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BACE-1, PS-1 and sAPPβ Levels Are Increased in Plasma from Sporadic Inclusion Body Myositis Patients: Surrogate Biomarkers among Inflammatory Myopathies

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Sporadic inclusion body myositis (sIBM) is a rare disease that is difficult to diagnose. Muscle biopsy provides three prominent pathological findings: inflammation, mitochondrial abnormalities and fibber degeneration, represented by the accumulation of protein depots constituted by β-amyloid peptide, among others. We aim to perform a screening in plasma of circulating molecules related to the putative etiopathogenesis of sIBM to determine potential surrogate biomarkers for diagnosis. Plasma from 21 sIBM patients and 20 age- and gender-paired healthy controls were collected and stored at –80°C. An additional population of patients with non-sIBM inflammatory myopathies was also included (nine patients with dermatomyositis and five with polymyositis). Circulating levels of inflammatory cytokines (interleukin (IL)-6 and tumor necrosis factor (TNF)-α), mitochondrial-related molecules (free plasmatic mitochondrial DNA (mtDNA), fibroblast growth factor-21 (FGF-21) and coenzyme-Q10 (CoQ)) and amyloidogenic-related molecules (beta-secretase-1 (BACE-1), presenilin-1 (PS-1), and soluble Aβ precursor protein (sAPPβ)) were assessed with magnetic bead–based assays, real-time polymerase chain reaction, enzyme-linked immunosorbent assay (ELISA) and high-pressure liquid chromatography (HPLC). Despite remarkable trends toward altered plasmatic expression of inflammatory and mitochondrial molecules (increased IL-6, TNF-α, circulating mtDNA and FGF-21 levels and decreased content in CoQ), only amyloidogenic degenerative markers including BACE-1, PS-1 and sAPPβ levels were significantly increased in plasma from sIBM patients compared with controls and other patients with non-sIBM inflammatory myopathies ($p < 0.05$). Inflammatory, mitochondrial and amyloidogenic degeneration markers are altered in plasma of sIBM patients confirming their etiopathological implication in the disease. Sensitivity and specificity analysis show that BACE-1, PS-1 and sAPPβ represent a good predictive noninvasive tool for the diagnosis of sIBM, especially in distinguishing this disease from polymyositis.

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INTRODUCTION

Although sporadic inclusion body myositis (sIBM) is considered a rare disease (ORPHA611), it is the most common myopathy in individuals over 50 years (1). This disease belongs to the group of inflammatory myopathies, together with dermatomyositis (DM) and polymyositis (PM). Its prevalence (4.5–35 per million) varies among countries and ethnic groups, although several studies have suggested that this value could be underestimated. Clinically, sIBM is characterized by insidious weakness in proximal and distal muscles, especially in the quadriceps and finger flexors. Neck flexors and extensors are frequently affected, and also dysphagia is present in up to 60% of patients with sIBM. The clinical progression is slow and often leads to severe disability (2–6).

The first diagnostic criteria for sIBM were proposed by Calabrese *et al.* (7) in 1987, but so far, muscle biopsy is

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still essential for diagnosis. Although the pathogenesis of sIBM is not well known, inflammatory, mitochondrial and degenerative pathogenic mechanisms have been described. Diagnosis is confirmed by characteristic findings on muscle biopsy demonstrating endomysial mononuclear cell infiltrates, rimmed vacuoles, amyloid deposits and mononuclear cell invasion of non-necrotic fibers. Although the presence of β-amyloid deposits in muscle of sIBM patients is classically accepted by the scientific community (8), some controversy arose regarding the methodology used to detect this molecule (9). The presence of increased β-amyloid peptide has also been described in plasma by Abdo *et al.* (10), confirming its implication in the disease. Recent studies also reported the presence of the TDP-43 protein in these inclusion bodies, which may be more specific in sIBM than β-amyloid peptide (11). In addition, COX (cytochrome c oxidase) negative and SDH (succinate dehydrogenase) positive fibers are present in most of the cases.

Because of the slow progression of the disease and the diagnostic difficulties, the diagnosis is often delayed or misdiagnosed commonly as PM (1,3,12). Salajegheh *et al.* reported the existence of circulating autoantibodies against a 43-kDa muscle protein called CN1A, highly specific to IBM (13,14), although recent investigations found this autoantibody in other autoimmune diseases (15). Apart from this report, there is no information about noninvasive circulating diagnostic biomarkers in sIBM.

