Original Article

Reciprocal Expression of Myostatin and Vitamin D Receptors in Sarcopenia

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Abstract

Background: Sarcopenia is the loss of skeletal muscle mass and strength or/and performance that occurs in concert with biological aging.

Objectives: To evaluate both myostatin (MST) and vitamin D_3 receptor (VDR) genes expression in Egyptian adults with their relation to each other and muscle state.

Methods: Two groups were involved; group A of sarcopenic patients and group B of non-sarcopenic subjects. Multiplex quantitative RT-PCR for MST and VDR mRNA in the peripheral lymphocytes of sarcopenic and non-sarcopenic (control) groups subjects was done.

Results: There was an inverse relationship between expression of myostatin and vitamin D3 receptor genes ($r = -0.345$, $P = 0.014$). Both MSTN and VDR showed a significant positive and negative association with muscle state respectively ($p<0.001$).

Conclusion: The reciprocal MSTN and VDR expression reflect their role in the regulation of muscle state, which opens a new challenge for them as preventive and therapeutic targets for sarcopenia.

Key words: MST, myostatin, RT PCR, Multiplex PCR, sarcopenia, VDR.

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INTRODUCTION

The term sarcopenia (in Greek, sarx for flesh and penia for loss), Ali & Garcia found that sarcopenia is increasingly being recognized as a geriatric syndrome and a key public health issue. Starting at the age of 30 years, individuals lose 1–2% of muscle per year, and by the age of 80 years, 30% of the muscle mass is lost.[1]

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It may be likewise helpful to recognize sarcopenia as a geriatric syndrome because this view promotes its identification and treatment even when the exact causes remain unknown.[2] The well-recognized functional consequences of sarcopenia include gait and balance problems and increased risk for fall, physical inactivity, decreased mobility, slow gait, and poor physical endurance.[3]

Geriatric syndromes are common, complex and costly states of impaired health in older individuals. Geriatric syndromes result from incompletely understood interactions of disease and age on multiple systems, producing a constellation of signs and symptoms.[4]

Emerging evidence has shown that vitamin D administration improves muscle performance and reduces falls in vitamin D-deficient older adults.[5]

On the other hand, Myostatin (growth and differentiation factor - 8 [GDF-8]) is a transforming growth factor superfamily member with importance as a negative growth regulator for skeletal muscle.[6]

Mutations in the myostatin gene result in a hyper-muscular phenotype in mice[7]. However, little is known of the underlying mechanism or the role 1, 25 $(OH)_2$ D_3 plays in promoting myogenic differentiation at the cellular and/or molecular level and its relation to myostatin gene expression. Garcia et al., declared that addition of vitamin D_3 to muscle cell culture decrease expression of myostatin.[8]

The aim of this study was to evaluate the association of both myostatin and vitamin D_3 receptor genes expression in the peripheral lymphocytes by Multiplex semi-quantitative RT-PCR relation to each other and muscle state.

SUBJECTS AND METHODS

A. Subjects

Fifty subjects were recruited for this study from Ain Shams University hospitals from May 2013- December 2016. They included 27 males and 23 females of age range from 30-84 years. Before any study procedures were initiated for any subject in the study, a written informed consent was properly executed and documented as approved by Research Ethical Community (REC) of Faculty of Medicine, Ain Shams University. The participants were subjected to anthropometric measurements for muscle state and then sub-divided into two main groups; *Group A*, including 25 sarcopenic subjects in which diagnosis was based on documentation of *criterion 1 (low muscle mass)* plus *criterion 2 (low muscle strength*) or *criterion 3 (low physical performance)* according to the European Working Group on Sarcopenia in Older People (EWGSOP, the Sarcopenia Working Group).[2]

The *Group B* included 25 non-sarcopenic subjects acting as a control group.

For all participants, full medical history and clinical examination were done. In addition, radiological and laboratory data were collected from their clinical files records. The patients were evaluated for any exclusion criteria as smoking, alcohol intake, malignancy, pregnancy/ lactation; current or recent history of hepatic or renal disease; supplementation of greater than 400 IUs vitamin D_2 or vitamin D_3; current anti-seizure medications or glucocorticoids; tanning for more than 8 hours within the past month; history of intestinal malabsorption and unwillingness to consent to the study.

