Microbial Pathogenesis

High prevalence of Merkel cell polyomavirus is associated with dysregulation in transcript levels of TLR9 and type I IFNs in a large cohort of CF patients from the Italian (Lazio) Reference Center for Cystic Fibrosis

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Abstract:	Merkel cell polyomavirus (MCPyV) has been detected in respiratory specimens including those from Cystic Fibrosis (CF) patients, raising questions about its immunological and clinical relevance in the respiratory tract. MCPyV might promote an inappropriate antiviral response contributing to a chronic inflammatory response and resulting in detrimental effects in CF. Respiratory samples (n=1138) were randomly collected from respiratory tract of CF patients (n=539) during July 2018 - October 2019. MCPyV-DNA detection was performed by Real Time-PCR and positive samples were characterized by sequencing of the NCCR genomic region. The transcript levels of Toll-like receptor 9 (TLR9) and type I interferon (IFN-I) genes (IFNα, IFNβ and IFNε) were examined by RT/Real Time-PCR assays. MCPyV-DNA was detected in 268 out of 1138 respiratory specimens (23.5%) without any difference in the prevalence of

	MCPyV-DNA according to age, gender or bacteriological status of CF individuals. Thirteen out of 137 CF patients remained positive for MCPyV-DNA over the time (a median follow-up period of 8.8 months). Detection of MCPyV-DNA in respiratory specimens was not associated with the occurrence of exacerbation events. Both MCPyV positive adolescents (11-24 years) and adults (>25 years) had lower mRNA levels of TLR9, IFNβ, IFNε and IFNα than the negative patients of the same age group, while MCPyV positive children produced increased levels of TLR9 and IFN-l genes ($p<0.05$ for TLR9, IFNβ, IFNε) with respect to the negative ones. There were significant differences in TLR9 levels ($p<0.01$), but not in those of IFNs, between MCPyV-DNA positive and negative patients with S. aureus , P. aeruginosa or both. Overall, these results indicate that MCPyV-DNA is frequently detected in the respiratory samples of CF patients and might influence the expression levels of IFN-related genes in an age dependent manner. The concomitant detection of MCPyV together with S. aureus and/or P. aeruginosa correlated with alterations in TLR9 levels suggesting that virus-bacteria coinfections might contribute to affect antiviral immunity in CF patients.

Highlights

- MCPyV is frequently detected in the respiratory samples of CF patients
- MCPyV prevalence does not vary according to age or gender or microbiology status
- MCPyV reduces TLR9 and IFN-I expression levels in CF adolescents and adults

High prevalence of Merkel cell polyomavirus is associated with dysregulation in transcript levels of TLR9 and type I IFNs in a large cohort of CF patients from the Italian (Lazio) Reference Center for Cystic Fibrosis

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Summary

Merkel cell polyomavirus (MCPyV) has been detected in respiratory specimens including those from Cystic Fibrosis (CF) patients, raising questions about its immunological and clinical relevance in the respiratory tract. MCPyV might promote an inappropriate antiviral response contributing to a chronic inflammatory response and resulting in detrimental effects in CF. Respiratory samples (n=1138) were randomly collected from respiratory tract of CF patients (n=539) during July 2018 - October 2019. MCPyV-DNA detection was performed by Real Time-PCR and positive samples were characterized by sequencing of the NCCR genomic region. The transcript levels of Toll-like receptor 9 (TLR9) and type I interferon (IFN-I) genes (IFNα, IFNβ and IFNε) were examined by RT/Real Time-PCR assays. MCPyV-DNA was detected in 268 out of 1138 respiratory specimens (23.5%) without any difference in the prevalence of MCPyV-DNA according to age, gender or bacteriological status of CF individuals. Thirteen out of 137 CF patients remained positive for MCPyV-DNA over the time (a median follow-up period of 8.8 months). Detection of MCPyV-DNA in respiratory specimens was not associated with the occurrence of exacerbation events. Both MCPyV positive adolescents (11-24 years) and adults (>25 years) had lower mRNA levels of TLR9, IFNβ, IFNε and IFNα than the negative patients of the same age group, while MCPyV positive children produced increased levels of TLR9 and IFN-I genes ($p<0.05$ for TLR9, IFNβ, IFNε) with respect to the negative ones. There were significant differences in TLR9 levels ($p<0.01$), but not in those of IFNs, between MCPyV-DNA positive and negative patients with *S. aureus*, *P. aeruginosa* or both. Overall, these results indicate that MCPyV-DNA is frequently detected in the respiratory samples of CF patients and might influence the expression levels of IFN-related genes in an age dependent manner. The concomitant detection of MCPyV together with *S. aureus* and/or *P. aeruginosa* correlated with alterations in TLR9 levels suggesting that virus-bacteria coinfections might contribute to affect antiviral immunity in CF patients.

1. Introduction

Progressive pulmonary disease is the primary cause of morbidity and mortality in cystic fibrosis (CF) patients. In recurrent pulmonary exacerbation, the role of chronic bacterial infections is well-known, whereas the role of viral infections is still debated [1,2]. Common viral infectious agents in CF individuals include Rhinovirus (HRV) [3, 4], Respiratory syncytial virus (RSV), and Influenza A and B viruses [5,6]. Despite being most studied in association with Merkel cell carcinoma (MCC) [7,8], Merkel cell polyomavirus (MCPyV) has also been detected in respiratory specimens, raising questions about its clinical relevance in the respiratory tract [9-13]. Several studies have reported different prevalence of MCPyV-DNA in the upper and lower respiratory tract samples of immunocompetent and immunosuppressed individuals with variable respiratory symptoms, respectively: 0.8%-0.6% (Australia, 2009) [9]; 3.5%-0.8% (Sweden, 2009) [10]; 2.1%-1.9% (Finland, 2009) [11]; 2.0%-1.3% (UK, 2011) [12]; 6.0%-NA (Bulgaria) [13].

