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Antibody recognition of the hyper-glucosylated adhesin protein HMW1Ct of non-typeable *Haemophilus influenzae* in Rett syndrome

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ABSTRACT

Rett syndrome (RTT) is an X-linked neurodevelopment disorder associated with the single monogenic mutation in methyl-CpG binding protein 2 (MeCP2) in up to 95 % of cases. The growing number of genome-wide association studies and incomplete concordance for autoimmune diseases in monozygotic twins concur to support the involvement of environmental factors, like infectious agents or chemicals, in the breakdown of tolerance leading to autoimmunity. In fact, the coexistence of a dysregulation of the immune system in RTT patients has been previously hypothesized. We herein explored the hypothesis that an autoimmune component derived from environmental bacterial infection of non-typeable *Haemophilus influenzae* may coexist in RTT. At this purpose we screened sera from RTT syndrome patients, non-RTT pervasive developmental disorders patients and healthy controls with the hyper-glucosylated adhesin protein HMW1Ct-Glc used as an antigen in ELISA in order to identify specific antibodies to N-glucosylation sites. Results showed that HMW1Ct-Glc is able to significantly discriminate antibodies among RTT sera and controls. Competitive ELISA confirmed the specific interaction between antibodies characteristic of RTT syndrome and the N-glucosylation motifs of the bacterial adhesin protein HMW1Ct-Glc.

1. Introduction

Rett syndrome (RTT) is a genetic X-linked neurodevelopment disorder associated with the single monogenic mutation in methyl-CpG binding protein 2 (MeCP2, Gene ID: 4204) in up to 95 % of cases,[1] more rarely by mutations in cyclin-dependent kinase-like 5 (CDKL5, Gene ID: 6792),[2] and forkhead box protein G1 (FOXG1, Gene ID: 2290) gene.[3] The typical RTT clinical symptoms include early neurological regression in 80 % of patients, afterward loss of acquired cognitive, social, and motor skills in a typical four-stage neurological regression. Since many of the core symptoms and neurological features can be shared with other neurodevelopmental pathologies, it is likely that the disorders share some critical molecular underpinnings.[4] Subsequently, advancements in neurobiological knowledge, together with an improved understanding of MeCP2 function within the central nervous system (CNS), have facilitated the development and evaluation of therapeutic strategies specifically targeting Rett syndrome.[5] In this context, despite RTT syndrome is a genetic disorder, it is increasingly considered a broad-spectrum pathology, the coexistence of a dysregulation of the immune system has been previously hypothesized.[6,7] Moreover, the growing number of genome-wide association studies and incomplete concordance for autoimmune diseases in monozygotic twins concur to support the role of environmental factors (including infectious agents and chemicals) in the breakdown of tolerance leading to autoimmunity through different mechanisms.[8] In particular, a derangement of microglia immune responsiveness, situation where the

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normal immune functions of microglia are disrupted or dysregulated, might be likely to occur in these patients, as neuroinflammation is a powerful modulator of the CNS immune system.[9] Actually, the nexus between MeCP2 and autoimmunity has been reported in the literature,[6,10,11] and in particular autoantibodies to brain proteins, i.e. nerve growth factor[12,13] and folate receptor.[14] Moreover, future research direction could involve targeting therapeutically immune dysfunction as a novel strategy for RTT management.[15]

In pathological conditions of the central nervous system, such as RTT and other neurological diseases (e.g., multiple sclerosis, MS) in which antibodies and/or antigens involved in the disease are unknown, specific antibody detection in biological fluids is a challenge for patient stratification. In this context, we previously demonstrated that anti-N-glucosylated peptide CSF114(Glc) antibodies in a multiple sclerosis (MS) patient subpopulation,[16–18] preferentially recognize the N-glucosylated adhesin protein of non-typeable *Haemophilus influenzae* (NTHi) and in particular the C-terminal portion HMW1Ct(1205–1526), termed HMW1Ct-Glc.[19] To the best of our knowledge, this was the first example of an N-glucosylated bacterial antigen that can be considered a relevant candidate for triggering pathogenic antibodies in MS to stratify patients. It is to note that, after the introduction of the *Haemophilus influenzae* vaccination, the majority of invasive infections is now caused by NTHi in all age groups in USA.[20] Since the NTHi cell-surface adhesins are heavily glucosylated on a relevant number of sequons (NXS/T) exposed on beta-turns, the N-glucosylated residues are likely to be exposed conceptually in vivo in a multivalent shape, thus potentially generating an immunological response.[18,21] Translating these considerations to RTT syndrome, since MeCP2 acts intrinsically upon immune activation affecting neuroimmune homeostasis by regulating the pro-inflammatory/anti-inflammatory balance,[11] the hypothesis that environmental factors like bacterial infections could be involved in RTT is gaining interest.

