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Assessment of plasma biomarkers for their association with Multiple Sclerosis progression

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ABSTRACT

In a cross-sectional study involving 160 Multiple Sclerosis (MS) patients and 70 healthy controls we set out to determine the association of five blood biomarkers with MS risk and progression scores. High levels of Semaphorin3A (SEMA3A) in females, and low levels of prolactin and estradiol in males associated with MS risk. High MS disability correlated with higher SEMA3A levels in females. Our findings suggest the clinical applicability of SEMA3A, and prolactin as biomarkers for MS progression. However, these biomarkers had sex-specific associations with MS, and any therapeutic approaches utilizing them should take that into consideration.

1. Introduction

Multiple Sclerosis (MS) is an autoimmune neuro-degenerative disorder resulting from an autoimmune reaction against myelin and myelin associated antigens. Neuro-axonal demyelination and degeneration, and oligodendrocyte cell death associated with MS manifest primarily as lesions in the white matter, and progressively in the gray matter of the central nervous system (CNS) (Filippi et al., 2013; Perez-Miralles et al., 2013). While MS has been extensively studied in search of risk and prognostic factors, very few factors have proven applicability and reproducibility in different MS populations. The prevalence of MS in the Kuwaiti population was considered very low in the 1990s, but it has increased from 4.4 in 1990 to 85 cases per 100,000 individuals in 2014 (al-Din et al., 1990; Alroughani et al., 2014). The Kuwaiti MS population is an understudied population where advances in investigative research of MS associated factors has been limited. The clinical characteristics of MS in the Kuwaiti MS population are similar to those reported in other populations, such as; sex bias at a ratio of 2:1 females to males, and male-specific progressive disease course. However, there is a deficiency in examining blood markers in Kuwaiti MS patients shown to associate with MS incidence and clinical variables in other MS populations. There are four blood markers reported to associate with MS progression that include; hormones (testosterone, and prolactin), sphingomyelin and Semaphorin3A (SEMA3A). Testosterone aside from its sexual regulatory functions also have an immunomodulatory function (Gubbels Bupp, 2015). Testosterone levels were found significantly lower in MS females, and associated with more CNS damage (Tomassini et al., 2005). Prolactin levels in MS patients have been studied in MS populations with conflicting results possibly due to small cohort size and type of sample used (Da Costa et al., 2011; Foroughipour et al., 2012; Markianos et al., 2010; De Giglio et al., 2015). Sphingomyelin is known to contribute to CNS myelination, and increased levels within the normal range are considered to be beneficial in neurodegenerative diseases. Sphingomyelin levels have been shown to associate with better outcome in MS (Hon et al., 2009). SEMA3A is a secreted protein that has dual function as either a chemo-repulsive agent inhibiting axonal outgrowth, or as a chemo-attractant agent stimulating the growth of apical dendrites. SEMA3A is critical for normal neuronal pattern development, and it can regulate synaptic transmission in the brain and promote regulatory cytoskeletal remodeling in the adult CNS (Sahay et al., 2005; Tran et al., 2009). In MS, SEMA3A was shown to have altered expression in MS lesions suggesting its involvement in MS progression (Gutierrez-Franco et al., 2016). No studies have been reported on

Abbreviations: CNS, central nervous system; DDI, Dasman Diabetes Institute; EDSS, expanded disability status scale; ELISA, Enzyme-Linked Immunosorbent Assay; HC, healthy controls; HRP, Horseradish peroxidase; MS, Multiple Sclerosis; MSSS, Multiple Sclerosis Severity Scale; SEMA3A, Semaphorin3A; TMB, Tetramethylbenzidine.

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plasma/serum levels of SEMA3A in MS samples. Here we set out to determine the association of these four markers with MS risk and progression in a sampled MS population. We also examined the sex dimorphism of these factors if present, in MS patients.

2. Methods

2.1. Case-control sample collection

A total of 160 Multiple Sclerosis patients were recruited for this study at Dasman Diabetes Institute (DDI), and 70 healthy control (HC) volunteers were recruited by social networking from the general Kuwaiti population. DDI ethical review committee, and Kuwait's Health Sciences Center's Joint Committee for The Protection of Human Subjects approved all study protocols. Both committees adhere to the declaration of Helsinki's Ethical Principles for Medical *Research* Involving *Human Subjects guidelines.* All information pertinent to study protocols were fully explained to all participants prior to procurement of their informed written consent. MS patients inclusion criteria were; a detailed clinical history (demographics, age of onset, disease duration, expanded disability status scale (EDSS) score, and treatment history), being a Kuwaiti born citizen, MS disease duration of ≥2 year, and the agreement to provide a blood sample. Exclusion criteria included; having an EDSS score of 0, and disease duration of 1 year or less. Healthy controls' exclusion criteria included; being a non-Kuwaiti, having a family history of MS, and a diagnosis of an autoimmune or neurodegenerative disorder. Blood samples (4 mL) were collected in EDTA vacutainers, and centrifuged at 3000 rpm at room temperature for 10 min. Plasma fractions (2–3 mL) were collected in appropriately labeled, sterile cryotubes, and stored at −80 °C until use.

