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MAP3738c and MptD are specific tags of *Mycobacterium avium* subsp. *paratuberculosis* infection in type I diabetes mellitus

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Abstract *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) is the causative agent of Johne's disease, a chronic inflammation of ruminants' intestine. Recent studies have linked *Map* to type I Diabetes mellitus (T1DM). We searched the presence of antibodies against two specific proteins of *Map* (MptD and MAP3738c) in sera of patients affected by T1DM and type II Diabetes mellitus (T2DM). MptD protein (MAP3733c) has been recognized as a *Map* virulent factor whereas MAP3738c has not yet been studied. Both proteins are encoded by genes belonging to a *Map* specific pathogenicity island. Forty three T1DM patients' sera, 56 T2DM patients' sera and 48 healthy subjects' sera were screened by ELISA to evaluate the immunoresponse against MptD or MAP3738c recombinant proteins. Results showed a positive response to both proteins in T1DM patients whereas no difference with controls was found for T2DM patients. Results suggest a potential relation between T1DM and the bacterial infection.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*Map*) is the etiological agent of Johne's disease (Paratuberculosis), a chronic granulomatous inflammatory pathology that affects big and small ruminants.

Since *Map* has been found in retail and commercialized milk, its consumption by children may be a risk factor for *Map* infection [1]. Moreover, different reports suggest that the bacterium may be involved in inflammatory disease development, such Crohn's disease in genetically susceptible people [2].

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Paratuberculosis is present in more than 50% of Sardinian herds, therefore Sardinia inhabitants may be exposed to potential contamination by *Map*, and it has been reported that occupational exposure is linked to increased humoral response against *Map* [3]. Moreover, genetic segregation of the Sardinian population [4] together with *NOD2/CARD15* [5] as well as *SLC11A1* [6] allele polymorphisms (both involved in pathogen detection and clearance) may create the ideal setting for *Map* infection in susceptible individuals.

Some authors speculated on the mimicry between bacterial and host heat shock proteins that share common epitopes, during infection [7], and it was assumed that host response triggered by microbial antigens would activate an immune response against self-epitopes.

In particular, infections caused by pathogens that share antigens with high homology to proteins of the host, may activate T cells specific immune response to self antigens, causing autoimmune disease. The mechanism is due to the properties of antigen recognition by a T cell receptor (TcR) that is degenerate and thus flexible. In this way, antigens belonging to infectious organisms that have cross-reactivity with self-epitopes can also activate T cells that have a low affinity for self-molecules and escaped negative selection [8].

In line with this view, experimental analysis of the correlation between humoral response against *Map* and T1DM by using immunological assays with specific proteins has been already proposed [9].

Within this scenario, the purification of mycobacterial antigens highly likely unique in sequence and preferentially located on the cell surface of the bacterium, may open up new horizons for the development of immunodiagnostic preparations or vaccines against *Map*.

For this reason, the goal of this study was to test Sardinian type I and type II diabetic sera compared to healthy controls in ELISA (Enzyme-Linked-Immunosorbent-Assay) experiments against two new specific recombinant proteins of *Map* [MAP3733c (MptD) and MAP3738c], respectively an expected membrane protein and a factor involved in the biosynthesis of cell surface components, in order to thoroughly investigate the linkage between *Map* and the autoimmune disease T1DM.

2. Materials and Methods

2.1. Diabetic patients' sera

Sera from 43 Sardinian T1DM patients, 56 T2DM subjects, and 48 healthy controls were collected to perform ELISA tests as already described [9]. All patient donors gave specific informed consent for the study.

2.2. Bacterial Cultures and Growth Media

Mycobacterium avium subsp. *paratuberculosis* strain 1515 (MAP ATCC43015) was grown in 7 H9 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% ADC enrichment (Sigma), 0.05% Tween80 (Sigma-Aldrich) and Mycobactin J (2 μg/ml) (Allied Monitors, Fayette, MO, USA) at 37 C.

E. coli strains *M15* (*pREP4*) (Qiagen, Hilden, Germany) and *BL21(DE3) codon plus* (Stratagene, La Jolla, CA, USA), were grown at 37 C in LB broth (Microbiol, Cagliari, Italy) or LB agar (Agar 1.5% (Invitrogen, Carlsbad, CA, USA) added to LB) with supplementation of desired antibiotics as kanamycin (Sigma) 30 μg/ml or ampicillin (Sigma) 100 μg/ml.