With all these considerations in mind, we have previously successfully used synthetic modified beta-turn peptide probes bearing an N-glucosylation exposed on the solid surface of the ELISA, to characterize specific antibodies in RTT patient serum.[22] In particular, RTT patients presented high antibody levels against the synthetic probe N-glucopeptide CSF114(Glc), thus suggesting an aberrant modification of protein N-glucosylation rate and the possible activation of autoimmunity processes in the syndrome.[23] A dysregulated N-glycosylation pattern in RTT pathogenesis was confirmed by a study on MeCP2-null mice, in which a reduced N-glycosylation in the protein involved in neuronal cell communication brain nucleotide pyrophosphatase-5, was characterized in both pre-symptomatic and symptomatic mice. Importantly, these N-glycosylation modifications were rescued by MeCP2 reactivation.[24] The underlying principle for this chemical modification stems in N-glucosylation recognition that can be a pervasive feature of biomolecules occurring in the central nervous system (CNS) pathologies characterized by neuronal disruption.

Consequently, we report herein the hypothesis that an autoimmune component derived from environmental bacterial infection may coexist in RTT. At this purpose we screened RTT syndrome patient sera in comparison with control sera from non-RTT pervasive developmental disorders (non-RTT PDD) patients and healthy subjects (normal blood donors, NBD), with a hyperglucosylated adhesin antigen in a solid-phase ELISA to identify specific antibodies to N-glucosylation sites. This antigen, consisting of a mixture of three N-glucosylated HMW1Ct variants containing 7, 8, and 9 glucose moieties on Asn, was obtained by simultaneous co-expression of adhesin HMW1Ct and N-glucosyltransferase HMW1C in *E. coli* following a previously described method.[19]

2. Materials and methods

2.1. Protein expression

2.1.1. General procedure for protein expression

The C-terminal fragment of the HMW1A adhesin (residues 1205–1536, termed HMW1Ct) and the glycosyltransferase enzyme HMW1C (PDB: 3Q3H) were expressed similarly to the previously described protocol,[19] using *E. coli* BL21 cells previously engineered with plasmid pET-45b (+) (Merck, Milano, Italy), encoding for the fragment HMW1Ct and equipped with the gene for carbenicillin resistance, and plasmid pET-24a (+), encoding for the glucosyltransferase enzyme ApHMW1C and equipped with the gene for kanamycin resistance. Cell cultures were prepared using Luria-Bertani (LB) liquid culture soils. Stock solutions of antibiotics were prepared in H_2O Milli-Q and stored at −20 °C. Working concentration in cell media is 50 μg/mL for kanamycin (only for N-glucosylated HMW1Ct-Glc and 100 μg/mL for carbenicillin. Lysis buffer (pH 7.5) was composed of 5.96 g of HEPES (50 mM), 2.92 g of NaCl (100 mM) and 50 mL of glycerol (10 %) dissolved in 0.5 L of H_2O Milli-Q. The HMW1Ct-Glc and/or HMW1Ct *E. coli* glycerol stocks were incubated overnight at 37 °C in a 5 mL culture containing the carbenicillin or kanamycin antibiotic(s) under shaking. Subsequently, the bacteria were grown in 1 L of the same LB liquid culture soil. The solution was incubated under shaking at 37 °C. Cell growth was monitored measuring the optical density at 600 nm (OD_{600}) with an UV instrument (Amersham Biosciences, Little Chalfont, UK). The same LB liquid culture soil was used as blank. When the OD value reached 0.6–0.8, the induction of the expression was performed adding 1 mM solution of isopropyl-β-D-1-thiogalactopyranoside (IPTG). Cell suspension was incubated overnight at 16 °C under shaking. Cells were recovered through centrifugation at 4000 rpm for 30 min at 4 °C. The supernatant was removed, and the final pellet was washed 3x with PBS buffer, recentrifuged and stored at −20 °C.

2.1.2. General procedure for protein purification

The pellet was suspended in 30 mL of lysis buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 10 % glycerol) adding 10 μL/g of cells of protease inhibitor (cocktail Set III EDTA-free, Merck, Milano, Italy). Mechanical lysis of the cell membrane was obtained by using an ultrasonic processor. The lysis solution was then centrifuged for 110 min at 35000 rpm and the supernatant containing the product(s) was recovered. The purification was performed using an Äkta FPLC system (Amersham Biosciences, Cytiva Italy Srl, Milano, Italy). During the first purification step a Hi Trap-His column (HisTrap HP 5 mL) was used with the binding buffer A1 for Hi Trap-His (30 mM imidazole, 50 mM HEPES pH 7.5, 300 mM NaCl, (pH 7.5, 5 % glycerol) and the elution buffer B1 for Hi Trap-His (300 mM imidazole, 50 mM HEPES pH 7.5, 300 mM NaCl, 5 % glycerol).