To the best of our knowledge, only two studies examined the prevalence of MCPyV infection in CF patients reporting different frequency values (6.8% [14] and 26% [15]). These results consolidate the hypothesis that the respiratory tract of CF patients might display a greater susceptibility to human polyomaviruses (HPyVs) infections.

Upon infection, HPyVs dsDNA can be sensed by Toll-like receptor 9 (TLR9), a key receptor in the host innate immune response that recognizes viral or bacterial dsDNA in the form of nonmethylated CpG motifs [16]. Upon ligand binding, TLR9 induces the transcription nuclear factor NF-kappaB (NF-kB), leading to increased production of inflammatory mediators and interferon (IFN) [17]. In order to escape from immune recognition responses, MCPyV, and other dsDNA oncogenic viruses such as Epstein-Barr virus, Hepatitis B virus, and the high-risk Human Papillomavirus 16 (HPV16), have developed evasion strategies to alter the production of TLR9 [18-21].

MCPyV possesses a non-enveloped icosahedral capsid that contains a circular double stranded DNA genome divided into the early and the late region [22]. The early and late regions are separated by the non-coding control region (NCCR) that contains the origin of replication (ORI) and bidirectional transcriptional elements that govern early and late viral gene expression [23]. Alternative splicing and alternative use of open reading frames (ORF) generate the proteins large tumor (LT), small T (sT), 57 kT antigens and LT open reading frame [22].

The expression of the MCPyV LT can downregulate TLR9 expression in epithelial and MCC-derived cells [21]. Moreover, it has been found that the antiviral and antitumor actions of IFN-β can be suppressed in cells transformed by wild-type MCPyV through a JAK1-MCPyV LT antigen interaction [24]. The antagonistic relationship between HPyVs and type I IFN (IFN-I) is also confirmed by studies evaluating HPyVs sensitivity to IFN-I action [25]. In particular, *in vitro* and *in vivo* experiments carried out in MCPyV infected MCC cell lines and mice respectively, showed that IFN-I modulated LT activity promoting the expression of promyelocytic leukemia protein, which interferes with the function of the LT [26].

In this study, we estimated the prevalence of MCPyV-DNA in respiratory samples of a large cohort of CF patients (n=539) from a single hospital in Rome, one of the Italian Reference Center for CF, analyzing viral load, and sequencing the viral region NCCR from all MCPyV-DNA positive respiratory samples. In addition, in order to shed light on the potential pathogenic role of MCPyV in CF, demographic, microbiological and clinical data collected from MCPyV-DNA positive and negative patients were compared.

Given that TLR9, through the binding of intracellular viral DNA, plays a major role in promoting the activation of antiviral pathways associated to IFN-I response [17,27], we hypothesized that alterations in the expression of TLR9 in the respiratory tract of MCPyV positive CF patients might lead to attenuated antiviral innate immune responses. Thus, the transcript expression of TLR9 and distinct

IFN-I genes (IFNα, IFNβ and IFNε) were examined in respiratory samples of MCPyV positive CF patients according to their bacteriological and clinical status.

2. Materials and methods

2.1 Study population

Respiratory samples [n = 1138 (oropharyngeal aspirate n=619 and sputum samples n=519)] were randomly collected from CF patients (n = 539) attending the Lazio Regional Reference Center for CF, Policlinico Umberto I Hospital, Sapienza University of Rome, for routine visits during July 2018 - October 2019. The type of respiratory sample, oropharyngeal aspirate or sputum, did not differ with respect to CF patients' age (age of patients with oropharyngeal aspirate samples: mean/standard deviation, 25.4/16.5 years; age of patients with sputum samples: mean/standard deviation, 25.4/16.5 years, $p > 0.05$). Although follow-up schedules can change depending on the health status of CF patients, most individuals were routinely seen in the hospital every 3 months: the median follow-up period was 2.8 months (range: 0.4-15.4 months). Moreover, one respiratory sample was collected from 173 (173/539, 32.1%), two respiratory samples from 197 (197/539, 36.5%), three respiratory samples from 105 (105/539 19.5%) and four respiratory samples from 64 (64/539, 11.9%) out of 539 CF patients, respectively (Table 1).

Demographic and clinical data, such as gender, age, genotype, BMI, pulmonary function, exacerbation events were collected from medical records. Following the usual microbiological investigation, MCPyV-DNA was tested by Real Time PCR in the residual respiratory samples. Then, the gene expression analysis of TLR9 and IFNα, IFNβ, IFNε was carried out in a subgroup of 95 MCPyV-DNA positive samples and 147 negative samples of CF patients for whom the respiratory specimens were enough to allow RT/Real Time PCR assays. The study was approved by the ethics committee of the Policlinico Umberto I Hospital, Sapienza University of Rome, and informed consent was obtained from patients suffering from CF.

