
<th>SDMA (µmol/L)</th>
<th>l-Arginine (µmol/L)</th>
<th>NO (µmol)</th>
<th>hs-CRP (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute CAD</td>
<td>0.73±0.063</td>
<td>0.778±0.2</td>
<td>61.28±6.84</td>
<td>28.025±2.71</td>
<td>35.33±3.21</td>
</tr>
<tr>
<td>Chronic CAD</td>
<td>(0.639–0.821)</td>
<td>(0.533–1.023)</td>
<td>(36.90–85.66)</td>
<td>(19.06–36.99)</td>
<td>(18.52–52.14)</td>
</tr>
<tr>
<td>p-value</td>
<td>0.010</td>
<td>0.0094</td>
<td>0.0378</td>
<td>0.462</td>
<td>0.057</td>
</tr>
<tr>
<td>Cohen’s d</td>
<td>1.31</td>
<td>0.74</td>
<td>0.92</td>
<td>0.34</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Statistical significance is found in the comparison of ADMA, SDMA and l-Arginine concentrations between the two groups. 95% CIs are indicated in parentheses following their corresponding means.

Fig 1. Linear regression analyses investigating the correlation of 25(OH)D concentrations with ADMA (A) and SDMA (B) levels. An R-square of 0.002800 was obtained for A and 0.006479 for B, demonstrating minimal correlation between the investigated parameters. F- and p-values were 0.049 and 0.783 respectively for A, and 0.038 and 0.846 respectively for B, thus illustrating a lack of significance in both cases.
and ADMA concentrations. Unlike our study, they investigated the hypothesis of vitD’s modulation of ADMA in an ambulant, ageing population whereas we recruited patients with verified CAD as well as including the other dimethylated arginine in our investigation. Contrary to the results provided by Ngo et al. (15) our results suggest a lack of association between 25(OH)D levels and either of the dimethylated arginines (Table 2 and Fig. 1), thus denying the potential molecular mechanism hypothesis of vitD affecting endothelial function via modulation of ADMA and SDMA. Moreover, the insignificant result obtained from the comparison of L-Arginine concentrations in CAD subjects with normal and suboptimal vitD levels suggests a lack of involvement of vitD with amino acid biosynthesis.

In light of the undeniable role of inflammation in atherosclerosis (24), numerous studies have examined the involvement of vitD in such a process. Among such studies is that of Jablonski et al. (25), who described an increase in endothelial cell expression of both proinflammatory transcription factor nuclear factor κB and cytokine interleukin-6 in vitD deficient subjects compared to sufficient ones. Additionally, studies have demonstrated a significant reduction in inflammatory markers after supplementation with vitD (26). Among these studies is that conducted by Witham et al. (9), which described a decline in hs-CRP concentration after supplementation with an oral dose of vitD.

Results presented in this study demonstrate that a lower hs-CRP concentration is associated with CAD subjects with a normal vitD status compared to those with suboptimal levels (Table 2), suggesting an anti-inflammatory effect, and thus complement similar previous research.

We acknowledge that the results of our study may be limited by the study cohort size and nature since only male subjects were recruited. We also realize that the nature of the study does not permit establishment of a relationship between vitD and CVD; however, it presents a promising explanation for the obscure association of vitD with endothelial function and, in turn, the disease.

CONCLUSION

In view of the results presented here, this study proposes the molecular mechanism linking vitD with endothelial dysfunction to be related to inflammation and not via modulation of the dimethylated arginines. Further investigations would unveil how vitD regulates inflammatory markers and, in turn, vascular function. Results of this study also add to existing data linking vitD with CVD on an observational level.

Acknowledgments

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17) Hollis BW, Frank NE. 1985. Solid phase extraction sys-