The conditioning of the column was performed using buffer A1 for 10 min. The supernatant containing the products was then injected and eluted with a gradient from 0 % to 100 % of buffer B1. The UV detector was set to 280 nm and 215 nm. All the fractions obtained were analyzed through Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis technique (SDS-PAGE).

The separation of HMW1Ct-Glc from ApHMW1C was obtained in the second purification step through the ion exchange technique. A Hi Trap Q-FF (5 mL, GE Healthcare) column was used. A buffer exchange in order to substitute buffer B1 with binding buffer A2 (20 mM Tris buffer, 20 mM NaCl, pH 8) for Hi Trap Q-FF was performed using Amicon Ultra Centrifugal Filters (MWCO = 10 kDa). The Hi Trap Q-FF column was then conditioned with buffer A2 for 10 min. The sample was injected and eluted using a linear gradient from 0 % to 100 % of elution buffer B2 (20 mM Tris buffer, 1 M NaCl, pH 8) for Hi Trap Q-FF. The UV detector was set to 280 nm and 215 nm. All the fractions obtained were analyzed by SDS-PAGE. The analytical characterization of the purified antigen HMW1Ct-Glc is reported in the Supplementary Materials (Figures S1-S3).

Both HMW1Ct and HMW1Ct-Glc were stocked in PBS buffer (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 dissolved in 1 L of H_2O Milli-Q) at −20 °C. Their concentration was calculated using the Lambert-Beer law after an absorption measure performed using an UV spectrometer (Varian Cary 4000, Agilent, Santa Clara, California, USA) set to a range from 320 and 240 nm, using the extinction coefficient $\varepsilon = 7052\ L\ mol^{-1}\ cm^{-1}$.

2.1.3. General procedure for SDS-PAGE

The SDS-PAGE gel was prepared by depositing between two glasses the running 16 % gel solution, composed of 1.6 mL H_2O Milli-Q, 4.27 mL 30 % acrylamide, 2 mL 1.5 M tris buffer pH 8.8, 80 µL 10 % SDS, 80 µL 10 % ammonium persulfate (APS), 10 µL tetramethylethylenediamine (TEMED). After the polymerization, the 4 % stacking gel solution (1.8 mL H_2O Milli-Q, 0.4 mL 30 % acrylamide, 0.750 mL 0.5 M TRIS buffer pH 6.8, 30 µL 10 % SDS, 30 µL 10 % APS, 6 µL TEMED) was deposited above the previous one inserting the comb for the formation of the wells. After polymerization, the gel was positioned inside the SDS-PAGE apparatus (Biorad, Segrate, Milano, Italy) and the tank buffer 1x (100 mL of Tris buffer/Glycine/SDS (10x) in 1 L) was added. 10 µL of each sample were combined with 5 µL of loading buffer 5x (200 mM of Tris-Cl (pH 6,8), 400 mM of DTT, 8 % of SDS, 0.4 % of bromophenol blue and 40 % of glycerol), treated at 100 °C for a few minutes and centrifuged. Each sample was then loaded in the dedicate well. The commercial marker PageRuler Plus Prestained Protein Ladder, 10–250 kDa, was used as reference. The electrophoresis was performed for 90 min at 140 mV and subsequently the gel subjected to stain using a Coomassie solution (25 mL H_2O Milli-Q, 20 mL MeOH, 5 mL AcOH, 0.05 g Coomassie blue dye) for 30 min. In order to remove the excess dye, the stained gel was treated overnight with a destaining solution (700 mL H_2O Milli-Q, 200 mL MeOH, 100 mL AcOH) under gentle shaking.

2.2. Solid-phase immunosorbent assay (ELISA)

All ELISA parameters, including plates, coating conditions, reagent dilutions, buffers, and incubation times were previously optimized.[19] Samples were tested in triplicates and blanks were tested using Fetal Bovine Serum (FBS) buffer instead of sample sera at identical conditions. The absorbance value for each serum was calculated as (mean Abs of serum triplicate) − (mean Abs of blank triplicate). One positive multiple sclerosis patient serum and one negative healthy control as references, were included in each plate for further normalization.