2.3. MAP3733c (MptD) and MAP3738c Cloning and Protein Purification

Nucleotide sequences were originally retrieved from the GenBank *MAP3733c* sequence (GeneID: 2717486) and the *MAP3738c* sequence (GeneID: 2721098) of *Map* strain K-10 whole genome (NC_002944) GenBank: AE016958 [10]. Genes were amplified using the genomic DNA of *Map* as template purified by phenol-chloroform extraction method [6]. Forward primer BamHI-*MAP3733c*-Fw (5′-GCGCGGATCCATGACGGC CACTAGCTCGACGACCCAGTCCAGTCGCCGC-3′), containing the BamHI restriction site (underlined sequence), and reverse primer PstI-*MAP3733c*-Rv (5′-GCCGCTGCAGTTATCAAGC TAGGCCGGCCCTCTG-3′), containing the PstI restriction site (underlined sequence), were used to amplify the complete *MAP3733c Map* gene using the proofreading Phusion™ High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) with the following PCR mix: 5x Phusion HF Buffer (10 μl), dNTPs (200 μM), Primer Forward *MAP3733c*-BamHI (0.4 μM), Primer Reverse *MAP3733c*-PstI (0.4 μM), Phusion DNA Polymerase (2U), genomic *Map* DNA (150 ng), H_2O until a volume of 50 μl; thermal cycling conditions were as follows: an initial denaturation step at 98 C for 30 s followed by 35 cycles consisting in 98 °C for 10 s, 61 °C for 20 s and 72 °C for 30 s. Eventually, a final extension of 72 °C for 5 min has completed the amplification.

Amplification product was cloned into the expression vector pQE-30 (Qiagen) after double digestion with BamHI and PstI (New England Biolabs) restriction enzymes. Ligation was performed with T4 DNA ligase (Invitrogen) for 2 h at 37 °C and construct was electroporated into *E. coli M15 (pREP4)*. Kanamycin/ampicillin resistant clones were confirmed by colony PCR and plasmid restriction analysis to assess *MAP3733c* fragment presence. Briefly, a PCR mix solution (10x Taq buffer (3 μl), $MgCl_2$ (1.5 mM), dNTPs (200 μM), forward primer BamHI-*MAP3733c*-Fw (0.3 μM), reverse primer PstI-*MAP3733c*-Rv (0.3 μM), DNAmize Taq Enzyme (1U) (Finnzymes, Woburn, MA, USA) in a final volume of 30 μl) was used to amplify the fragment with the following thermocycler parameters: initial denaturation at 95 °C for 3 min and 35 cycles formed by 95 °C for 40 s, 65 °C for 40 s and 72 °C for 1 min, ending with a 72 °C final extension for 10 min.

Selected clones were grown until an exponential phase of OD_{600} = 0.4 and 100 ml of culture were induced for expression at 27 °C for 8 h by the addition of 0.5 mM isopropyl-α-D-thiogalactopyranoside (IPTG) (Sigma). Cells were harvested by centrifugation at 4000×*g* for 20 min at 4 °C and pellet was resuspended in 16 ml of denaturing Ni-NTA lysis buffer (Invitrogen) (6 M Guanidine Hydrochloride, 20 mM NaH_2PO_4, pH 7.8, 500 mM NaCl) supplemented with Triton X-100 (0.25%). Sample was homogenized by sonication (Bandelin, UW2070) and lysate was then clarified at 4.000×*g* for 30 min at 4 °C. Protein purification was performed by immobilized metal affinity chromatography (IMAC) following the protocol of Ni-NTA resin purification system (Invitrogen) under hybrid conditions with some modifications. Briefly, the lysate was

incubated in a column with the resin for 16 h at 4 °C with gentle agitation. The next day, the supernatant was removed and the resin was washed 2 times with 6 volumes of Denaturing Binding Buffer (8 M urea, 20 mM NaH_2PO_4 pH 7.8, 500 mM NaCl), 2 times with 5 volumes of Denaturing Wash Buffer (8 m Urea, 20 mM NaH_2PO_4, pH 6.0, 500 mM NaCl) and 5 times with 7 volumes of Native wash buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 20 mM imidazole). Finally, the recombinant protein was eluted with 10 ml of Native Elution Buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 250 mM imidazole) splitting the eluate in fractions of 1 ml each. Elution fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to assess protein yield, purity and size by BSA standard (Invitrogen) comparison on gel. Fraction containg purified recombinant protein was concentrated and dialyzed against PBS supplemented with Phenylmethanesulfonyl fluoride (PMSF) 1 mM (Fluka, Biochemika) in Amicon centrifugal filter devices (Millipore, Billerica, MA, USA).

Forward primer NdeI-*MAP3738c*-Fw (5′-GCGCCATATGAGCGT GACACCTGGCGCCGATC-3′), containing the NdeI restriction site (underlined sequence), and reverse primer XhoI-*MAP3738c*-Rv (5′-GCGCCTCGAGCCGTGGGTGCAGCACACCGATGA-3′), containing the XhoI restriction site (underlined sequence), were used to amplify *MAP3738c* gene as mentioned above for *MAP3733c* gene. The *MAP3738c* PCR product was cloned into the expression vector pET-28a+(Novagen) and ligated construct was then electroporated into *E. coli* BL21(DE3)cp cells as host for expression. Positive clones were selected by kanamycin resistance and confirmed by colony-PCR and restriction analysis.