2.2 Microbiological investigations

Respiratory tract samples were analyzed for common microorganisms by standard methods and procedures for the traditional microbiological investigation (BD *BBLTM* Stacker Plates, Heidelberg, Germany). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Inc., Billerica, MA, USA) was used for bacterial identification where necessary. Since *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most prevalent bacterial species involved in respiratory tract infection in CF, patients were stratified according with the presence of these bacteria for the viral and immunological analysis.

2.3 MCPyV DNA Extraction and Quantification by qPCR

Total DNA was extracted from respiratory samples using the DNeasy® Blood & Tissue Kit (QIAGEN, Milan, Italy), according to the manufacturer's instructions. Specific qPCR assays were performed using TaqMan-based qPCR, employing primers and probes targeting MCPyV sT, as previously described [15]. All samples were tested in triplicate, and the number of viral copies was calculated from standard curves constructed using a ten-fold dilution series of plasmid pMCV-R17a containing the entire genome of MCPyV (Addgene, #24729) (dilution range: $10^{\wedge 8}$–10 copies/mL). The lower detection limit of the assay was 10 DNA copies of the target gene per amplification reaction, corresponding to 10 copies per reaction (10 copies/reaction). The amount of cellular DNA was quantified simultaneously using a SYBR Green PCR for the housekeeping β-globin gene and used to normalize the MCPyV DNA.

2.4 Nested PCR for NCCR analysis and Sequencing

MCPyV-positive DNA samples were subjected to nested PCR for the amplification of the NCCR region. Two sets of primers, ORIF1/ORIR1 (nucleotide positions 4832–4853 and nucleotide positions 5334–5314) and ORIF2/ORIR2 (nucleotide positions 5077–5100 and nucleotide positions 5280–5261), were employed to generate an NCCR fragment of 504 and 203 base pair (bp). Numbering of nucleotides was based on the sequence of MCC350, a strain of North American origin (GenBank: EU375803) [28]. In detail, PCR reactions were carried out following a published protocol [29].

PCR products were purified and sequenced in a dedicated facility (Bio-Fab research, Rome, Italy). DNA sequencing was performed with a Sanger protocol (Big Dye Terminator Sequencing, Life Technologies), using an ABI 3730 System (Life Technologies, BioFab research s.r.l., Rome, Italy). The obtained sequences were compared to the reference strain (EU375803) and sequence alignment was performed using ClustalW2 [http://www.ebi.ac.uk/Tools/msa/clustalw2/] available on the EMBL-EBI website using default parameters.

2.5 TaqMan-Based Real-Time RT-PCR Assays for mRNA Expression

The mRNA levels of TLR9 (Hs.PT.58.40576968, Integrated DNA Technologies, IDT, Coralville, IA, USA), IFNα (Hs.PT.58.24294810.g, IDT), IFNβ (Hs.PT.58.39481063.g, IDT), and IFNε (Hs.PT.58.4812867.g, IDT) were measured by quantitative RT-Real time PCR assays carried out with the LightCycler 480 instrument (Roche, Basel, Switzerland) as previously described [30]. Briefly, total RNA was extracted from respiratory samples using the RNeasy Plus Universal Tissue Mini Kit (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), according to the manufacturer's protocol. Primers and probes for each gene were added to the Probes Master Mix (Roche, Basel, Switzerland) at 500 and 250 nM, respectively, in a final volume of 20 μL. The housekeeping gene β-glucuronidase/GUS was used as an internal control. Gene expression values were calculated by the comparative cycle threshold value (Ct) method (2^-ΔCt).

2.6 Statistical methods

Data analysis involved a process of indexing, coding, and data categorizing. All statistical analyses were performed with Statistical Package for Social Science (SPSS) version 25, which is a graphical user interface for MATLAB (ver. 2021). Categorical variables were summarized as proportion, absolute frequency (and/or percentage), and continuous variables were summarized as mean and Standard Deviation (± SD) or median and inter-quartile range (IQR: 25th and 75th percentile). Baseline demographic and outcome variables were compared to the presence/absence of MCPyV-DNA and exacerbation using Chi-square ($\chi 2$). Differences in the levels of TLR9 and IFNs between

MCPyV-DNA positive and negative CF patients were evaluated using the Mann–Whitney test. The same test was used to compare the levels of mRNA of TLR/IFNs between MCPyV-DNA positive and negative CF patients according to the microbiological status. Spearman's rho coefficient (r) was calculated to assess the correlation between TLR9 and IFNs levels, and age. A series of repeated measures analysis of variance (RM-ANOVA) were carried out to assess whether there was a difference in viral load over four assessment time points (baseline, T1, T2 and T3 follow-up period). RM-ANOVA F-scores at p-value less than 0.05 for each subscale were considered significant. Intervention was compared on baseline characteristics as well as baseline measures of the outcomes. Logistic regression analysis was fitted to develop models of risk factors for exacerbation occurrence in MCPyV-DNA positive CF patients.

3. Results

3.1 MCPyV prevalence, viral load and seasonality

One thousand hundred thirty-eight respiratory samples were randomly collected from 539 CF patients and analyzed for MCPyV-DNA. Overall, 137/539 (25.4%) CF patients were positive at least one time (Table 1), for a total of 268/1138 MCPyV-DNA positive respiratory specimens (23.5%) (Table 2). Considering CF individuals with at least one follow-up sample (at least two samples), 82/539 (15.2%) patients were MCPyV-DNA positive at least two times; 36/539 (6.7%) patients were MCPyV-DNA positive at least three times; 13/539 patients (2.4%) had four MCPyV-DNA positive samples (Table 1).