2.2.1. Sample collection

In this study, a total of 88 samples including patients and healthy controls were enrolled. The RTT group consisted of 35 patients (mean age 17.74 ± 8.99 years) subdivided into n = 25 (71 %) with classical clinical presentation with proven MeCP2 gene mutation and n = 10 (29 %) atypical presentation. All the patients were anonymous and recruited in the Child Neuropsychiatric Unit, University Hospital "Azienda Ospedaliera Universitaria Senese", Siena (Italy), during the study. Participants were eligible for inclusion in the study if they presented a clinical diagnosis of Rett Syndrome and a confirmed genetic test indicating the presence of MeCP2, CDKL5, or FOXG1 genes mutation. A group of non-RTT PDD group consisted of 29 patients (mean age 8.8 ± 8.4 years) diagnosed based on well-established criteria. Patients were recruited from those attending the unit for routine clinical follow-up. Blood samplings in the patient group were performed during the routine follow-up study at hospital admission, while the samples from the control group were carried out during routine health checks, sports, or blood donations, obtained during the periodic clinical checks. The healthy control subjects were 24 samples (mean age 11.62 ± 4.63 years) age-matched. After collection, blood was immediately centrifuged at 4 °C, 1700 g for 20 min then the sample was frozen to −20 °C until analysis.

The study was approved by the Institutional Review Board of "Azienda Ospedaliera Universitaria Senese" Study ID number 11615 code OMEGA3 SIENA title "Supplementazione con acidi grassi polinsaturi omega-3 in pazienti con sindrome di Rett" principal investigator Joussef Hayek. Parents, tutors, or guardians of all the participants provided their written informed consent to participate in this study.

2.2.2. Solid-phase ELISA

ELISA conditions were previously optimized employing the synthetic antigens for the detection of antibodies in MS sera.[19,25]

Briefly, 96-Well activated polystyrene ELISA plates (NUNC Maxisorp, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) were coated with 1 µg/100 µL/well of the antigens HMW1Ct and HMW1Ct-Glc in coating buffer and incubated at 4 °C overnight. After 3 washes with 0.9 % w/w saline solution containing 0.05 % v/v tween 20 (saline tween), non-specific binding sites were blocked with blocking buffer (10 % of fetal bovine serum in saline tween) at room temperature (r.t.) for 60 min. Sera diluted 1:100 in blocking buffer (100 µL/well) were added and incubated at 4 °C for 16 h. After 3 washes, 100 µL/well of secondary antibody solution [alkaline phosphatase conjugated anti human IgM or IgG Fab2-specific affinity purified antibodies (A3437 or A3312 respectively, Merck, Milano, Italy) diluted 1:500 in blocking buffer] were added. After 3 h incubation at r.t., plates were washed 3 times and then 100 µL/well of substrate solution (p-nitrophenylphosphate 0.1 % w/v in carbonate buffer and $MgCl_2$ 10 mM) were added. After 15 min (IgG plates) or 40 min (IgM plates), the reaction was blocked with 50 µL of 1 M NaOH and the absorbance read in a plate reader (SUNRISE, TECAN, Cernusco Sul Naviglio, Italy) at 405 nm. Mean absorbance values at 405 nm subtracting the blank are reported.

2.2.3. Competitive ELISA

Stock solution of the coating antigen (1 mg/mL) was diluted 1:100 in PBS pH 7.2 (HMW1Ct and HMW1Ct-Glc. 100 µL of coating antigen solution were added to each well (1 µg antigen/well) in a 96-well ELISA plate (NUNC Maxisorp, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), and incubated overnight at 4 °C. The plate was washed three times with 0.9 % w/w saline solution containing 0.05 % v/v tween 20 (saline tween). 100 µL of Fetal Bovine Serum solution (FBS 10 % in saline tween) well were added and the plate was incubated 2 h at room temperature (r.t.). Then the plate was emptied and a mixture of sera and competing antigen was added in each well. Serum concentration was constant (dilution 1:200) while competing antigen concentrations were between 10^{-11} and 10^{-5}M.

The plate was incubated 1 h at r.t., washed three times, and then 100 µL of secondary antibody solution (alkaline phosphatase conjugated anti human IgM or IgG Fab2-specific affinity purified antibodies) diluted 1:500/well were added and incubated 3 h at r.t. Plates were washed three times. 100 µL/well of substrate solution (p-nitrophenylphosphate 1 mg/mL in carbonate buffer and $MgCl_2$ 10 mM) were added. After 30–60 min, the reaction was blocked by adding 50 µL NaOH 1 M/well and the final absorbance value was measured with a plate reader at 405 nm (Tecan-Sunrise spectrophotometer, Cernusco Sul Naviglio, Italy).

Peptide concentration-absorbance relationship was represented graphically as signal inhibition percentage, and half-maximal response concentration values (IC50) were calculated with GraphPad Prism software version 6.01.