B. Methods

1. Anthropometric Measurements for Muscle State

Specific for assessment of muscle state by mid arm muscle circumferences (MAMC) = mid-arm circumference (MAC) – (3.14 × TSF triceps skin fold thickness), muscle strength by Hand Grip Dynamometer (Barbosa et al., 2005)[9] and Muscle State Performance by Timed Get Up and Go Test "TUGT").[2]

2. Biochemical and Molecular Investigations

Blood Sample Collection and Handling: 10ml fresh venous blood treated with EDTA anticoagulant was taken from each subject.

Lymphocytic separation by Ficoll Hypaque Plus technique: lymphocytes were isolated by density gradient centrifugation with Ficoll-Hypaque Plus from heparinised blood (*Pharmacia Biotech, Sollentuna, Sweden)* as described previously Gawad et al.[10] All samples were stored at −80°C until assays were performed.

RNA extraction: Total RNA was extracted according to TriFast™ Total RNA Purification Kits, based on a modified salt precipitation procedure in combination with highly effective inhibitors of RNase activity (PeQLab Biotechnologie GmbH Corporation, Erlangen, Germany). Then measurement of RNA concentration and purity Using UV-Spectrophotometer was done.

Optimization of Semiquantitative Multiplex RT-PCR: It is a demanding amplification technique which allows simultaneous detection of several RNA targets in a single tube. A ß actin primer was selected to be suitable for multiplex RT PCR with both MSTN and VDR primers and optimization of multiplex RT-PCR was done for both. RNA was converted to cDNA using QIAGEN One Step RT-PCR Kit (QIAGEN, USA), using MSTN primers 25 pm/µl: sense primer: 5'- TGGTCATGATCTTGCT-GTAAC-3', and antisense primer 5'-TGTCTGTTACCTTGACCTCTA-3', with the product of 80 bp.[11] Housekeeping ß actin primers 3 pm/µl: the sequences of these primers were chosen according to Smith and co workers.[12] Sense primer: 5'-CTACGTCGCCC-TGGACTTC-GAGC-3', antisense primer: 5'-GATGGAGCCGCCGATCCA-CACGG-3'. These primers product bands were detected at 385 bp. VDR primers 25 pm/ µl: Sense primer: 5'- AGCCTCAATGAGGAGCA- CTCCAAG -3', and antisense primer 5'- GATGGAGCCGC-CGATCCACACGG -3', with product of 206 bp.[13] This was also done by Multiplex RT PCR with the

Housekeeping ß actin primers 3 pm/µl. The first step of RT was at 60ºC for 60 minutes, PCR activation was at 95ºC for 15 minutes, then repeated 40 cycles of denaturation at 95ºC for 1 minute, annealing at 55ºC for 1 minutes for MSTN (60ºC for VDR primers), then 72ºC for 10 minutes & then held at 4ºC till use.

ß actin products bands were used as reference bands for the semi quantitation of the MSTN or VDR RNA bands. The signal intensities in agarose gel of MSTN and VDR RNA in each sample was determined *relative* to that of ß actin in the same sample using Quantity one *Gel pro* (computer program version 4.6.3) Bio-Rad Laboratories, USA. That determined the relative amount of MSTN or VDR in different samples in the form of ratio of the gene expression relative to the ß actin.[14-16]

All contaminated wastes included in this study were sealed and discarded in strong, impermeable biohazard bags for further safe transport of them according to Ain Shams University hospitals infection control biohazard waste disposal policy.

Statistical Analysis

The T-test analysis used to compare between the normally distributed data (mean ± standard deviation) subjects' age among the two studied groups. The Non-parametric analysis Kruskal-Wallis test and the median test was used for statistical comparison of variables between various groups. Chi-square analysis was used to compare between and find out the relationship between various qualitative data. Variables were cross tabulated in all possible combinations against each other. The values of $p \leq 0.05$ and $p \leq 0.001$ were considered statistically significant and highly significant respectively. ROC curves also were used to determine the cutoff values for optimal sensitivities and specificities. The Pearson correlation was used to correlate parametric values. All statistical analyses were performed with the software package SPSS for Windows, version 15.0 (SPSS Inc., Chicago, Illinois).