Inflammatory processes, as well as mitochondrial dysfunction and degeneration, are pathologic processes that are widely known to play a role in sIBM. These pathological features found in muscle of sIBM patients may involve different molecules that might be altered in this disease. Regarding inflammation, it has been demonstrated that myoblasts produce interleukin (IL)-6 and tumor necrosis factor (TNF)-α in response to inflammatory stimuli of T-lymphocytes (16), and T-lymphocyte infiltrates are commonly present in PM and in sIBM (17). In relation to mitochondrial and inflammatory lesions, it was also recently reported that circulating mitochondrial DNA (mtDNA) in plasma released by injured cells causes a powerful innate immune response that triggers inflammation through the recognition of damage-associated molecular patterns (DAMPs) by toll-like receptors (TLRs) (18,19). Since sIBM presents both mitochondrial and cell damage accompanied by chronic inflammation, this parameter may also be potentially involved in the development of sIBM.

Parallelly, fibroblast growth factor-21 (FGF-21) was reported to be a plasmatic biomarker for mitochondrial muscle disease (20), which would increase as a protective compensatory mechanism in response to mitochondrial damage. Additionally, coenzyme-Q10 (CoQ) is widely known as a key molecule in mitochondrial respiratory chain function. The plasma levels of this coenzyme are reportedly related to myopathy, especially with statin-induced myopathies (21). In addition, CoQ10 seems to be related to the production of tau-aggregation present in Alzheimer's disease (AD) as well as in sIBM biopsies (22). However, to our knowledge, this is the first time that these mitochondrial markers have been quantified in the plasma of sIBM patients.

With regard to amyloidogenic protein deposition leading to cell degeneration, many studies have recently described parallelisms between sIBM and AD (23,24). These studies suggest that sIBM and AD may share a common etiology. In fact, amyloid-β deposition and the presence of phosphorylated tau protein have been detected in both brain tissue and muscle biopsy from patients with AD and sIBM disorders, respectively (25). These amyloid-β depositions are caused by the amyloidogenic processing of amyloid precursor protein (APP) by β-secretase-1 (BACE-1) and γ-secretase (presenilin-1 [PS-1]) leading to cell injury. Non-amyloidogenic processing of APP by the α-secretase does not cause amyloid-β depositions. Wu *et al.* (26) have proposed the measurement of plasma BACE-1 activity as a potential biomarker for AD, and Nogalska *et al.* (27) found increased BACE-1 mRNA levels in sIBM muscle fibers. Another product of this amyloidogenic processing of APP is sAPPβ. This fragment is released when BACE-1 cleaves the APP protein, releasing soluble Aβ precursor protein (sAPPβ) and the amyloid-β fragment. Although sAPPβ does not oligomerize and causes depositions, its presence indicates that APP has been cleaved by BACE-1, and a fragment of amyloid-β has also been released. Thus, increased levels of plasma sAPPβ indicate a higher amount of amyloidogenic particles that will lead to an increased amount of inclusion bodies (Figure 1). This molecule is used to monitor AD patients to demonstrate the efficacy of new therapeutic drugs (28). However, as far as we know, none of these amyloidogenic degeneration molecules have ever been measured in plasma of sIBM patients.

Because information about circulating biomarkers in sIBM is scarce, the aim of the present study was to evaluate if inflammatory, mitochondrial and degenerative circulating molecules potentially involved in the etiopathogenesis of sIBM may be altered in plasma of sIBM patients and if they may be useful as diagnostic tools.

MATERIALS AND METHODS

Study Design

We performed a multicenter, cross-sectional, case-controlled, observational study.

Study Population

The respective cases of sIBM, DM and PM were diagnosed by clinical and pathological tests in the Department of Pathology and Internal Medicine of the Hospital Clínic of Barcelona (Barcelona, Spain) and in the Hospital Vall d'Hebron (Barcelona, Spain). All the patients fulfilled the criteria proposed by the European Neuromuscular Centre (29,30),