As far as MCPyV-DNA persistence in respiratory samples was concerned, 50 CF patients were positive in two consecutive samples (median follow-up period: 4.67 months, range: 0.7-10.2), 19 CF patients were positive in three consecutive samples (median follow-up period: 6.8 months, range: 2.8-11.4), 13 CF patients were positive in four consecutive respiratory samples (median follow-up period: 8.8 months, range: 2.2-11.8) (Table 1).

Moreover, no statistically significant differences were found in the prevalence of MCPyV infection and in the levels of MCPyV-DNA between females and males and also analyzing CF patients stratified in three age groups (0-10 years, 11-24 years, >25 years) (Table 1).

Analysis by qPCR showed a MCPyV-DNA titer in the respiratory samples ranged from $9*10^1$ to $1.2*10^5$ copies/mL, mean value of $8.2*10^3$ copies/mL (± SD $7.8*10^3$) and median Ct value of 30.2 (IQR: 28.1–32.2). There was no difference in MCPyV-DNA comparing aspirate and sputum samples in CF patients and also between different age groups ($p>0.05$ for all the analysis) (Table 2).

Considering CF patients who remained positive for MCPyV-DNA detection in four consecutive respiratory specimens (n=13, median follow-up period: 8.8 months, range: 2.2-11.8), by carrying out repeated MCPyV-DNA measures at different time intervals (T0-T1: 3.5 months; T1-T2: 2.3 months; T2-T3: 2.4 months), we found that the number MCPyV-DNA copies/mL decreased over the time ($p<0.001$).

Then, we evaluated the trend of monthly positivity rates of MCPyV-DNA in CF patients during the year analyzed (July 2018 - October 2019). MCPyV-DNA was detected every month, with the greatest number of MCPyV positive respiratory samples found in February, October and December. The highest MCPyV prevalence was observed in February (18.2% of the month's samples), suggesting a winter seasonality for this DNA virus (data not shown).

3.2 MCPyV NCCR Analysis

Analysis of MCPyV NCCR regions obtained from the total of the positive MCPyV respiratory samples of CF subjects was carried out. The amplified NCCRs, spanning from nucleotide position 5077 to 5280, were compared with the reference sequence of the prototype North American strain MCC350 (Figure 1). Results showed a NCCR characterized by a high degree of homology with the prototype strain, despite the presence of some deletions, insertions, or mutations. Overall, a deletion of 3 bp (CCC), 3 TTT, 2 bp (CC) and 2 bp (AA), was frequently observed sited in positions 5163–65, 5260–62, 5157–58 and 5178–79, respectively (Figure 1). Interestingly, T to C transitions were recurrently observed in positions 5148, 5162, 5220 and 5280 (Figure 1); instead, A to T transversions

were found in position 5171, 5176, 5218 and 5270. Finally, a GTTGA insertion at positions 5210–5211 was identified. Analysis of putative binding sites was also carried. The NCCR sequence of the prototype MCC350 [28] contains binding sites transcriptional factors such as non-histone protein 1 (NHP-1), forkhead box N2 (FOXN2), basic transcription element binding protein 3 (BTEB3), forkhead box O3a (FOXO3a), nuclear factor of activated T cells 3 (NF-AT3), forkhead box J2 (FOXJ2), GATA-binding factor 1 (GATA1), activator protein 1 (AP1) and paired box gene 2 (PAX2) (Figure 1). Comparison of the cellular transcription binding motifs reported above with all the NCCR sequences recovered from MCPyV-DNA positive samples, evidenced that mutations, deletions, and insertions changed some putative binding sites in v-rel avian reticuloendotheliosis viral oncogene homolog A (RELA), signal transducer and activator of transcription 4 (STAT4), transformation-specific-1 transcription factor (ETS-1), Androgen Receptor (AR), Hepatic leukemia factor (HLF) and specificity protein-3 (Sp3) as reported in Figure 1.

3.3 Microbiology in MCPyV positive and negative CF patients

Within the CF patients' respiratory samples (n=1138), the most common bacteria detected were *S. aureus* (n=397), *P. aeruginosa* (n=268) or both bacteria (n=227) (Table 2). The concomitant detection of MCPyV-DNA with *S. aureus* or *P. aeruginosa* was observed in 103/1138 samples (9.1%) and in 61/1138 samples (5.4%) respectively, whereas the co-detection of MCPyV DNA, *S. aureus* and *P. aeruginosa* was found in 48/1138 samples (4.2%). No significant association was found between MCPyV-DNA detection and the microbiological status of CF patients and also analyzing MCPyV positive individuals stratified by age (0-10 years, 11-24 years, >25 years; $p>0.05$) (Table 2).

Given that mucoid *P. aeruginosa* has been associated with more severe pulmonary disease [31], we examined the phenotype of *P. aeruginosa* according to the MCPyV positivity. We did not find any difference in the prevalence of the mucoid phenotype of *P. aeruginosa* between MCPyV positive (26.5%) and negative (24.8%) CF patients and also examining MCPyV positive patients stratified by age (0-10 years, 11-24 years, >25 years; $p>0.05$) (Table 2).