RESULTS

Mean age of the sarcopenic patients was 68.84± 5.71 (ranged from 60 to 84 years) and in the non-sarcopenic control group was 45.28± 14.62 (ranged from 30 to 73 years) p=0.000.

The relation of age and muscle anthropometrical parametric measurements to the different studied groups is indicated in Table 1. There was a positive association between the age of patients and sarcopenia with the high significant difference (P=0.00). The relation of the Relative Quantities of MSTN and VDR genes expression to the different studied groups is shown in Table 2. There was increase in the MSTN relative quantity in sarcopenic patients more than the healthy subjects. On the other hand, VDR expression decreased in sarcopenic patients relative to healthy subjects with high significant differences between both groups in the two markers (P=0.00).

The correlation of the relative quantity of MSTN and VDR to each other, to age and to muscle anthropometrical parametric measurements in the different studied groups showed significant positive correlation between MSTN and age, and Timed Get Up & Go Test, and significant negative correlation between it and VDR, mid arm muscle circumference and hand grip test. The correlation between VDR and the same parameters used with MSTN is illustrated in Table 3.

The relation of the relative quantity of MSTN and vitamin D receptor to sex in different studied groups showed no significant differences as shown in Table 4.

Multiplex RT-PCR analysis by agarose gel electrophoresis and ethidium bromide staining for Beta actin bands (385 bp) with MSTN product (80 bp) and Beta actin bands with VDR product (206 bp) is shown in Figure 1.

The ROC curves analysis for Myostatin (MSTN) values and Vitamin D receptor down regulation (VDRD) in sarcopenic group versus control group shows the best cut off value for MSTN at 120.29 with absolute sensitivity and 88% specificity and the best cut off value for VDRD is 81.83 with absolute sensitivity and 96% specificity is shown in Figure 2.

The positivity rates using the corresponding cutoff values for both MSTN and VDR genes expression among the studied groups were shown in Table 5. The positivity rate of MSTN was 100% in sarcopenia and 12% only in the control group. While VDR positivity rate was 100% in control group and only 4% in the sarcopenic patients with high significant differences (P=0.00 for both). The sensitivity, specificity, predictive values and accuracy of MSTN and VDR in the studied groups showed that there was absolute sensitivity for both markers; 88% specificity for MSTN & 96% specificity for VDR. VDR also took the upper hand in PPV & accuracy (96.15% & 98%, respectively). While MSTN PPV & accuracy were 89% & 94% respectively as shown in Table 6.

DISCUSSION

The term sarcopenia is now commonly used to describe the loss of skeletal muscle mass and strength that occurs in concert with biological ageing. This loss of skeletal muscle fibres is usually secondary to decreased numbers of motor neurons, but other factors including decreased physical activity, altered hormonal status, decreased total caloric and protein intake, inflammatory mediators, and factors leading to altered protein synthesis, must also be considered.[17]

Table 1. Relation of age and muscle anthropometrical parametric measurements to the different studied groups

Group		n	Median	Mean Rank	χ^2	p
Age (years)	**Sarcopenia**	25	68	35	22.018	**0.000****
	Control	25	41	15		
MAMC (cm)	**Sarcopenia**	25	20	13.24	35.377	**0.000****
	Control	25	25	37.76		
Hand grip (Kg)	**Sarcopenia**	25	10	16.04	21.142	**0.000****
	Control	25	25	34.96		
TUGT (sec)	**Sarcopenia**	25	15	34.88	20.883	**0.000****
	Control	25	10	16.12		
Total	50					

**p≤0.001=highly significant p-value, MAMC: mid arm Muscle circumference, TUGT: Timed Get Up and Go Test.

Table 2. Relation of the relative quantities of MSTN and VDR genes expression to the different studied groups

	Group	n	Median	Mean Rank	χ^2	p
MSTN Relative quantity	**Sarcopenia**	25	435.59	37.68	34.974	**0.000****
	Control	25	86	13.32		
VDR Relative quantity	**Sarcopenia**	25	60.35	13.08	36.392	**0.000****
	Control	25	145.14	37.92		
Total	50					

**p<0.001=highly significant p-value

Table 3. Correlation of relative quantity of MSTN and VDR to each other, to age and muscle anthropometrical parametric measurements in the different studied groups.