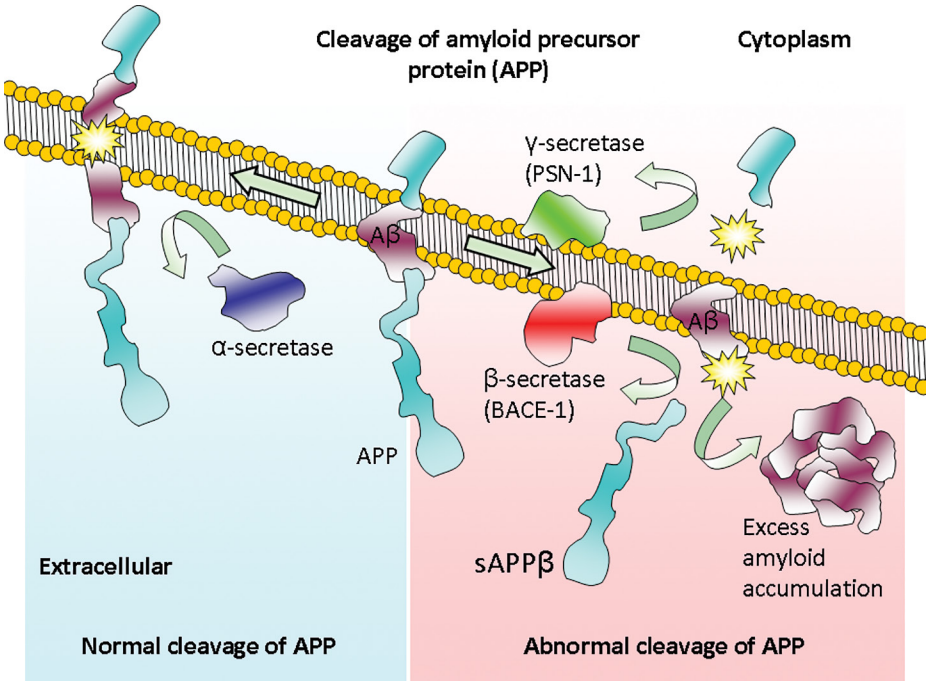

Figure 1. Normal and abnormal cleavage of the amyloid precursor protein. Both pathways are physiologic, but an increase in the abnormal pathway mediated by BACE-1 and PS-1 leads to amyloid-β oligomerization and the accumulation of amyloid-β plaque.

representing definite forms of each disease. Twenty-one patients of sIBM were prospectively and consecutively included in the present study at the time of diagnosis after signing the informed consent previously approved by the ethical committee of the Hospital Clinic of Barcelona. On inclusion, all the sIBM patients completed the inclusion body myositis functional rating scale (IBMFRS), a validated disease-specific functional rating scale (31), scoring 23.6 ± 1.2 out of 40 and presenting clinical features of moderate to advanced sIBM. Parallelly, we included 20 age-gender–paired healthy controls to determine significantly altered biomarkers in sIBM patients. The inclusion criteria for the healthy controls were as follows: age >40 years, absence of family history of mitochondrial disease, absence of muscle disease, viral infection, drug abuse or contact with mitochondrial toxic agents. In addition, patients with inflammatory myopathy different from sIBM (nine patients with DM and five patients with PM) were included to determine the sensitivity and specificity of the biomarkers selected versus sIBM patients.

Sample Collection and Processing

A total of 20 mL peripheral blood were collected from both patients and controls by antecubital vein puncture in EDTA tubes. Plasma was isolated by centrifugation at 1,500*g* during 15 min and stored at –80°C until analysis.

Inflammatory Molecules Analysis

The concentration of soluble inflammatory molecules was determined using a Human Cytokine Plex (Bio-Rad) according to the manufacturer's instructions. The molecules determined were IL-6 and TNF-α. Plates were analyzed on a Luminex 100™ instrument (Luminex) by using Bio-Plex Manager™ Software (Bio-Rad). Concentrations were obtained by standard calibration curves. All measurements were performed in duplicate. Results were expressed in picograms per milliliter (pg/mL).

Mitochondrial-Related Molecules Analysis

Circulating mtDNA was isolated from plasma with a QIAGEN Amp Blood Mini Kit and stored at 4°C for further analysis for a maximum of 24 h after extraction. Free circulating mtDNA was assessed by quantitative real-time polymerase chain reaction in an Applied Biosystems 7500 Real Time PCR System by the amplification of a fragment of the mitochondrial *12SrRNA* gene as reported previously (32). Circulating mtDNA content was expressed as the number of copies of mtDNA per milliliter of plasma. Circulating FGF-21 was measured by an enzyme-linked immunosorbent assay (ELISA) (Biovendor R&D) using an internal curve of standards run in duplicates, and the results were expressed as picograms per milliliter (pg/mL) (33). Plasma levels of CoQ were assessed by high-pressure liquid chromatography (HPLC) in reverse form with electrochemical detection of the reduced and oxidized molecule. Values were expressed as micromoles per liter (μmol/L) (34).