3.4 Gene expression of TLR9 and IFN-I subtypes MCPyV positive and negative CF patients

Considering that patients' age has an impact on the innate immune response to respiratory viruses [32,33], we compared TLR9 and IFN-I gene expression levels between positive and negative MCPyV patients stratified in age groups (0-10 years, 11-24 years, >25 years).

Demographic and clinical characteristics of CF patients analyzed are reported in Table 3. MCPyV positive children produced increased levels of TLR9 and IFN-I genes compared to the negative ones ($p=0.02$, $p<0.001$, $p=0.002$, $p=0.27$) (Figure 2, Panel A), whereas both MCPyV positive adolescents and adults had lower mRNA levels of TLR9, IFNβ, IFNε and IFNα than the negative patients (adolescents: $p<0.001$, $p=0.01$, $p=0.02$, $p=0.26$; adults: $p=0.04$, $p=0.001$, $p<0.001$, $p=0.12$) (Figure 2, Panel B and C). Moreover, in the MCPyV negative group, IFNβ, IFNε and IFNα mRNA levels were lower in children compared to adolescents and adults ($p<0.001$, $p<0.001$, $p=0.002$), while TLR9 was increased in adolescents and reduced in children and adults ($p=0.03$, Figure 3, Panel A). On the other hand, analyzing the MCPyV positive group, we found that TLR9 levels were reduced in adolescents and adults compared to children ($p<0.001$) while there were no significant differences in IFNβ, IFNε and IFNα mRNA levels between age groups ($p>0.05$ for all genes, Figure 3, Panel C). In agreement with these results, transcript levels of TLR9 were found to be inversely correlated with age in MCPyV positive patients ($r=-0.34$, $p=0.001$) (Figure 3, Panel D), differently from that observed in MCPyV negative patients ($r=-0.10$, $p=0.22$) (Figure 3, Panel B).

3.5 Gene expression in MCPyV positive and negative CF patients according to the microbiological status

Given that bacterial colonization might differently activate TLRs and IFN response [30,34], we further explored whether transcript levels of TLR9, IFNβ, IFNε and IFNα in MCPyV positive CF patients could be influenced by the microbiology status. Thus, CF patients were stratified according to the detection of *S. aureus*, *P. aeruginosa* or both. We found that TLR9 levels decreased in MCPyV-DNA and *P. aeruginosa* positive CF patients compared to those negative for MCPyV-DNA detection ($p=0.009$, Figure 4). By contrast, TLR9 levels were higher in *S. aureus* colonized MCPyV positive CF patients than in the negative ones ($p=0.006$, Figure 4). Differently, when CF patients were infected

by *P. aeruginosa* and *S. aureus*, TLR9 levels were higher in those negative for MCPyV-DNA than in those positive for MCPyV-DNA (p=0.003, Figure 4). There were no differences in mRNA levels of IFNβ, IFNε and IFNα between MCPyV positive and negative CF patients suffering from *S. aureus*, *P. aeruginosa* or both bacterial infections (p>0.05, data not shown).

3.6 Exacerbation events

We assessed whether there was an association between the detection of MCPyV-DNA in respiratory specimens, the alteration in TRL9 and IFN-I levels and the frequency rates of acute exacerbations observed in CF patients. Exacerbation data were available for 1108/1138 respiratory samples (97.4%) collected from 516/539 CF patients (95.7%): 89 CF patients presented exacerbation. The number of CF individuals with respiratory exacerbation was similar between those positive (n=27/266, 10.1%) or negative (n=62/842, 7.4%) for MCPyV-DNA (Table 4). Exacerbation events were higher in the adults compared to adolescents and children in MCPyV negative patient's group (p=0.04, Table 4), while they did not differ according to the age of MCPyV positive patients (p=0.12, Table 4). Comparing exacerbated and non-exacerbated CF patients, we found a higher frequency of acute respiratory events in MCPyV negative patients with *P. aeruginosa* infection (p=0.003, Table 4), while a similar frequency of exacerbation events was recorded in MCPyV positive individuals with *S. aureus*, *P. aeruginosa* or both bacteria (p=0.20, Table 4).

A logistic regression analysis was carried out to develop a model of risk factors for respiratory exacerbation occurrence in MCPyV positive CF patients. The detection of *S. aureus* (OR 2.45, 95% CI 0.89-6.68; p=0.08) or *P. aeruginosa* (OR 0.69, 95% CI 0.26-1.78; p=0.446) was not associated with a higher frequency of pulmonary exacerbations episodes in MCPyV positive individuals. We also found no difference between exacerbated and non-exacerbated CF patients in both MCPyV positive and negative patients according to TLR9, IFNβ, IFNε and IFNα mRNA levels (p>0.05, data not shown).

4. Discussion

Although MCPyV is known as a critical factor in the development of MCC [28], MCPyV-DNA has been detected in a variety of non-MCC cancers, including chronic lymphocytic leukemia, malignant tonsillar tissues, cervical carcinomas, nonmelanoma skin cancers, and lung cancer [35] and recently in diverse specimens, including respiratory tract samples [9-13]. As hypothesized for HPyVs [36,37], MCPyV could be transmitted by inhalation through the respiratory route and then spread to other sites, such as epidermal tissue. The role of MCPyV as a respiratory pathogen remains highly speculative since patients generally remain asymptomatic or exhibit nonspecific upper respiratory tract symptoms. Beside the role as respiratory pathogen, MCPyV infection in the respiratory tract mucosal cells may cause a dysregulation in the innate immune sensing and response to other microorganisms.