Parametric Measurements	MSTN (r)	p	VDR (r)	P
VDR	-0.345*	**0.014**	-	-
Age (years)	0.332*	**0.019**	-0.603**	**0.000**
MAMC (cm)	-0.389**	**0.005**	0.536**	**0.000**
Hand grip (Kg)	-0.415**	**0.003**	0.571**	**0.000**
TUGT (sec)	0.283*	**0.046**	-0.458**	**0.001**

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed). MAMC: mid arm muscle circumference, TUGT: Timed Get Up & Go Test

Table 4. Relative quantity of MSTN and VDR in relation to sex in different studied groups.

Clinical Factors		MSTN		χ^2 (P)	VDR		χ^2 (P)
		MR	Median		MR	Median	
Sex	Male (N=27)	25.19	179.9	0.027 (0.868)	26.69	93.82	0.389 (0.533)
	Female (N=23)	25.87	129.1		24.11	80.84	
Total no.	50						

**p≤0.001=highly significant p-value. *p≤0.05= significant p-value. MR: mean rank

Table 5. Positivity rate of MSTN and VDR genes expression in different studied groups.

Positivity rate of MSTN (≥ 120.29)				χ^2	p	Positivity rate of VDR (≥ 81.83)		χ^2	p
		Positive	Negative			Positive	Negative		
Group	Sarcopenia no.%	25 100%	0 0%	39.29	**0.000****	1 4%	24 96%	46.15	**0.000****
	Control no. %	3 12%	22 88%			25 100%	0 0%		
Total no. %		28 56%	22 44%	50 100%		26 52%	24 48%	50 100%	