3. Results and discussion

3.1. Solid-phase ELISA

Sera from 35 Rett syndrome (RTT) patients, 29 non-RTT pervasive developmental disorders (non-RTT PDD) patients and 24 normal blood donors (NBD) have been screened in solid phase ELISA using the N-glucosylated protein HMW1Ct-Glc and the corresponding unglucosylated protein HMW1Ct as antigens. Clinical characteristics of the selected patients are summarized in the experimental section.

Antigens have been coated in 96-well microplates to identify antibodies in sera. The protocol employed was previously described[19] with minor modifications. Data distribution of IgM and IgG antibody reactivity to proteins HMW1Ct-Glc and HMW1Ct in solid-phase ELISA are summarized in Fig. 1.

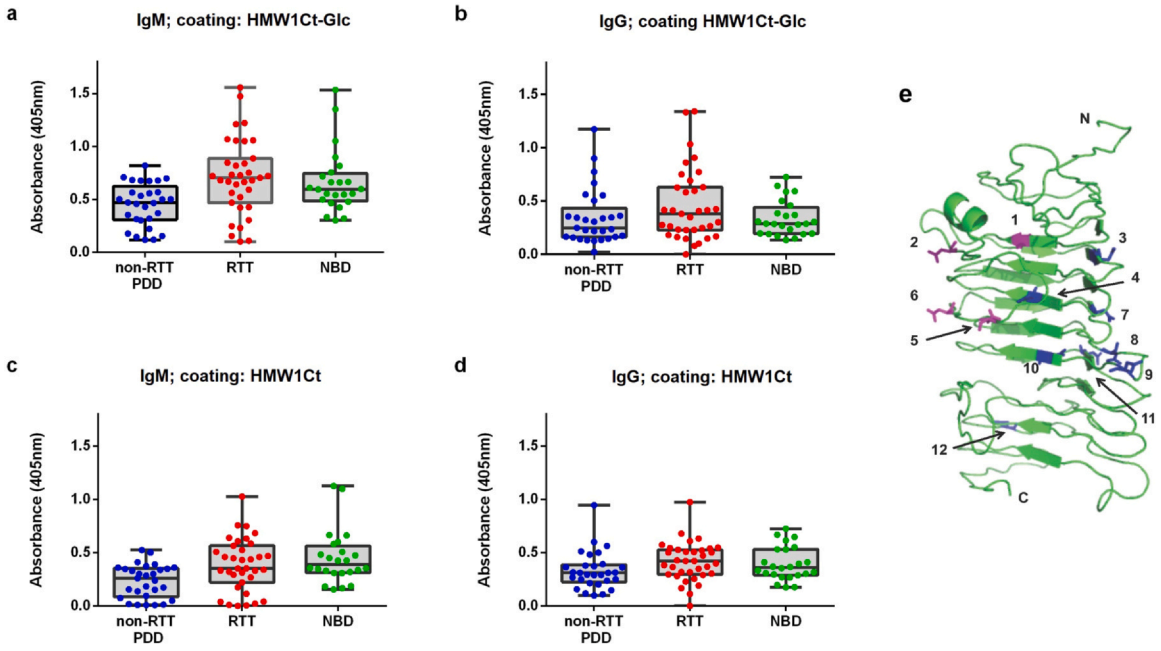

Fig. 1. Antibody reactivity in non-RTT PDD, RTT, and NBD sera identified by SP-ELISA to the N-glucosylated adhesin protein HMW1Ct-Glc (panel a: IgM; panel b: IgG) and to the unglucosylated protein HMW1Ct (panel c: IgM; panel d: IgG). Antibody titers are expressed as absorbance at 405 nm. Data are presented as mean values with 25th and 75th percentile from minimum to maximum values. Kruskal-Wallis test statistics 12.5 and 2.65; P value = 0.0024 and 0.2665 for IgM and IgG, respectively. Panel e: I-TASSER computed model of HMW1Ct with predicted N-glucosylation sites 1,2,5,6 highlighted in magenta and all other predicted N-glucosylation sites are depicted in blue.