The impact of respiratory viruses has been only recently recognized in CF patients [38]. Increased respiratory symptoms, decline of respiratory function, higher frequency and duration of hospitalization have been reported in CF for HRV, RSV and Influenza A/B viruses [39,40]; however microbiological and clinical implication of respiratory viruses in CF remains still a poorly understood issue.

In this study, the prevalence of MCPyV-DNA in respiratory samples collected from CF patients reached 23.5%, a rate higher than those reported in previous studies in non CF individuals [8-13], validating the hypothesis that microenvironment conditions in CF respiratory tract could promote a higher rate of MCPyV infection and/or the frequency of its reactivation from latency [14,15]. We did not observe a statistically significant difference in MCPyV prevalence and viral load stratifying CF patients according to age and gender or microbiology status.

These data confirm what is already hypothesized for CF patients: the rate of positivity for respiratory viruses is similar among CF children and adults [1,41], although data about viral prevalence remain highly under-reported, especially in the adults, because not all CF patients have severe symptoms during viral infections and also for technical difficulties as the use of incomplete PCR panels to routinely detect the respiratory viruses [42]. Adults with CF have a lower prevalence of respiratory

viral infections associated with pulmonary exacerbations compared to children and infants [43]. Also, there was no clear seasonal trend for MCPyV-DNA detection in respiratory samples from CF patients. However, in agreement with what was already described in literature [14,15], a higher frequency of MCPyV positivity was reported in February, October and December suggesting that cold weather and low relative humidity could promote MCPyV replication and spreading. Moreover, our data showed that MCPyV could persist for extended periods in the respiratory tract of CF patients: thirteen patients remained MCPyV-DNA positive in four consecutive respiratory samples for a median follow-up period of about 9 months. Although HPyV reactivation in the respiratory tract has been observed in hematology/oncology and AIDS patients [44]; this result suggests that CF patients might have a greater risk of MCPyV reactivation, perhaps as a result of a persistent microbial stimulus in their respiratory tract [14].

In order to improve the knowledge of NCCR alterations in MCPyV strains circulating in CF, the NCCR variability was also analyzed. It has been established that modifications in NCCR structure are the main event in the onset of HPyVs-related pathology, as demonstrated for other HPyVs such as JCPyV and BKPyV, in which NCCRs control gene expression and, harboring the origin of DNA replication and transcription factor binding sites, represent a key determinant in viral replication [45]. On the other hand, relatively little is known about the role that NCCR plays in MCPyV infection, and limited data are available on the relationship between MCPyV NCCR variability and pathogenesis. NCCR sequence analysis revealed a high degree of homology with the prototype MCC350 strain, although transitions, transversions, single or double deletions and insertions were randomly observed. Differently to JCPyV and BKPyV, in which the upstream or 5'side of NCCR is highly conserved and the downstream or 3'side undergoes rearrangements [46], we found that mutations occurred both in early and in late proximal side of the MCPyV NCCR.

Rearrangements in MCPyV NCCR have been associated with genotypes that vary with ethnicity [47]. Specifically, two major subtypes, I and II, were identified based on the presence or absence of a 25 bp tandem repeat into nucleotide positions 5177–5178 of the MCPyV NCCR. Based on the

occurrence of two additional insertions (2 bp, TT, and 5 bp insertions, GTTGA, between nucleotide positions 5199–5200 and 5210–5211, respectively), MCPyV strains have been further assigned to five genotypes. In our analyzed strains, we found the MCPyV NCCR IIa-2 strain which contains the 5 bp insertion GTTGA (nucleotide positions 5210–5211) and represents the predominant strain among white persons of European descent, as expected for our cohort of CF patients [47].

In this study, it was evaluated whether the nucleotide changes observed in MCPyV NCCRs involved the structure of putative binding sites for different cellular transcription factors [45,47-49]. Sequence analysis showed that the MCC350 NCCR sequence contains multiple binding sites such as NHP-1, FOXN2, BTEB3, FOXO3A, NF-AT3, FOXJ2, GATA1, AP1 and PAX2, already described within the NCCRs of other HPyVs [45,47-49]. In several strains obtained from MCPyV-positive samples, mutations fell within these putative binding sites, changing some of these motifs in RELA, STAT4, ETS-1 AR, HLF and Sp3.

The relevance of different cellular factors, including Tst-1, Neurofibromatosis type 1 (NF-1), Specificity protein 1 (Sp1), NF-kB and Purine element binding protein α (PURα), that specifically determine JCPyV tropism for glial cells and play an important role in favouring efficient HPyVs DNA replication, has been described [50]. Moreover, a potential association, between a C/G mutation in the NCCR Sp1 site and increased BKPyV virulence in hemorrhagic cystitis patients, has been proposed [51]. Considering these evidences, further studies are warranted in order to define the importance of NCCR binding sites and understand how these changes may drive MCPyV replication and pathogenicity *in vitro* and *in vivo*.

In the attempt to understand whether changes in NCCR sequence could be correlated with an increased MCPyV replicative capacity, the viral load was analyzed considering the occurrence of these mutations. In CF patients who had positive results for MCPyV-DNA detection in four consecutive respiratory samples and in which no mutations were observed, the number of MCPyV-DNA copies/mL decreased over the time. Conversely, a higher number of MCPyV-DNA copies/mL was reported in CF patients with GTTGA insertion. As previously reported for BKPyV and JCPyV,

rr-NCCRs conferred a higher replication rate to these viruses, contributing to disease progression [52]. Consequently, also for MCPyV, it is possible to speculate that the shift from canonical NCCR to rr-NCCR could determine higher replication capacity. It is also likely that high MCPyV-DNA levels might enhance the chance of viral integration into the cell genome and then its oncogenic properties in a context different from that of MCC.