2.2. Enzyme Linked Immunosorbent Assays (ELISA)

Total human testosterone (Abcam, Cambridge, UK) ELISA kit used here is a solid phase competitive colorimetric immunoassay. In summary, fixed amounts of target molecule were labeled with horseradish peroxidase (HRP) that competes with unlabeled target present in standards or samples for a limited number of binding sites of a target specific antibody. After 2 h incubation at room temperature with shaking, the microtiter plate was washed to stop the competition reaction. The chromogen solution tetramethylbenzidine (TMB) was added and incubated for 30 min at room temperature. The reaction was stopped with H_2SO_4 and absorbance was measured at 450 nm wavelength. The intensity of this colored product is inversely proportional to the concentration of target present in the original specimen. Testosterone levels were reported in ng/mL (sensitivity 0.07 ng/mL, detection range 0.02–16 ng/mL). Inter- and intra-assay coefficients of variability (CV) were 9.6% and 5.8%, respectively.

Prolactin ELISA kit (Abcam, Cambridge, UK) and Semaphorin3A ELISA kit (LifeSpan Bioscience, Inc., WA, USA) used here employ a sandwich colorimetric immunoassay principle. In brief, a 96-well plate is supplied precoated with anti-tag antibody. Diluted plasma samples and supplied standards were added into each well. An antibody cocktail was prepared for the prolactin ELISA by adding a tag labeled capture antibody and a reporter conjugated detector antibody at a 1:1 ratio, this cocktail will immunocapture prolactin and immobilize it into the wells, whereas SEMA3A ELISA only used a single biotinylated detection antibody. The plate was incubated at room temperature for 1 h with constant shaking at 400 rpm. After incubation, the wells were washed three times to remove unbound material. TMB substrate was added to wells and incubation was set for 10 min with constant shaking. TMB was catalyzed by HRP, generating a blue color proportional to analyte in the sample/standards. This reaction was then stopped by addition of stop solution inhibiting any color change from blue to yellow. The amount of bound analyte was measured at 450 nm and concentrations were recorded in both assays in ng/mL. Detection range for prolactin was 0.39–50 ng/mL with a sensitivity of 70 pg/mL, and 0.16–10 ng/mL for SEMA3A with a detection sensitivity of 0.16 ng/mL. Inter- and intra-assay CV for prolactin ELISA were 5.5% and 1.4%, whereas Inter- and intra-assay CV for SEMA3A ELISA were 6.5% and 5.5%, respectively.

2.3. Sphingomyelin fluorometric assay

Sphingomyelin levels were ascertained in plasma fractions using Cell Biolabs sphingomyelin assay kit according to manufacturer protocol (Cell Biolabs, Inc., CA, USA). In brief, kit supplied sphingomyelinase hydrolyzes plasma sphingomyelin into phosphorylcholine and ceramide. Supplied alkaline phosphatase breaks down phosphorylcholine into choline, which is oxidized by supplied choline oxidase to produce hydrogen peroxide. A highly specific fluorescence probe detected hydrogen peroxide, and horseradish peroxidase catalyzed the reaction between the probe and hydrogen peroxide bound at a 1:1 ratio. Samples and standards were incubated for 60 min at 37 °C with the probe. A standard fluorometric plate reader was used to record fluorescence emission at 590–600 nm after excitation at 530–570 nm. An average zero standard fluorescence value was subtracted from all standards and samples, and a standard curve was plotted. The slope was calculated for each standard curve/plate and sphingomyelin levels in samples were calculated using the following formula; sphingomyelin (mg/dL) = (Corrected sample fluorescence / Slope) × Sample dilution (50). Detection range was 31–125 mg/dL.

2.4. Statistical analysis

D'Agostino and Pearson normality test was performed for all data and all were non-normally distributed, therefore non-parametric analyses were performed. For statistical association analysis non-parametric Mann-Whitney test was used. For correlation analysis non-parametric Spearman correlation test was used. All statistical analyses were performed using GraphPad Prism software v.7.02 (GraphPad Software, Inc., CA, USA).

Table 1
Demographics, clinical characteristics, and median levels of biochemical markers assessed in MS and healthy controls cohorts.

Criteria	MS cohort (n = 160)	Healthy cohort (n = 70)
Demographics		
Age in years [median (IQR[a])]	31.5 (25.75–39)	26 (24–33)
Sex (females/males)	105/55	48/22
Type of MS (%)		
RRMS	143 (89.38)	NA[b]
SPMS	10 (6.25)	
PPMS	3 (1.87)	
Benign	4 (2.5)	
Disability/severity indices		
EDSS	1.75 (1–2.5)	NA
MSSS	3.34 (2.078–5.11)	
MS medication		
Fingolimod	63	NA
Tysabri	30	
Interferon beta-1a	25	
Interferon beta-1b	12	
Other (rituximab, aubagio, tecfidera)	11	
None	19	
Biochemical marker levels [median (IQR)]		
Testosterone (ng/mL)	0.04 (0.03–1.26)	0.03 (0.03–1.05)
Prolactin (ng/mL)	4.41 (3.15–5.98)	5.24 (4.23–6.66)
SEMA3A (ng/mL)	353.7 (279.4–509.6)	268.9 (190.7–379.5)
Sphingomyelin (ng/mL)	68.3 (56.33–85.13)	65.54 (53.26–77.15)

[a] IQR is interquartile range.
[b] NA is not applicable.