Degenerative Molecules Analysis

Plasma levels of BACE-1, PS-1 (concretely PS-1-NTF) and sAPPβ were analyzed with the ELISA sandwich enzyme immunoassay technique using an internal curve of standards and run in duplicates following the manufacturer's instructions (reference SEA718Hu, SEC200Hu and MBS165363, respectively; USCN-Life-Science), and values were expressed as nanograms per milliliter (ng/mL).

Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM) either as absolute units or as a percentage of increase or decrease between groups. Odds ratio and Fisher test were used to calculate gender distribution and its statistical differences among groups. Nonparametric statistical analysis was performed to select candidate

biomarkers with significantly altered expression in sIBM patients with respect to healthy controls by using the independent sample Mann-Whitney *U* test. In addition, correlations were assessed using Spearman linear regression analysis. Selected biomarkers were further tested among the different groups of patients with inflammatory myopathies using the independent sample Mann-Whitney *U* test. Additionally, for evaluating the predictive capacity of these selected biomarkers to discriminate between sIBM subjects and either healthy controls or the group of patients with inflammatory myopathies other than sIBM, binary logistic regression was performed to assess the sensitivity and specificity of each molecule tested. Furthermore, the Omnibus test, the Hosmer-Lemeshow goodness-of-fit, the ROC curve and the area under the curve (AUC) were also performed to assess the reliability of these molecules for diagnosis. In all cases, a p value <0.05 was considered statistically significant.

RESULTS

There were no differences in terms of age and gender among the groups. Table 1 shows all the clinical and demographical data of the patients included. IBMFRS test confirmed moderate to advanced level of severity for sIBM disease, but did not render statistical significant association to further evaluate molecular biomarkers.

We found remarkable trends toward altered plasma expression of inflammatory and mitochondrial biomarkers in the plasma of sIBM patients. The plasma levels of IL-6 and TNF-α in sIBM patients were higher compared with healthy controls (43.9 ± 29% versus 18.2 ± 23%, respectively). The circulating mtDNA and FGF-21 values were also greater in the plasma of sIBM patients compared with healthy controls (10.2 ± 49% versus 52 ± 40%, respectively), whereas plasma CoQ levels were lower at 3.7 ± 8%. However, none of these alterations was significant (Figure 2).

Table 1. Demographic and clinical data of the three study populations.

	Sporadic inclusion body myositis (n = 21)	Controls (n = 20)	Dermatomyositis and polymyositis (n = 14)	Statistical significance
Demographic data				
Male (n (%))	10 (47.6)	13 (65)	6 (43)	NS
Female (n (%))	11 (52.3)	7 (35)	8 (57)	NS
Age (years) (mean ± SEM)	67.7 ± 2.3	68.5 ± 1.4	59.3 ± 2.8	NS
Disease progression data				
IBMFRS test	23.6 ± 1.66	–	–	–

NS, nonsignificant; SEM, standard error of the mean. The IBMFRS is a disease-specific, 10-point functional rating scale for patients with sIBM. This test classifies the clinical features of sIBM (with a maximum score of 40) according to the impossibility to perform daily activities such as dressing, personal hygiene and swallowing (0 score).

Interestingly, all the plasma biomarkers of amyloidogenic degeneration were significantly increased in the plasma of sIBM patients compared with healthy controls. BACE-1 was significantly increased (102 ± 29.6%) in plasma of sIBM patients compared with healthy controls (38,409 ± 5,629 versus 18,999 ± 2,487; $p = 0.003$). PS-1 was also significantly increased in these patients (31.58 ± 26.9%) compared with the healthy control cohort (1.82 ± 0.18 versus 1.33 ± 0.10; $p = 0.003$), and sAPPβ levels also showed a strong trend to an increase in sIBM patients compared with healthy controls; however, they were not statistically significant (19.8 ± 4.1 versus 15.2 ± 2.5; $p = 0.054$). In addition, a positive correlation was found between BACE-1 and PS-1 plasma levels ($R^2 = 0.087$; $p < 0.05$). The biomarkers showing significant differences between sIBM patients and healthy controls (amyloidogenic molecules) were further evaluated in a third study population composed of patients with DM and PM, the non-sIBM inflammatory myopathy group. This third study population showed similar results compared with the healthy control group, with only 1.2 ± 13.1% in the case of BACE-1 (19,244 ± 2,494 versus 18,999 ± 2,487, respectively), increasing to 1.5 ± 6% for PS-1 values (1.35 ± 0.08 versus 1.33 ± 0.1, respectively) and decreasing by 12.7 ± 6.5% in the case of sAPPβ (13.33 ± 1 versus 15.26 ± 2.53, respectively). None of these differences were statistically significant