Antibody titers to the N-glucosylated HMW1Ct-Glc appeared slightly increased in the RTT group only in the case of IgM antibody titers (Kruskal-Wallis test statistics 12.5 and 2.65; P value = 0.0024 and 0.2665 for IgM and IgG, respectively). This result is particularly interesting and supports the hypothesis that the immune system developed specific IgMs to the N-glucosylated HMW1Ct-Glc after a possible non-typeable *H. influenzae* (NTHi) infection, also confirmed by the lower absorbance signals detected with the unglucosylated protein HMW1Ct. We also examined the differences between classical and atypical RTT variants, but no conclusive evidences emerged. In fact, antibody reactivity remains low in non-RTT PDD group, reinforcing the observation that antibodies directed to N-glucosylated residues are specific to RTT group. This result is in agreement with the diagnostic value of IgM antibodies to a designed N-glucosylated synthetic type I' beta-turn peptide probe that was previously described both in MS and RTT.[16,22,23]

On the other hand, it is noteworthy that the moderately high titers observed for both antigens HMW1Ct-Glc and HMW1Ct in normal blood donors sera (NBD) is not surprising since NTHi is a rather ubiquitous human pathogen to which most people have been exposed. In fact, the presence of antibodies against unglucosylated epitopes shared by both proteins has been previously reported and unequivocally demonstrated in the case of MS. In any case, patient sera revealed to be highly populated with antibodies against the N-glucosylated protein HMW1Ct-Glc, suggesting the recognition of an epitope specifically displayed on the glucosylated HMW1Ct-Glc protein as in the previously described case of a multiple sclerosis subpopulation.[19]

The power of IgM antibody binding levels to HMW1Ct-Glc was also investigated by the Receiver Operator Curve (ROC) analysis comparing different values as sensitivity, specificity, and likelihood ratios.[26] ROC-curve for IgM antibody activity was calculated based on the 35 RTT cases versus both the non-RTT PDD and the healthy samples for a total of 53 controls (Fig. 2). The area under the curve was 0.6663 (0.5420–0.7906, 95 % confidence interval, P value < 0.008561). The optimal cut-off absorbance value was set-up at 0.7193 with 48.57 % (31.38–66.01 %, 95 % CI) sensitivity, 86.79 % (74.66–94.52 %, 95 % CI) specificity with a likelihood ratio of 3.678. Applying the selected cut-off, the presence of specific IgM to HMW1Ct-Glc was quantified in 17 out of 35 (48.6 %) RTT patient sera. Then, protein antigen HMW1Ct-Glc was able to recognize both specific IgM antibodies in RTT patients suggesting the involvement of a bacterial infection in the immune response.

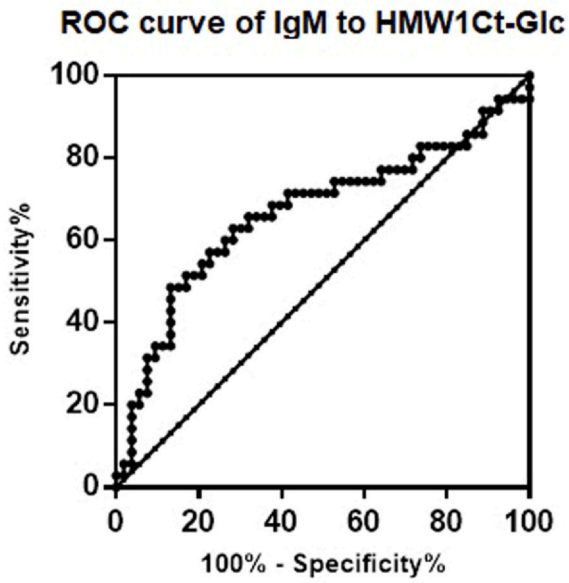

Fig. 2. ROC curve analysis of IgM antibodies to HMW1Ct-Glc in RTT patients versus non-RTT PDD patients and controls determined by SP-ELISA. The area under the curve is 0.6663 (0.5420–0.7906, 95 % confidence interval (CI), P value < 0.008561). The optimal cut-off absorbance value was set-up at 0.7193 with 48.57 % sensitivity (31.38–66.01 %, 95 % CI), 86.79 % specificity (74.66–94.52 %, 95 % CI) with a likelihood ratio of 3.678.