**p<0.001=highly significant p-value

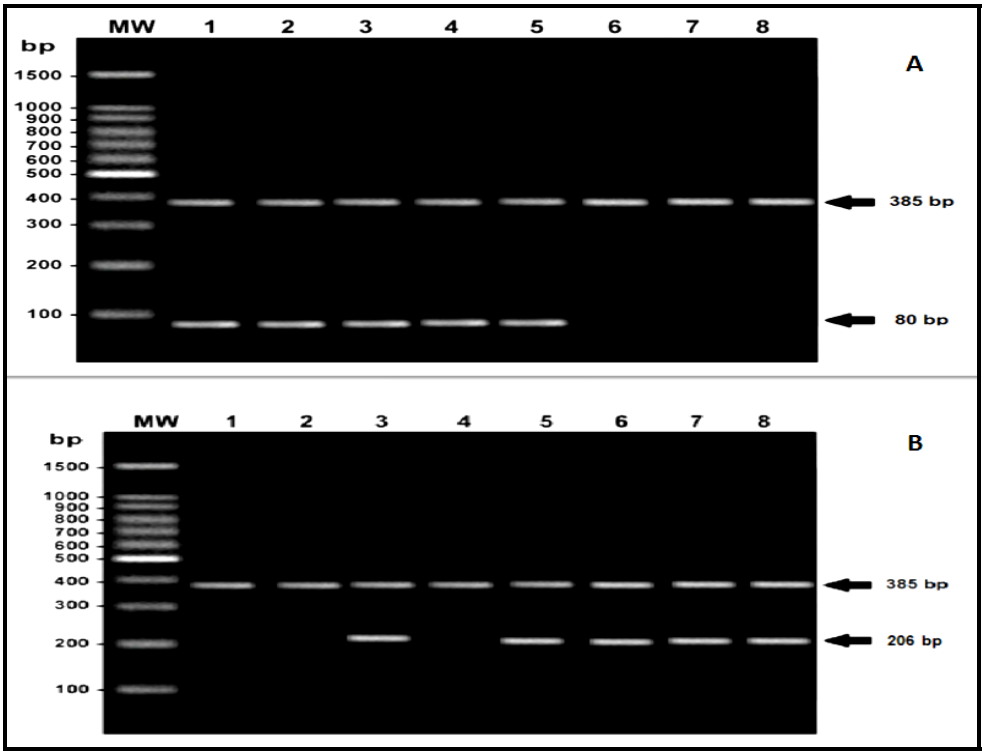

Fig. 1: Multiplex RT-PCR analysis by agarose gel electrophoresis and ethidium bromide staining for Beta actin bands (385 bp) with MSTN product (80 bp) in **A: MW:** Molecular weight ladder standard (100-1500 bp) lane, **Lanes 1-5:** Positive MSTN samples and **Lane 6-8:** Negative MSTN samples. In **B:** beta actin bands (385 bp) with VDR product (206 bp). **MW:** Molecular weight ladder standard (100-1500 bp) lane, **Lanes 1, 2, 4:** Negative VDR samples and **Lanes 3, 5-8:** Positive VDR samples.

Table 6. Sensitivity, specificity, predictive values and accuracy of MSTN and VDRD in all studied groups.

	Sensitivity	Specificity	Predictive value		Accuracy
			Positive	Negative	
MSTN	100%	88%	89 %	100%	94%
VDRD	100%	96%	96.15%	100%	98%

VDRD: Vitamin D receptor down regulation.

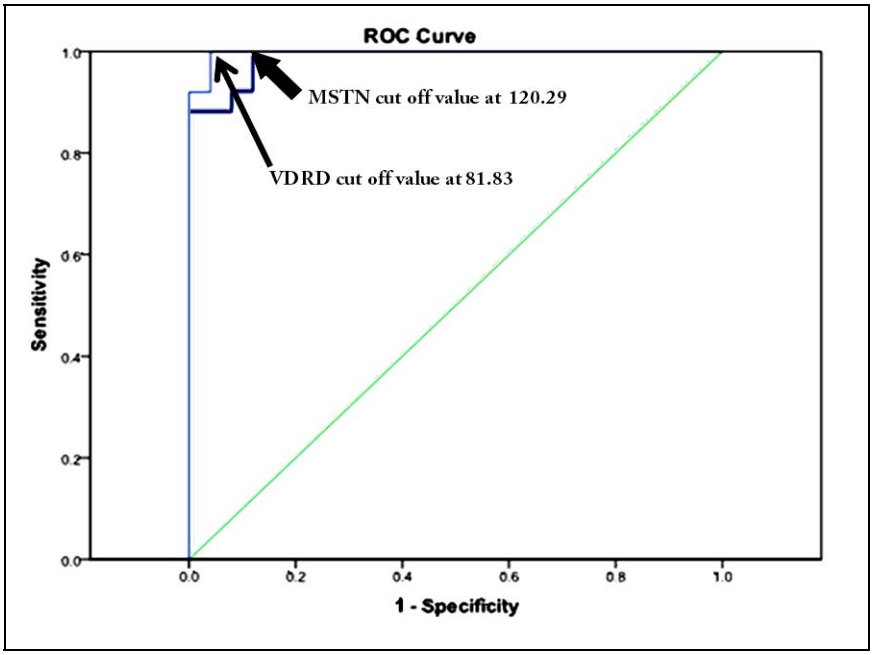

Fig. 2: ROC curves analysis for Myostatin (MSTN) values and Vitamin D receptor down regulation (VDRD) in the sarcopenic group versus control group shows the best cut off value for MSTN at 120.29 with absolute sensitivity and 88% specificity with the area under the curve= 0.987 and p value = 0.000. The best cut off value for Vitamin D receptor is 81.83 with absolute sensitivity and 96% specificity, the area under the curve is 0.997 with p value =0.000.

The prevalence of sarcopenia ranged from 13 to 24% in persons aged 65 to 70 years and was over 50% for those older than 80 years. As a major public health problem, the health care cost of sarcopenia in the United States alone was estimated at 18.5 billion dollars in the year of 2000.[18] This estimation took into consideration the direct costs of sarcopenia, including hospital, out-patient, and home health care expenditures, and did not include the indirect costs of sarcopenia such as loss of productivity. Therefore, developing strategies to prevent and treat sarcopenia are of great importance.[19]

Accordingly, age, sex and muscle anthropometrical parametric measurements among Egyptian subjects of the current study were evaluated and related to sarcopenia in comparison to non-sarcopenic control group.