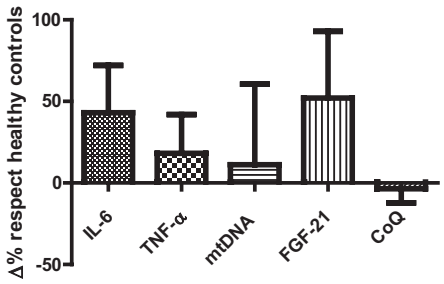

Figure 2. Plasma levels of potential inflammatory (IL-6 and TNF-α) and mitochondrial (free mtDNA, FGF-21 and CoQ) biomarkers in plasma of sIBM patients. Values are expressed as percentage of increase or decrease with respect to healthy controls. No statistically significant differences were found between sIBM patients and controls. However, increased levels of these molecules in sIBM (especially IL-6, TNF-α and FGF-21) suggest evidence of their implication in the pathogenesis of sIBM.

(p = NS) (Figure 3). Specificity of the ELISA kits for the amyloidogenic molecules were confirmed by Western blot in both BACE-1 and PS-1. Similar patterns of expression were found in those experiments regarding differences between sIBM patients and controls (data not shown).

Consequently, when sIBM patients were compared with the non-sIBM group (healthy controls + DM + PM), BACE-1, PS-1 and sAPPβ showed a significant increase of 99.5 ± 29.2%, 34.8 ± 13.3% and 36.5 ± 28.2%, respectively (38,409 ± 5,629 versus 19,244 ± 2,494, $p = 0.001$; 1.82 ± 0.18