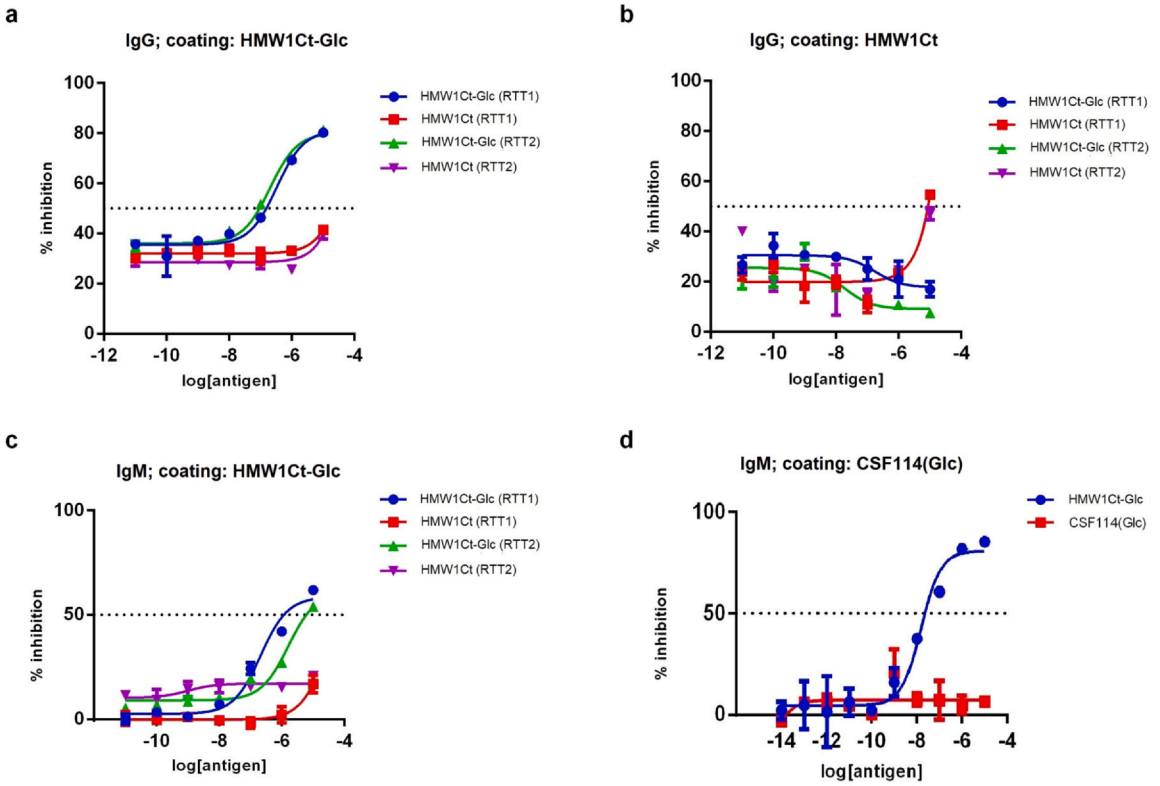

Fig. 3. Inhibition curves. a) IgG antibodies to HMW1Ct-Glc antigen on the plate; b) IgG antibodies to HMW1Ct antigen on the plate; and c) IgM antibodies to HMW1Ct-Glc antigen on the plate in two representative sera RTT1 and RTT2, using as inhibitors the glucosylated HMW1Ct-Glc adhesin antigen and the corresponding unglucosylated analog HMW1Ct. d) IgM antibodies to CSF114(Glc) antigen on the plate in the representative RTT1 serum, using as inhibitors CSF114(Glc) and HMW1Ct-Glc. The results are shown as % inhibition activity of the sera (ordinate axis) versus antigen concentrations in logarithmic scale.

3.2. Competitive ELISA

The autoantibody recognition, using HMW1Ct-Glc and HMW1Ct as antigens, was also evaluated by competitive ELISA on two representative RTT patient sera using the proteins as both inhibitors and coating agents. The selection of sera was based on a previous titration by SP-ELISA with proteins, to identify those presenting high IgG and IgM antibody titers. Competitive ELISA results are summarized in Fig. 3.

Results clearly showed that the hyper N-glucosylated adhesin HMW1Ct-Glc was able to inhibit the binding of IgG and IgM antibodies in sera from a selected population of representative RTT patients (mean IC50 = 2.64 ± 0.9 $\times 10^{-7}$ M and 9.01 ± 0.9 $\times 10^{-7}$ M, non-linear fit R square 0.9875 and 0.9732 for IgG and IgM respectively, Fig. 3 panels a and c). In contrast, the non-glucosylated protein HMW1Ct did not inhibit, or only inhibited RTT1 serum binding at significantly higher concentrations (Fig. 3, panel b), confirming the fundamental role of the N-glucosyl moieties on the adhesin protein for the interaction with anti-N-glucosyl antibodies in RTT patient sera. Moreover, results using the N-glucosylated peptide CSF114(Glc) as antigen in competitive ELISA experiments showed that HMW1Ct-Glc was able to inhibit IgM anti-CSF114(Glc) antibodies, whereas the peptide CSF114(Glc) was not able (Fig. 3, panel d). On the other hand, sera from healthy controls were also tested and no inhibition was observed (data not shown). Indeed, generally speaking in the case of anti-N(Glc) antibodies in multiple sclerosis, the evidence that only competition tests for IgGs, but not those for IgMs, were successful when peptide sequences were investigated as antigens may be due to two different reasons.[27,28] From a practical point of view, anti-N(Glc) IgGs, which possess higher affinity compared to IgMs, are often present in sera, therefore hampering the outcome by binding free target antigens and preventing IgM inhibition. Secondly, high-avidity, pentameric IgMs, possessing ten identical antigen binding sites, may be difficult to displace without a very high affinity ligand.[27] Then, the presence of N-glucosylations in the HMW1Ct-Glc protein was enough to inhibit anti-CSF114(Glc) IgM binding in serum RTT1, assessing the cross-reactivity between the bacterial HMW1Ct-Glc protein and the previously developed synthetic antigenic probe, i.e., the structure-based designed β-turn N-glucosylated peptide CSF114(Glc).