Protein expression was induced at OD_{600} = 0.5 for 3 h at 37 °C by the addition of 1 mM IPTG to 400 ml of cell culture. Cells were harvested by centrifugation, resuspended in 30 ml of Profinia® native lysis buffer (Biorad, Hercules, CA, USA) (300 mM KCl, 50 mM KH_2PO_4, 5 mM imidazole, pH 8.0) and lysozime (1 mg/ml) to be subsequently sonicated as above. Lysate was then clarified and MAP3738c fusion protein was purified by Profinia® protein purification system (Biorad) according to the manufacturer's protocol for native conditions using solutions provided with the commercial kit. Gel electrophoresis, concentration and dialyzation to assess yield and size were performed as above.

2.4. ELISA, Bioinformatics and Statistical Analysis

ELISA experiments to evaluate the humoral responses in T1DM and T2DM sera against the two recombinant antigens MptD and MAP3738c were performed as already described [9].

"*In silico*" protein analysis was performed with Sequence Analysis software (Informagen, Inc.) to assess proteins' sizes and physical-chemical properties. ELISA's statistical analysis was carried out with a two-tailed Student's *t*-test using www.graphpad.com online tool to evaluate the difference between the OD_{405} average value of diabetic patients and healthy controls. A level of *P*-value <0.05 has been considered statistically significant among averages. All data were also compared using a Chi-square with Yate's correction assuming a cut-off value derived from the average of healthy control plus two standard deviations (SD) as well as with a Receiver Operating Characteristic (ROC) analysis curve to convalidate the experimental accuracy.

3. Results

3.1. Cloning and Expression of MAP3733c (MptD) and MAP3738c Proteins

A gene encoding a putative bacterial integral membrane protein belonging to a *Map* ABC transporter was cloned. A 650 bp PCR product containing the *MAP3733c* ORF flanked by BamHI and PstI restriction sites was amplified and inserted into the pQE-30 expression vector, producing a construct that carried the *MAP3733c* ORF with upstream codons for a 6xHis tag which size was predicted in 24 kDa. MptD recombinant protein was purified by Ni-NTA affinity chromatography under modified hybrid conditions because native purification with automated chromatography systems such as Profinia® failed. On PAGE analysis the 6xHis tagged MptD fusion protein migrated at the expected molecular mass of 24 kDa (Fig. 1). Due to the high content of hydrophobic amino acids (about 51%), the expression yield of this protein was low, nevertheless eluted fraction was found to be very high in purity without any non-specific band. Concentration and dialyzation gave an approximate yield of 300 μg/L of culture.

A second gene (*MAP3738c*) encoding a hypothetical protein was expressed. "*In silico*" analysis of MAP3738c has shown its involvement in mycolic acid biosynthesis as cyclopropanation enzyme or methyltransferase on methoxy-mycolic acids.

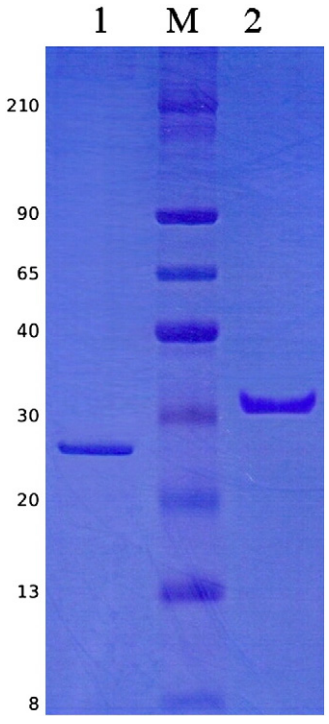

Figure 1 (SDS-PAGE) Purified recombinant proteins MAP3733c (MptD) and MAP3738c. Lane 1: purified protein MptD from induced *E.coli*: pQE30-*MAP3733c* at 24 kDa. Lane M: ColorBurst (Sigma) molecular weight marker. Lane 2: purified protein MAP3738c from induced *E.coli* BL21(DE3): pET28a-MAP3738c at 31 kDa.

MAP3738c was efficiently cloned into the pET-28a+vector. The cloned ORF was preceded at N-terminal by a sequence encoding for MGSSHHHHHHSSGLVPRGSH with a histidine tag and followed by a second 6xHis tag at the C-terminal. The expression of the 272 aa resulting protein was efficiently performed with high purity and optimal yield in native conditions. After concentration and dialyzation a yield of 600 μg/L culture was roughly assessed. SDS-PAGE analysis confirmed purity and an expected size of 31 kDa (Fig. 1).