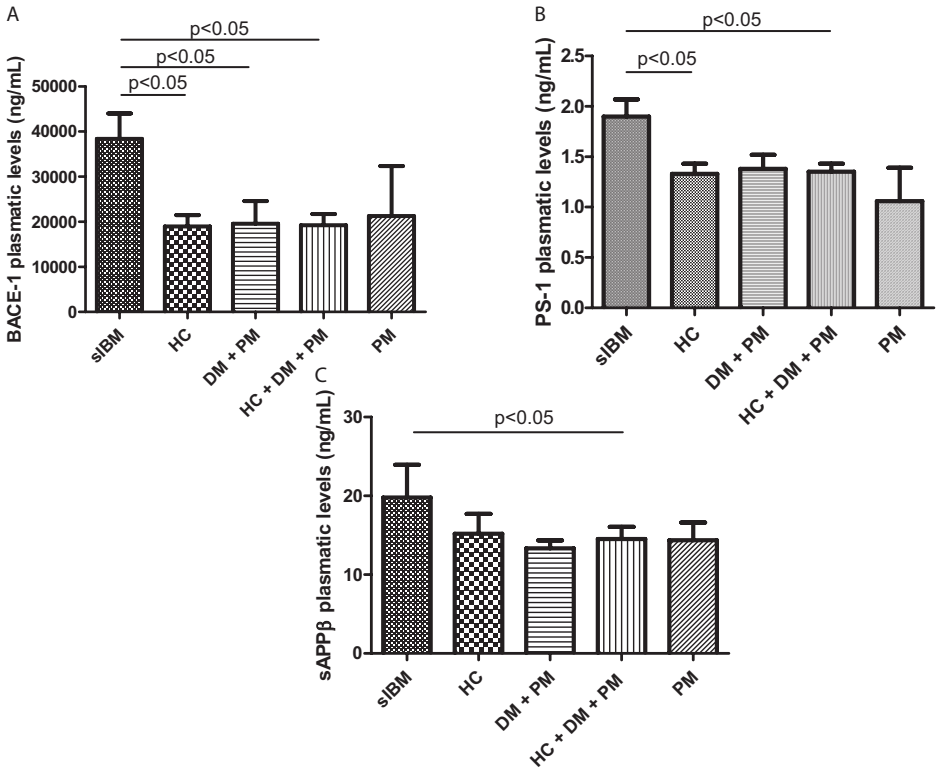

Figure 3. BACE-1 (A), PS-1 (B) and sAPPβ (C) plasma levels in sIBM patients compared with healthy controls (HC), non-sIBM inflammatory myopathy patients (DM + PM) and non-sIBM patients (HC + DM + PM). HC, healthy controls; DM, dermatomyositis; PM, polymyositis. Increased levels of these amyloidogenic molecules in plasma from sIBM patients compared with healthy controls, DM and PM demonstrate their implication in sIBM disease and also strengthen their possible use for diagnostic purposes.

versus 1.35 ± 0.08, $p = 0.024$, and 19.8 ± 4.1 versus 14.5 ± 1, $p = 0.03$, respectively) (Figure 3).

Finally, sIBM patients were compared with PM patients alone, with BACE-1, PS-1 and sAPPβ levels showing the same increased pattern, although these differences did not reach statistical significance, probably because of small sample size of the PM group (38,409 ± 5,629 versus 21,269.6 ± 11,068, $p = 0.103$; 1.82 ± 0.18 versus 1.06 ± 0.33, $p = 0.085$; 19.8 ± 4.1 versus 14.3 ± 2.2, $p = 0.173$, respectively) (Figure 3).

The Omnibus test and the Hosmer-Lemeshow goodness-of-fit test revealed that BACE-1, PS-1 and sAPPβ were suitable as a predictive tool to discriminate sIBM from the other study cohorts. Overall, the sensitivity and specificity were 74.5, 65.5 and 66.7%, respectively, with AUC of 0.77, 0.62 and 0.68, respectively. Combined analysis of these molecules did not show better sensitivity and specificity with respect to the overall values (Table 2).

DISCUSSION

At present, sIBM disease entails two main problems: difficulty for achieving early diagnosis and the lack of effective treatment (7,12,35). The aim of this study was to perform the first screening of circulating molecules potentially involved in the etiopathology of sIBM to promote both advances in understanding the etiology of this disease as well as the development of diagnostic tools. The understanding of the etiopathology of sIBM is crucial to find effective treatment, and improvements in the diagnosis of this disease are essential to reduce the invasiveness of the current approaches, the need for a second or third biopsy to ensure diagnosis and the potential confusion with similar diseases and to facilitate early detection and follow-up. The data provided by the present study demonstrate evidence of plasma biomarkers in a peripheral tissue that is by far more accessible than the target tissue of sIBM; also, these data prevent the need to perform a second muscle biopsy to confirm the diagnosis.

Despite being reported as key molecules in inflammatory processes and mitochondrial bioenergetics, the plasma levels of IL-6, TNF-α, free mtDNA, FGF-21 and CoQ were not significantly altered at plasma level. However, most of these molecules showed strong trends to being altered in the plasma of these patients, for example, IL-6 and TNF-α,

Table 2. Logistic binary regression results in patients with sporadic inclusion body myositis (sIBM) compared with the remaining cohorts (healthy controls + DM + PM).

Molecule	Sensitivity (%)	Specificity (%)	Overall	AUC
Separately				
BACE1	45.0	91.2	74.1	0.77 ± 0.07
PS1	38.1	82.4	65.5	0.62 ± 0.08
sAPPβ	11.1	97.0	66.7	0.68 ± 0.7
Combined				
BACE1 + PS1 + sAPPβ	50.0	87.9	74.1	
BACE1 + PS1	55.0	85.3	74.1	
BACE1 + sAPPβ	44.4	87.9	72.5	

Logistic binary regression results in patients with sporadic inclusion body myositis (sIBM) compared with the remaining cohorts (healthy controls + DM + PM). The sensitivity and specificity of each molecule are shown separately and combined in addition to the AUC of each molecule.

which are also reportedly altered in muscle (16,17). There may be a parallelism between the muscle and plasma levels of these molecules, and since these molecules are not useful to discriminate among other inflammatory myopathies, they may provide information as to the inflammatory status of the patients. Additionally, this inflammation does not seem to be triggered, at least significantly, by circulating mtDNA released from chronically injured muscle cells.