Viral infections in combination with colonizing polymicrobial communities may differentially contribute to alteration in the airway inflammation and innate immune response in CF [53]. TLRs, involved in viral detection and microbial recognition, play a key role in orchestrating the inflammatory response in respiratory epithelial cells of healthy individuals and in those with chronic respiratory diseases, including CF individuals [30]. Hence, the presence of an excessive or reduced TLR expression and activation can interfere with inflammatory response [54]. Because the respiratory tract in CF is most frequently colonized by *S.aureus* and *P. aeruginosa* that have been both involved in causing alterations in TLR and IFN pathways [55-57] we considered their presence as a relevant factor to understand if MCPyV could have a role in influencing IFN response.

Remarkably, the results presented here are the first, to our knowledge, to indicate that detection of MCPyV-DNA in respiratory samples of CF patients is associated with alteration in the transcript expression of TLR9. Surprisingly, MCPyV positive children had higher transcript levels of TLR9 and IFNs compared to the negative ones, while a reduction in TLR9 and IFNs mRNAs was found in adolescents and adults with MCPyV. The increased TLR9 and IFNs levels found in children with MCPyV might be caused by the activation of innate immune response as response to the first MCPyV infection occurring at the early ages; on the other hand, CF adolescents and adults might probably experience different MCPyV reactivations, resulting in abundant production of viral early genes able to modulate the innate immune responses [58]. Thus, MCPyV-mediated alteration of TLR9-mRNA production could confer a benefit against the intrinsic dysregulated inflammatory response observed during CF, in which the dysfunctional CFTR is known to trigger high proinflammatory cytokine levels either at basal or as consequence to viral or microbial infections [59]. Moreover, the finding

that TLR9 levels were higher in MCPyV positive children compared to those observed in MCPyV positive adolescents and adults could be partially explained by a more frequent detection of *S. aureus* in children: in this regard *in vitro* studies have identified TLR9 as one of the most important pattern recognition receptor involved in the induction of IFN-I signaling in response to *S. aureus* infection [60,61]. However, on one hand TLR9 was found to be inversely correlated with age in MCPyV positive CF patients, on the other hand TLR9 was higher in MCPyV negative adolescents, suggesting the existence of a potential combined effect of MCPyV and *S. aureus* co-detection on TLR9 expression in children. Indeed, CF adolescents had *S. aureus* as the most frequent bacterium in their respiratory tract, highlighting the complexity of the phenomenon analyzed. This hypothesis seems to be supported by the fact that TLR9 levels were enhanced in *S. aureus* colonized MCPyV positive CF patients than in the negative ones, while TLR9 levels decreased in those with *P. aeruginosa* and MCPyV compared to those negative for MCPyV-DNA detection. Indeed, the role of TLR9 played in response to *P. aeruginosa* remains not yet characterized thoroughly, although its contribution to the inflammatory response, up-regulation of TLRs, and bacterial clearance has been reported [62]. However, neutrophils appear to be capable of responding to *P. aeruginosa* DNA in a TLR9-independent manner [63], highlighting a complex interplay between bacteria, virus and innate immunity.

Some studies have reported a low prevalence of HPyV in the respiratory secretions from patients with acute respiratory symptoms [9,37]. Our results point out that the detection of MCPyV-DNA in CF respiratory samples did not affect the frequency of pulmonary exacerbation events. In relation to the microbiology status of CF patients analyzed, exacerbation events in MCPyV negative patients were more frequent in individuals with *P. aeruginosa* infection, while a trend toward a major number of acute respiratory events was found in those positive for both *S. aureus* and MCPyV. In agreement with this data, while acute respiratory events in MCPyV negative patients were more frequent in adults, who usually present *P. aeruginosa* colonization [64], we did not find a significant difference in the frequency of exacerbation events among the age groups in MCPyV positive patients.

In conclusion, MCPyV-DNA is frequently detected in the respiratory samples of CF patients, with a higher prevalence during cold months. Moreover, these results suggest that MCPyV could be involved in the reduction of TLR9 and IFNs transcript levels in both adolescents and adults with CF. By contrast, the detection of MCPyV-DNA and *S. aureus* is associated to an increase of TLR9 and IFNs expression levels in respiratory samples collected from CF children.

Overall, these observations indicated that MCPyV might differentially alter the expression of TLR9-mRNA in the respiratory tract of CF patients according to their bacteriological status, affecting indirectly the IFN-I production. Because analogous to MCPyV, both KIPyV and WUPyV have been observed in respiratory samples, but clear evidence for a causal association with respiratory illness has yet to be identified [65], further studies examining and comparing the prevalence of these HPyVs in CF and their relationship with IFN pathways are required to better evaluate whether these viruses can promote changes in microbial environment and mucosal immunological response.

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Fig. 1 Sequence analysis of the MCPyV NCCR PCR products. The alignment is shown between the nucleotide sequence from 5077 (proximal to the early genes) to 5280 (just upstream of the start codon of the VP2 gene) of the published sequence of MCPyV in GenBank (NCBI) (EU375803) [29] and that obtained from the sequencing of positive samples containing the observed mutations and deletions. Putative binding sites for transcriptional factors in the MCC350 are reported as well as the changed-putative binding motifs resulted from the mutated NCCR.