3.2. ELISA: Immunoreactivity of T1DM and T2DM Patients' Sera Against MAP3733c (MptD) and MAP3738c Recombinant Fusion Proteins

Due to the lack of immunogenicity by the 6xHis tag, as these residues are uncharged at pH 7.2–7.4 and result in a poorly immunogenic action towards the majority of species [11], both fusion proteins were tested in ELISA for evaluation of immune response in diabetic sera against MptD and MAP3738c proteins without removing tags as well. Moreover, T1DM and control sera were analyzed with a specific recombinant protein belonging to *Helicobacter pylori* (HP0986) tagged with 6xHis tag, which gave a negative result for the presence of a significant immune response against it (data not shown).

We performed ELISA tests with 43 T1DM sera, 56 T2DM sera and 48 healthy control sera against MAP3733c (MptD) and MAP3738c recombinant proteins to investigate the presence of humoral response against these antigens. ELISA results showed an increased humoral response in T1 diabetic sera against both MAP3733c and MAP3738c proteins compared to healthy sera (Fig. 2) [MAP3733c (MptD); *t* student = 7.70; FD = 89; p = 0.000000000017 (P < 0.0001); 95%]. [MAP3738c; *t* student = 6.49; FD = 89; p = 0.0000000045; (P < 0.0001); 95%]. Area Under ROC Curve (AUC) (Fig. 2) confirmed the accuracy of the difference between averages for both proteins' experiments [MAP3733c (MptD); AUC = 0,8576]. (MAP 3738c; AUC = 0,8278).

Assuming a cut-off value of 0.4 absorbance units at OD_{405} (average of healthy controls + 2SD) (Table 1), much greater than the ROC analysis curve's cut-off, set to approximately 0.2 absorbance units, MptD protein's ELISA results shown as a statistical analysis by the Chi-square with Yate's correction, generated χ equal to 21.657 with 1 degree of freedom and a two-tailed P value of 0.0001 showing a statistically significant difference between T1 diabetic patients and healthy controls. In