On considering the implication of mitochondria in sIBM, it was also suggested that FGF-21 is altered in these patients. Although the liver is the main producer of FGF-21 (36), muscle was also described to secrete this endocrine factor, and its production is known to be increased as a consequence of primary but not secondary mitochondrial disorders (37). In this regard, we found circulating FGF-21 levels to be increased, albeit not significantly, in these patients. Because all the subjects studied were free of metabolic disorders involving hepatic lesions, muscle may be directly involved in this trend to an increase in sIBM patients. Although it was not possible to evaluate liver and muscle biopsies from the subjects of this study for ethical reasons, this increase in FGF-21 levels seems to reinforce the assumption of mitochondrial implication in the etiopathogenesis of sIBM.

Coenzyme Q was evaluated because of its important involvement in mitochondrial respiratory chain function, as well as its implication in the formation of Tau aggregates (22). However, this molecule was not found to be altered in plasma. Nonetheless, further studies should evaluate the levels of this coenzyme in muscle biopsy where tau aggregation occurs.

On analyzing the molecules related to muscle degeneration and the formation of β-amyloid depositions, we found a significant increase in these biomarkers in plasma of sIBM patients. PS-1 and especially BACE-1 were dramatically increased, suggesting that the increase in these levels is responsible for the formation of β-amyloid depositions in sIBM. In physiological conditions, the processing of APP is carried out by the two pathways: the non-amyloidogenic pathway with α-secretase and the amyloidogenic pathway with BACE-1 and PS-1. However, in nonpathologic conditions, the amyloidogenic pathway is so diminished that there is no relevant formation of β-amyloid. In sIBM, we propose that the amyloidogenic pathway is altered and the levels of the molecules involved in this process (BACE-1 and PS-1) are dramatically increased, pathologically accelerating the formation of β-amyloid depositions. Additionally, the increase of these molecules in muscle (27), where they exert their function-causing protein depots, is also transferred to an increase in plasma levels of these molecules. Likewise, overexpression of sAPPβ, the resulting fragment of APP processed by BACE1, also demonstrated increased BACE1 activity, thereby reinforcing this theory.

The clinical onset of sIBM is similar to that of other inflammatory myopathies. Years of disease evolution are often required, and second biopsies are needed to confirm the diagnosis of sIBM. Consequently, in clinical practice, patients diagnosed with DM and especially PM are the best subjects to test the sensitivity and specificity of the biomarkers selected for sIBM diagnosis.

In the present study, the plasma expression of amyloidogenic markers (BACE-1, PS-1 and sAPPβ) in patients with inflammatory myopathies including DM and PM were similar to those of healthy controls, thereby strengthening the amyloid theory, since, despite sharing some clinical and pathologic features similar to sIBM, these other inflammatory myopathies do not present inclusion bodies. Based on these findings, we can conclude that increased expression of amyloid-related molecules in plasma is specific of sIBM. These findings strengthen the possibility of using those selected molecules as appropriate candidates for the diagnosis of sIBM and as potential biomarkers for discriminating between sIBM and other inflammatory myopathies. Regarding the sensitivity and specificity, we can conclude that, among the biomarkers selected, BACE1 levels are the best parameter for discriminating between sIBM patients and controls or other inflammatory myopathies, since the specificity and sensitivity achieved with the addition of PS-1 or sAPPβ did not increase the diagnostic precision compared with the use of BACE-1 quantification alone. sAPPβ and especially PS-1 levels are also altered in sIBM patients, confirming their involvement in the etiology of this disease.

These findings also strengthen the idea that sIBM is related to AD, at least with respect to its pathogenic mechanisms, showing the same kind of lesion in different tissues (muscle fiber and neurons, respectively). That is why sIBM is also known as muscle AD (23,24,38,39).

CONCLUSION

Considering the difficulties in diagnosing sIBM on the basis of clinical and anatomo-pathological findings, we propose that plasma BACE-1 levels may be a potential circulating biomarker for helping to achieve the diagnosis of sIBM. Because the clinical onset of sIBM and other inflammatory myopathies may be similar, and PM is often misdiagnosed as sIBM, further research should be done to validate in bigger sample size cohorts if those biomarkers could be suitable to ensure sIBM diagnosis in case of ambiguity for differential diagnosis.

The limitation of this study is the sample size, especially of the DM and PM groups. Further studies including more patients are needed to evaluate the usefulness of amyloidogenic biomarkers to establish the severity and evolution of sIBM through the follow-up of a cohort over time.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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