In the present study, sarcopenia is significantly related to aging ($p<0.001$); this is consistent with the findings of previous studies.[20, 21]

Roubenoff and Boirie & co workers explained the relationship of sarcopenia to aging that it may be due to the age associated altered central and peripheral nervous system innervations, altered hormonal status, inflammatory effects, and altered caloric and protein intake.[22,23] In addition to this, a previous study also reported that sarcopenia is usually accompanied by physical inactivity, decreased mobility, slow gait, and poor physical endurance which are also common features of the frailty syndrome.[24]

The prevalence of sarcopenia was reported to be higher for men over age 75 years (58%) than for women (45%).[25] The prevalence based on total skeletal mass determined by DXA was 10% for men and 8% for women between 60 and 69 years and 40 and 18%, respectively, for men and women over 80 years[26]. Ali & Garcia found that sarcopenia is increasingly being recognized as a geriatric syndrome starting at the age of 30 years, individuals lose 1–2% of muscle per year, and by

the age of 80 years, 30% of the muscle mass is lost.[1] Abdel Rahman et al., found that the prevalence of sarcopenia among nursing home older residents in Cairo was 17.7%; 22.2% in elderly men and 14.4% in elderly women.[27] Similarly, in the current study, the positivity rate of sarcopenic patients is higher (52%) among males than females (48%) with no apparent significance which may be because it needs a larger scale of a population to be observed.

The current study showed a significant decrease in mid-arm circumference, muscle mass among sarcopenic patients ($p<0.001$) this goes well with the other reports that compared muscle wasting with bone loss and suggested that decline in muscle mass could predict disability or mortality, and it is important to identify this decline before functional loss was severe to prevent or treat sarcopenia. [28, 29]

Also, in the present study, there is a significant ($P<0.001$) decrease in hand grip using Dynamometer (testing muscle strength), a significant ($p<0.001$) increase in timed get up and go test (TUGT) to which the patients take more time to reach the required 3m point (testing muscle performance) among sarcopenic patients. These findings coincide with those of Cruz-Jentoft and Rom and coworkers.[2,19] Several explanations for these changes were proposed to explain the inability of muscles to generate strength and endurance, slowness in activities, one of these explanations is that the absence of adequate nutritional intake activates the immune system and increases synthesis of inflammatory cytokines amplifying the chronic catabolic conditions reducing muscle mass and, consequently, affecting body function.[30] In summary, Alexandre et al. suggested that all these changes related to aging increase muscle fatigue and consequently the protein catabolism that can reduce both muscles mass and function.[31]

Vitamin D, which has an important role in the maintenance of muscle function for older adults, is produced endogenously.[32] In the present study, a novel evaluation of vitamin D receptor gene expression using multiplex semi-quantitative RT-PCR was done. A significant negative association ($p<0.001$) between vitamin D receptor gene expression and sarcopenia was found i.e. down regulation. Tan et al. studied vitamin D concentration only not its receptor and found a negative association between it and sarcopenia suggesting that it may be an indirect reflection to VDR.[33] On the other hand, another important molecule that is related to muscle state is also evaluated using multiplex semi-quantitative RT-PCR in this study which is the MSTN gene. A positive association was found between MSTN gene expression and sarcopenia which coincides with another study.[34]

In the current study, the Receiver Operating Characteristics (ROC) curve was constructed for the unique determination of the best cut off values for the relative quantities of both VDR down regulation (VDRD) and myostatin expression using RT-PCR. The best cut off value for MSTN and VDR genes are 120.29, 81.83 with the area under the curve 0.987, 0.997 respectively.

By applying these cutoff values, the positivity rate of MSTN among sarcopenic was (100%) ($p<0.001$) while the positivity rate of VDR among sarcopenic (4%) ($p<0.001$). Up to our knowledge this is the first study that analyses ROC curve results of both Mysostatin & Vit D receptors as biomarkers of sarcopenia.