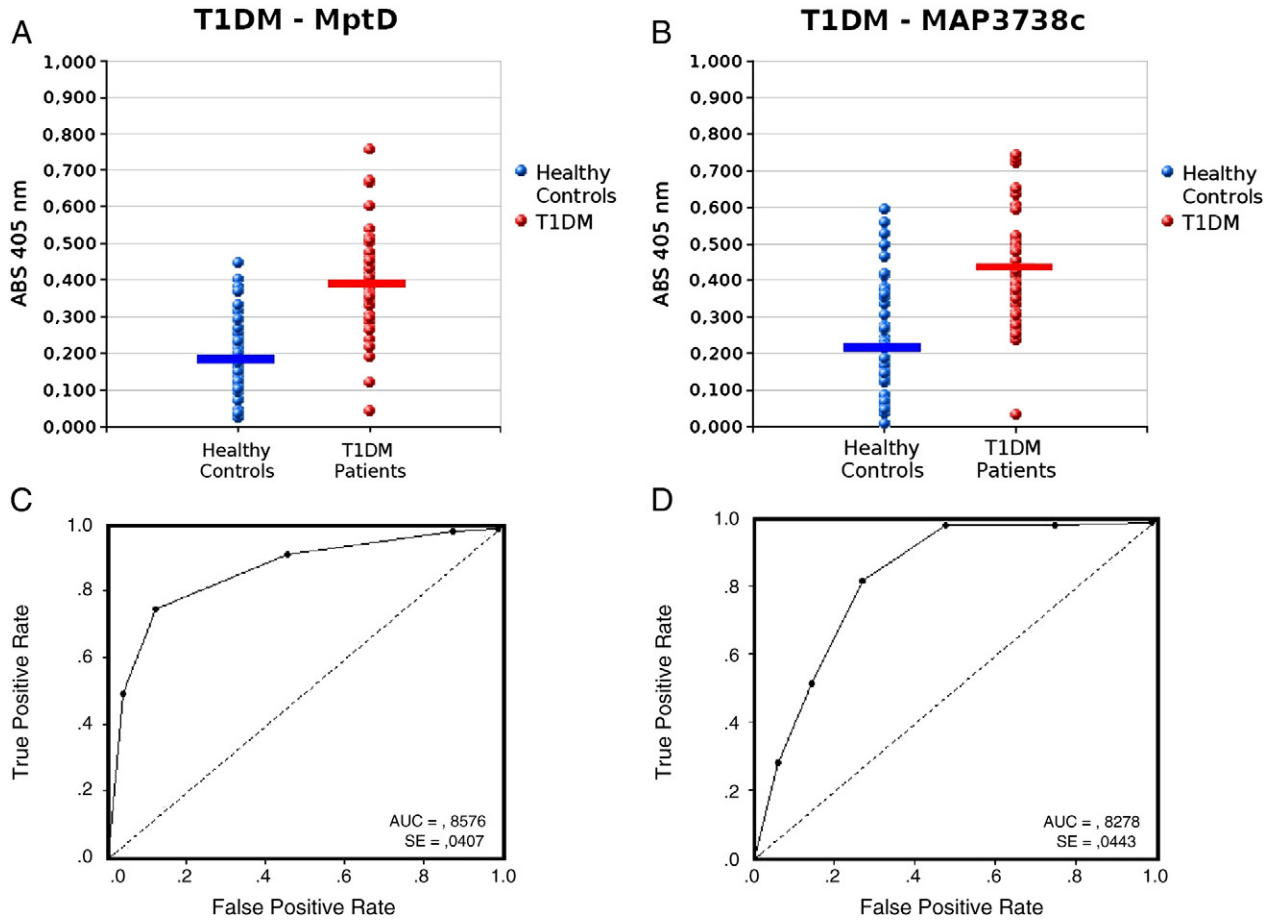

Figure 2 Evaluation of immunoreactivity against MAP3733c (MptD) recombinant protein (A), and MAP3738c recombinant protein (B) in sera from 43 patients with type I diabetes mellitus and 48 healthy controls. Data are shown as values of OD405 nm for each serum in performed ELISA. The average value for each population is indicated by the bolded horizontal line. ROC curve statistical analysis indicates the AUC value for experiments with MptD (C) and MAP3738c (D).

the same way, data for MAP3738c recombinant protein with a 0.4 cut-off gave a Chi-square with Yate's correction equal to 12.344 with 1 degree of freedom and a two-tailed P value equals to 0.0004.

Data from T2 diabetic sera pool (Fig. 3) (Table 1) showed no difference between T2 sera and controls for both proteins: [(MAP3733c (MptD); *t* student = 1,89; FD = 102; p = 0.0612; 95%; AUC = 0,5822] and (MAP3738c; *t* student = 0,11; FD = 102; p = 0.9108; 95%; AUC = 0,5456). Similarly, Chi-square analysis of T2DM ELISA data gave a χ value of 0.329 (MptD) and 1.581 (MAP3738c) with a P value of 0.5662 and 0.2086 respectively, showing no correlation between humoral immune response against *Map* and T2DM.

In conclusion, both ELISA data showed an increased antibody presence against the two *Map* specific antigens in T1DM sera, unlike sera of healthy controls and T2DM patients.

4. Discussion

Iron uptake is essential for microbial growth because the acquisition of this metal is an important feature for the process of bacterial pathogenesis [12]. Metal absorption is carried out by uptake factors such as the ABC transporters among which is numbered a specific operon denominated *Mpt*.

The *Mpt* operon belongs to a specific 38 kb pathogenicity island of *Map* and a constitutive element of its sequence, the *MAP3733c* gene, encodes for the protein MptD that was firstly described as a membrane protein expressed during infection stages [13] and further identified as a virulent factor as well [14].

Because the process of iron acquisition might be a possible target for chemotherapeutic agents [12] or immunoprophylactic tools, Heinzmann and collegues cloned mpt operon's fragments into the integrative pMV306 expression plasmid. Among these fragments also mptD ORF was present and has been expressed in *BCG* host with the aim of creating a vaccine candidate against *Map* [14]. Despite the authors were successfull to demonstrate a surface exposure, MptD protein was not purified.

We had tried to clone the single MptD ORF in a pMAL-series plasmid or in pMV261 vector with *M. smegmatis* as host; unfortunately, purification of expressed protein failed (data not shown). Subsequently, we cloned it into pQE-30 expression vector, with a 27 °C expression temperature and a lesser IPTG concentration during induction to avoid the formation of inclusion bodies, making it easy to perform the 6xHis tag-mediated purification although under modified hybrid conditions with Triton X-100 detergent which led to a low purification yield.

The second cloned protein, MAP3738c, seems to be the smallest among the hypothetical proteins of the specific 38 kb pathogenical island of *Map* [13]. The protein contains a putative cyclopropane mycolic acid synthase motif (CMAS) involved in biosynthesis of methoxy and cyclopropyl mycolic acids as for a similar domain found in *Mycobacterium tuberculosis* [15].

The analysis of the primary structure of MAP3738c showed a less hydrophobic character compared to the MptD protein, a detail that might explain the ease of its expression and purification. Enzymes involved in addition or degradation of mycolic acid components alter the lipidic composition and the hydrophobic balance of cell walls leading to a change in the ability to adhere to host cell surfaces [16], furthermore cyclopropanated mycolic acid is involved in host cell entry.