4. Conclusions

Rett syndrome (RTT) is considered and classified as a genetic disease related exclusively to a specific mutation on the MeCP2 directly linked to the X chromosome. RTT is in any case characterized by a very complex phenotype, suggesting the involvement of several factors in its pathogenesis and development. The hypothesis that not only genetics but also dysregulation of the immune system is involved in the syndrome (allowing to stratify all variants of patient populations) is increasingly accepted.[6,7] Because of the reported similarities between RTT syndrome and well-established autoimmune diseases such as multiple sclerosis, we investigated the possible role of the N-glucosylated bacterial protein HMW1Ct-Glc from non-typeable *Haemophilus influenzae* in the onset of Rett syndrome, which antigenic role in a multiple sclerosis subtype population has been previously demonstrated.[19]

These results highlight that HMW1Ct-Glc is able to significantly detect specific antibodies in a cross-sectional single cohort of RTT patient sera, thus discriminating RTT patients from controls. Competitive assays on two RTT patient sera featuring higher IgG and IgM antibody titers confirmed the specific interaction between antibodies characteristic of RTT syndrome and the N-glucosylation motifs of the bacterial protein HMW1Ct-Glc. These results pave the way for the final assessment that a bacterial infection of non-typeable *Haemophilus influenzae*

(NTHi), for which differently from *Haemophilus influenzae* no vaccine is available, can trigger an aberrant immune response associated with MECP2 gene mutations that are the cause of most cases of Rett syndrome, a progressive neurologic developmental disorder and one of the most common causes of cognitive disability in females. We hypothesize that early NTHi infection in Rett syndrome induces an autoimmune response via molecular mimicry involving a hyper-glucosylated adhesin. This aligns with evidence that microbial infections can remain latent and contribute to long-term neurodevelopmental or neurodegenerative effects, even in asymptomatic individuals.[19]

It should be noted that these findings are derived from a hypothesis-generating investigation, and a larger study will be necessary to assess these results. A limitation of this study can be the heterogeneity in the age distribution between the patient cohort and the control group, which could introduce a potential bias in the interpretation of the results. Future research should address this aspect by ensuring age-matched cohorts or by statistically adjusting for age-related effects to improve the robustness of the findings. However given that the involvement of antibodies in Rett syndrome remains an open and under-documented area of research, the characterization of antibodies involved in Rett syndrome is extremely relevant despite the P value being at the threshold of statistical significance. Such findings provide essential insights into an underexplored aspect of the disease immunological mechanisms, since MeCP2 acts intrinsically upon immune activation affecting neuroimmune homeostasis by regulating the pro-inflammatory/anti-inflammatory balance.[9] Thus, the hypothesis that environmental factors like bacterial infections could be involved in RTT is herein assessed. Inhibition experiments supported the ELISA results, emphasizing that the N-glucosylation motif is likely the key element in the recognition of antibodies against the bacterial adhesin protein.

CRediT authorship contribution statement

Mattan Hurevich: Writing – review & editing, Writing – original draft, Validation, Conceptualization. **Nir Qvit:** Writing – review & editing, Validation, Investigation, Conceptualization. **Eduardo Ponticiello:** Writing – review & editing, Resources, Methodology, Conceptualization. **Paolo Rovero:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Investigation, Data curation, Conceptualization. **Joussef Hayek:** Writing – review & editing, Validation, Conceptualization. **Anna Maria Papini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Feliciana Real Fernández:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Francesca Nuti:** Writing – review & editing, Methodology, Investigation, Data curation. **Lorenzo Altamore:** Methodology, Investigation, Formal analysis.

Informed Consent Statement

Informed consent was obtained from all parents, tutors, or guardians of the subjects involved in the study.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of “Azienda Ospedaliera Universitaria Senese” Study ID number 11615 code OMEGA3 SIENA title “Supplementazione con acidi grassi polinsaturi omega-3 in pazienti con sindrome di Rett” (principal investigator Joussef Hayek).

Supplementary Materials

Analytical characterization of HMW1Ct-Glc expressed antigen.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.glycos.2026.100028.

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