The relative quantity of VDR and MSTN among Egyptian subjects of the current study were evaluated and related to age, BMI, MAC, muscle mass, hand grip, TUGT and different studied parameters (sex, occupation, diabetes, hypertension, hyperlipidemia).

VDR seems not to be affected by sex in the current study ($p>0.05$), but seems to be affected by aging as a significant negative high concordance is found in the current study ($p<0.001$), suggesting that this might explain the reduction of the functional response of the muscle fibers to vitamin D.[35]

In the present study, there was a significant positive correlation between VDR and muscle mass, mid arm circumference, handgrip and subsequently a negative correlation with TUGT ($p\leq0.001$). It was reported that Vitamin D deficiency associated with a substantial decline in physical performance. Observational studies support a positive association between vitamin D levels and muscle strength and/or lower extremity function in both active and inactive older adults.[36] The vitamin D receptor (VDR) is expressed in human muscle tissue, which provides a rationale for a direct role of vitamin D in muscle function.[8]

In this study, there is a significant negative association between the expression of VDR gene and diabetes mellitus this result come in agreement with another study.[37]

Bid et al. also suggested that VDR gene polymorphism in a combination of genotypes is associated with the risk of T2DM and thus requires further studies as a probable genetic risk marker for T2DM.[38] Also, in the present study, both

hypertensive and hyperlipidemic patients show lower levels of VDR gene expression than non-hypertensive and non-hyperlipidemic patients. This was in agreement with Weng et al. who reported that vitamin D deficiency induces high blood pressure and accelerates Atherosclerosis in mice.[39]

As regard the MSTN in this study, it seems not to be affected by sex in the current study ($p>0.05$), but a significant ($p<0.05$) positive correlation between MSTN and age is found as previously was supported by Kovacheva et al. who found that higher molecular weight precursor myostatin (inactive MSTN), was detected only in the young mice but not the old animals.[40]

In the current study, there was a significant negative correlation ($p<0.05$) between MSTN and both mid-arm circumference and muscle mass' It looks like as if the expression of MSTN was blocked experimentally and there was an increase in skeletal muscle hypertrophy.[41] Patel et al. also reported that MSTN showed lower relative expression in those men who were stronger compared with those who were weaker.[42]

As regards the muscle performance in the current study, a significant association ($p<0.05$) between increased MSTN gene expression and increased TUGT (indicating poor muscle performance) was observed. MSTN gene mutations have significantly greater racing ability than homozygote wild-type dogs.[43] In this study, patients with diabetes mellitus, hypertension and hyperlipidemia had higher significant levels ($p<0.05$) of MSTN gene expression than non-diabetic, non-hypertensive and non-hyperlipidemic patients these results come in agreement with another study.[44] Guo et al. reported that altering metabolism in skeletal muscle can have profound effects on the metabolism of other tissues.[45] Increased glucose uptake in muscle, reduced adiposity, and increased hepatic insulin sensitivity are found in mice that become muscular by MSTN inhibition.[46]

There was no significant correlation between VDR as well as MSTN and BMI as it may need further study on the wider scale of patients. Finally, a significant negative correlation between VDR and MSTN gene expression was determined ($r= 0.345$), ($p\leq0.05$) in the current study.

As regards limitations of the study, the idea of preventing sarcopenia and the early management remains a challenge, so there is a need for a powerful screening tool for sarcopenia for clinical practice. Although human measurements are easy to obtain in clinical practice, the ability to predict the disease is still limited. Several important biomarkers appear to be associated with skeletal muscle mass, strength, and function. However, these biomarkers may not be specific to the skeletal muscle and are likely to be only weakly associated with clinically relevant outcomes. The use of MSTN & Vit D receptors as biomarkers in detecting sarcopenia need further investigations in large scale multicentric studies. However, a preliminary conclusion from our study that both MSTN and VDR may be of importance in the future considering both as potential mediators of sarcopenia so they may be used as therapeutic targets in a trial to pave the way for gene silencing therapeutics in patients with sarcopenia such as activator micro RNA and interference RNA based therapies on VDR & MSTN genes, respectively. The present results warrant further studies with larger sample size and more sophisticated molecular biology studies, to better evaluate and understand the role of both markers in sarcopenia pathogenesis and diagnosis.

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