Miltner and collegues cloned an homologue of *Rv3720*, a cyclopropane fatty acyl-phospholipid synthase gene, assessing that its constitutive expression in *M. avium* host was fundamental for the invasion of epithelial cells [16] while in a similar study the inactivation of *pcaA*, another cyclopropane synthase, dramatically decreased the virulence of *M. tuberculosis* [17].

With the emergence of reports about the zoonotic danger of *Map*, many investigations had focused on the presence of humoral response against different *Map* antigens in patients afflicted by autoimmune diseases [1,8,18]. In this study, a strong humoral immune response in T1DM sera against MAP3733c (MptD) and MAP3738c proteins was detected. An earlier study following a similar approach found this evidence using different specific antigens involved in bacteria survival and virulence [9]. The proteins of this study were not recognized by sera from patients with T2DM and controls. These results support the hypothesis proposed where *Map* is indicated as a potential trigger of the autoimmunity that characterize T1DM rather than to a non-autoimmune disease as T2DM.

Immunological assays on autoimmune diseases have already focused on MptD protein using a small peptide called aMptD derived from a phage display study [13]. This peptide mimed an ideotype sequence that recognized a small part of the MptD protein, and its use in serological assays for autoimmune diseases such as T1DM [19] showed results similar to the present results.

The direct identification of MptD protein has demonstrated the presence of intact *Map* cells in infected bulk milk [13], in addition electron microscopy experiments [20] revealed that in human host *Map* is characterized as having a spheroplastic form with a partial degradation of cell wall, which is also corroborated by the loss of the Ziehl-Neelsen staining positiveness. This does not exclude the presence of the MptD antigen in *Map* infecting humans; therefore MptD remains a potential surface marker in the immunodiagnostic of *Map*.

Concerning immune response of T1DM patients against MAP3738c, proteins previously screened that are similar in function or cellular localization would make easier the understanding of our results. Wu and colleagues showed the importance of pstA protein in the addition of amino acids to the lipopeptidic core of *Map* demonstrating a significant role for pstA in the development of an immune response in infected cattle [21]. Differently, Bannantine and Stabel had cloned the HspX protein of *Map* and revealed its immunogenicity in infected cows, although it was not a secreted antigen but rather a protein belonging to the soluble fraction [22].

As for HspX antigen, MAP3738c protein may not be exposed, it is in any case involved in the biosynthesis of cell surface components as the pstA antigen. This line of evidence suggests that even if MAP3738c is not exposed on the surface of *Map*, it could arouse a humoral response in T1DM patients as MptD, suggesting that the presence of the antigen on the outer surface of a cell is not an absolute prerequisite for the induction of an immune response by the host.

Our data showed a strong humoral response against two specific *Map* antigens strengthening the hypothesis about a correlation between T1DM and *Map*, as previously reported

Table 1 Characteristics and ELISA results of healthy controls' sera (left), type I diabetes patients' sera (center), and type II diabetes patients' sera (right) against recombinant antigens MAP3733c (MptD) and MAP3738c. M, male; F, female; ND, not determined; I, type I diabetes; II, type II diabetes. Arbitrary values were taken depending on the reading values in relation to a cut-off set at OD405 = 0.4 (control average plus 2 SD). Values are the following: − indicates a value less than 0.4, + indicates a value of 0.4–0.5, ++ indicates a value of 0.5–0.6, +++ indicates a value of 0.6–0.7, and ++++ indicates a value of 0.7–0.8.

Healthy control (sample name)	Sex	Age at blood sample (yr)	Family history of diabetes (type)	Seropositivity for:		Diabetic patient T1DM (sample name)	Sex	Age at blood sample (yr)	Family history of diabetes (type)	Seropositivity for:		Diabetic patient T2DM (sample name)	Sex	Age at blood sample (yr)	Family history of diabetes (type)	Seropositivity for:	
				MAP3733c (MptD)	MAP3738c					MAP3733c (MptD)	MAP3738c					MAP3733c (MptD)	MAP3738c
1c	F	33	ND	−	−	1d1	M	21		−	−	1d2	M	62		−	−
2c	M	25	ND	−	++	2d1	F	31		++	++	2d2	F	66		−	−
3c	F	50	ND	−	−	3d1	M	36	I	+++	+++	3d2	M	66	II	−	−
4c	F	36	ND	−	−	4d1	F	36		−	−	4d2	M	81		−	−
5c	F	67	ND	−	−	5d1	M	37		−	−	5d2	M	74	ND	−	−
6c	M	45	ND	−	−	6d1	M	26	II	−	−	6d2	M	62	II	−	−
7c	M	45	ND	−	−	7d1	M	30	I/II	+	+	7d2	F	50	II	−	−
8c	M	53	ND	−	−	8d1	F	37	I	−	−	8d2	M	53	II	−	−
9c	M	37	ND	−	−	9d1	F	37	I	+	++	9d2	M	75		−	−
10c	M	63	ND	−	−	10d1	F	27	I	+	+	10d2	F	72	ND	−	+
11c	M	63	ND	−	−	11d1	M	31	I	−	−	11d2	F	40	ND	−	−
12c	M	45	ND	−	−	12d1	M	40	I	−	+	12d2	F	76	ND	−	−
13c	F	60	ND	−	−	13d1	M	38		++	−	13d2	M	57	ND	−	−
14c	F	43	ND	−	−	14d1	F	37	I	+	++	14d2	M	62	II	−	−
15c	F	34	ND	−	−	15d1	F	35		+	+	15d2	F	77		−	−
16c	F	25	ND	−	−	16d1	F	40	II	−	−	16d2	M	56		−	−
17c	M	57	ND	−	−	17d1	F	34		−	+	17d2	M	71	I	−	−
18c	F	26	ND	−	−	18d1	M	41	I	−	−	18d2	M	65		−	−
19c	F	41	ND	−	+	19d1	F	36	I	−	−	19d2	F	66		−	−
20c	M	37	ND	−	−	20d1	F	37		+	−	20d2	M	69		−	−
21c	M	48	ND	−	−	21d1	F	32		+	+	21d2	M	75		−	−
22c	F	57	ND	−	−	22d1	M	43		−	−	22d2	M	63	ND	−	−
23c	M	31	ND	−	++	23d1	F	33	I	++	++	23d2	F	60		−	−

24c	M	37	ND	–	–	24d1	F	33		+++	+++	24d2	M	68	I	–	–
25c	M	39	ND	–	–	25d1	M	38		+	+	25d2	M	57	II	–	–
26c	M	28	ND	–	–	26d1	M	33		++	++++	26d2	M	66	ND	–	++
27c	F	35	ND	–	+	27d1	M	32		–	–	27d2	M	76	II	–	–
28c	F	21	ND	–	–	28d1	M	26	II	+	+++	28d2	F	74	ND	–	–
29c	M	45	ND	–	–	29d1	F	32	I	++	+++	29d2	F	76	ND	–	–
30c	M	39	ND	–	–	30d1	F	38		+	–	30d2	F	64	ND	–	–
31c	M	46	ND	–	+	31d1	M	34		–	+	31d2	M	69		–	–
32c	F	19	ND	–	–	32d1	M	94		+++	++++	32d2	M	66	ND	–	–
33c	M	35	ND	–	–	33d1	M	36		–	–	33d2	F	65	ND	+	–
34c	M	49	ND	–	–	34d1	M	27	I	+++	++++	34d2	M	65	ND	–	–
35c	F	25	ND	–	–	35d1	M	33	II	+	++	35d2	F	73	II	–	–
36c	M	42	ND	–	–	36d1	F	33		–	–	36d2	M	80	ND	–	–
37c	F	61	ND	–	++	37d1	F	23	I	–	+	37d2	M	62	II	–	–
38c	M	31	ND	–	–	38d1	M	43	II	–	+	38d2	M	70	ND	–	–
39c	F	29	ND	–	–	39d1	M	34		+++	–	39d2	M	74	ND	–	–
40c	M	53	ND	–	–	40d1	F	59	I	–	–	40d2	M	78		+	–
41c	M	25	ND	+	+	41d1	F	42	I	–	–	41d2	M	78	ND	–	–
42c	M	23	ND	–	–	42d1	F	47	II	–	–	42d2	F	53		–	–
43c	M	28	ND	–	–	43d1	ND	ND	ND	–	–	43d2	F	49		–	–
44c	F	35	ND	–	–							44d2	M	69		–	–
45c	M	21	ND	–	–							45d2	M	70		–	–
46c	M	28	ND	+	–							46d2	M	72		++	–
47c	M	29	ND	–	–							47d2	M	60	II	–	–
48c	M	23	ND	–	–							48d2	F	67	ND	–	–
												49d2	M	53	II	–	–
												50d2	M	56		–	–
												51d2	M	62	ND	+	+
												52d2	F	73	ND	–	–
												53d2	M	66		–	–
												54d2	F	60		–	–
												55d2	F	62	ND	+	–
												56d2	F	55	ND	–	–

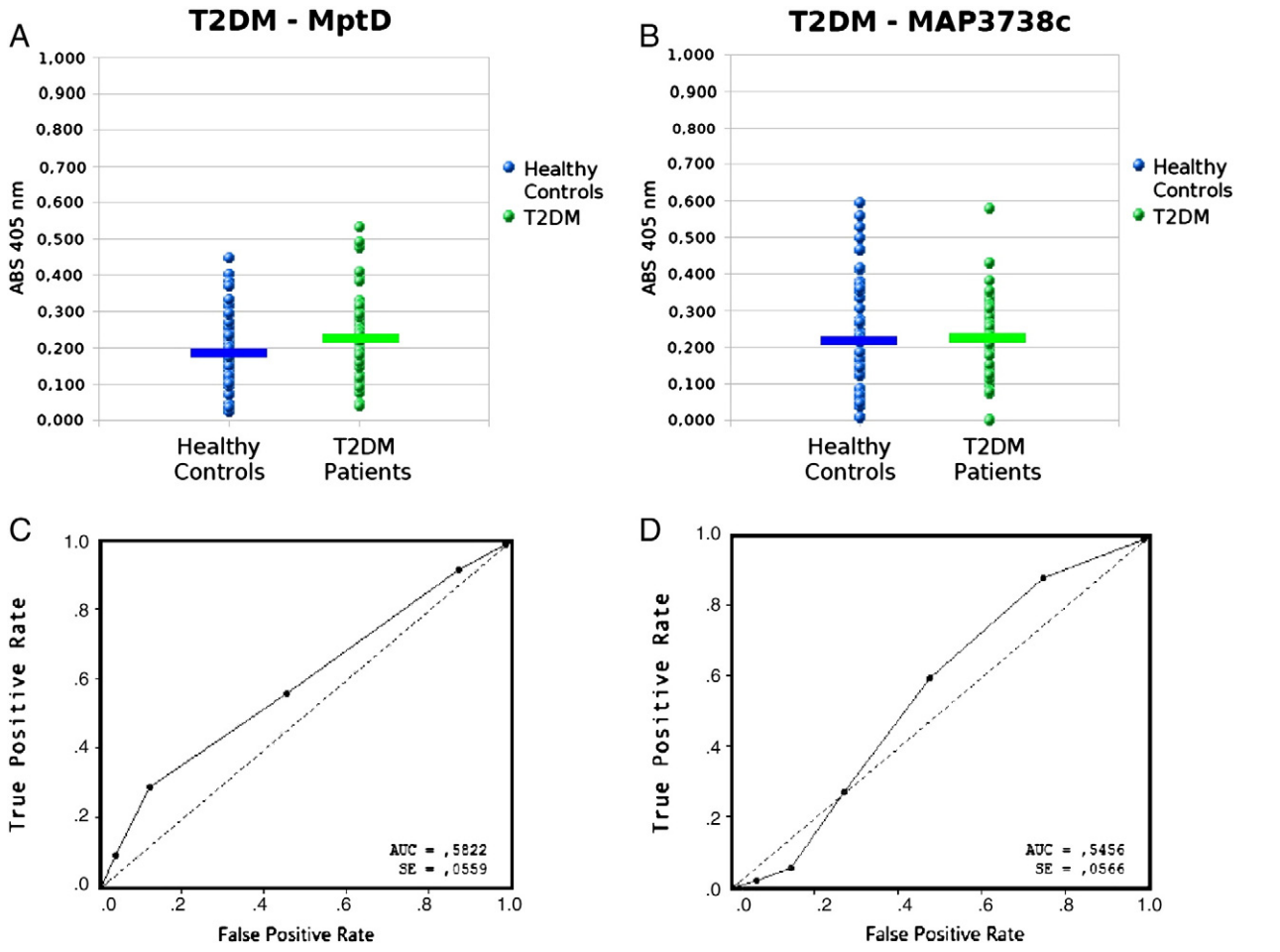

Figure 3 Evaluation of immunoreactivity against MAP3733c (MptD) recombinant protein (A), and MAP3738c recombinant protein (B) in sera from 56 patients with type II diabetes mellitus and 48 healthy controls. Data are shown as values of OD405 nm for each serum in performed ELISA. The average value for each population is indicated by the bolded horizontal line. ROC curve statistical analysis indicates the AUC value for experiments with MptD (C) and MAP3738c (D).

by other studies [6,9,19], confirmed by *IS900* PCR results for the presence of *Map* DNA in the blood of T1DM patients. Furthermore, a similar study with T2DM patients did not show any significant link between T2DM and *Map* [23] as corroborated by this study.

In conclusion, we presented data suggesting that two specific Map proteins, (MAP3733c) MptD and MAP3738c, may be involved in triggering an humoral immune response only in T1DM patients and not in T2DM subjects. These results reinforce the hypothesis that the bacterium has a role, as an environmental factor in triggering T1DM and other autoimmune diseases [24], however, further studies should evaluate if infection is an etiological factor for the onset of the autoimmune disease or is merely a consequence of the pathology.

5. Transparency Declaration

All authors have read and agreed to this version of the manuscript. The study does not present any conflict of interest for the authors.

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