Fig. 2 Expression levels of genes encoding TLR9, IFNβ, IFNε and IFNα in respiratory samples collected from cystic fibrosis (CF) patients with or without Merkel cell polyomavirus (MCPyV) infection. Data for children (Panel A), adolescents (Panel B) and adults (Panel C).

*p≤0.05 **p≤0.01 ***p≤0.001

Fig. 3 Expression levels of genes encoding TLR9, IFNβ, IFNε and IFNα in respiratory samples collected from cystic fibrosis (CF) patients stratified by age (0-10 years, 11-24 years, >25 years) without Merkel cell polyomavirus (MCPyV) (Panel A) or with MCPyV (Panel C). Scatterplot showed the correlation between age (years) and TLR9 mRNA levels expressed as relative expression in MCPyV negative patients (Panel B) and in MCPyV positive patients (Panel D).

*p≤0.05 **p≤0.01 ***p≤0.001

Fig. 4 Expression levels of TLR9 mRNA in respiratory samples collected from cystic fibrosis (CF) patients with or without Merkel cell polyomavirus (MCPyV), stratified according to the bacteriological status: *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and both bacteria.

*p≤0.05 **p≤0.01 ***p≤0.001

Figure 1

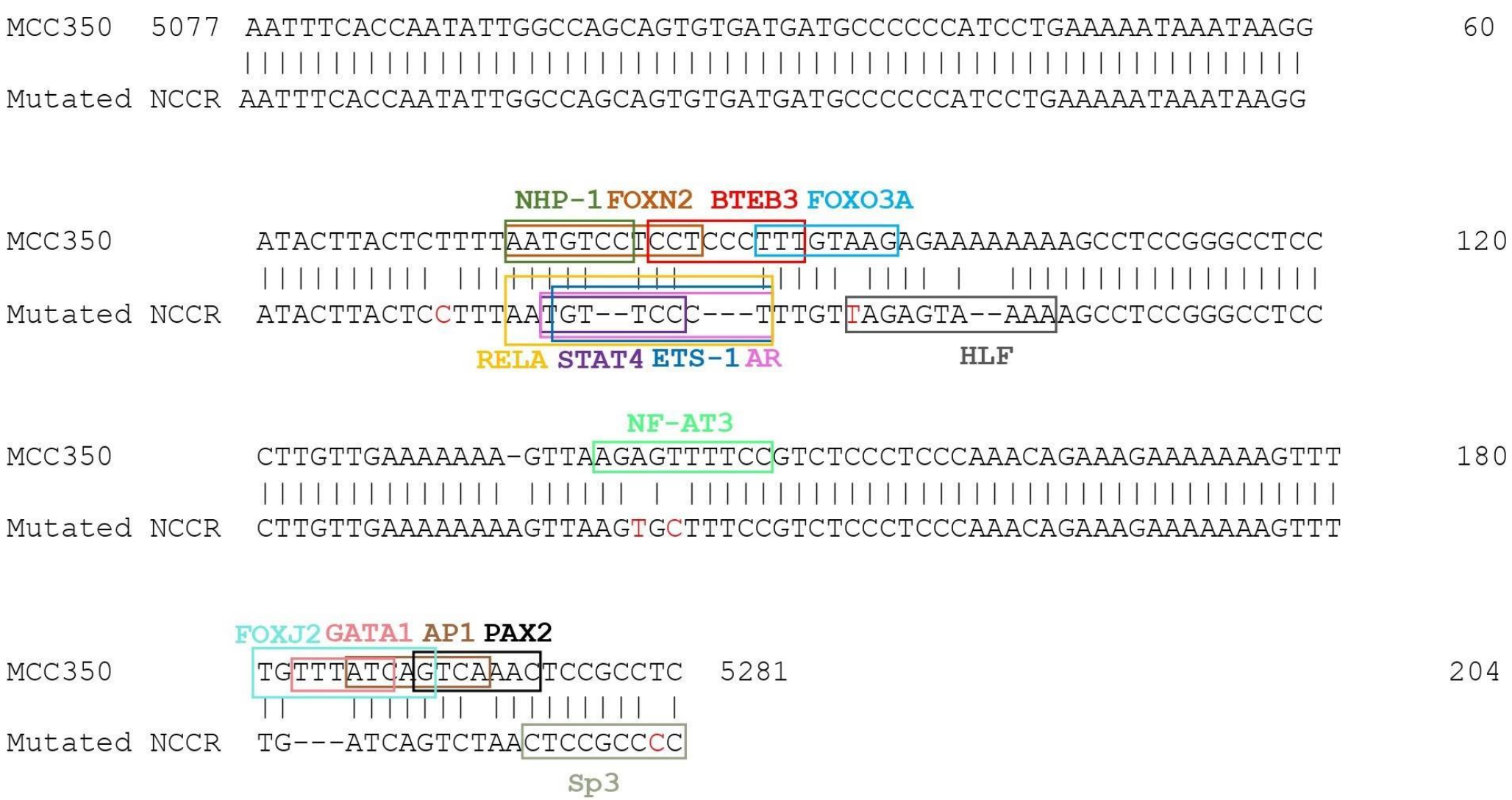

Figure 2 Panel A

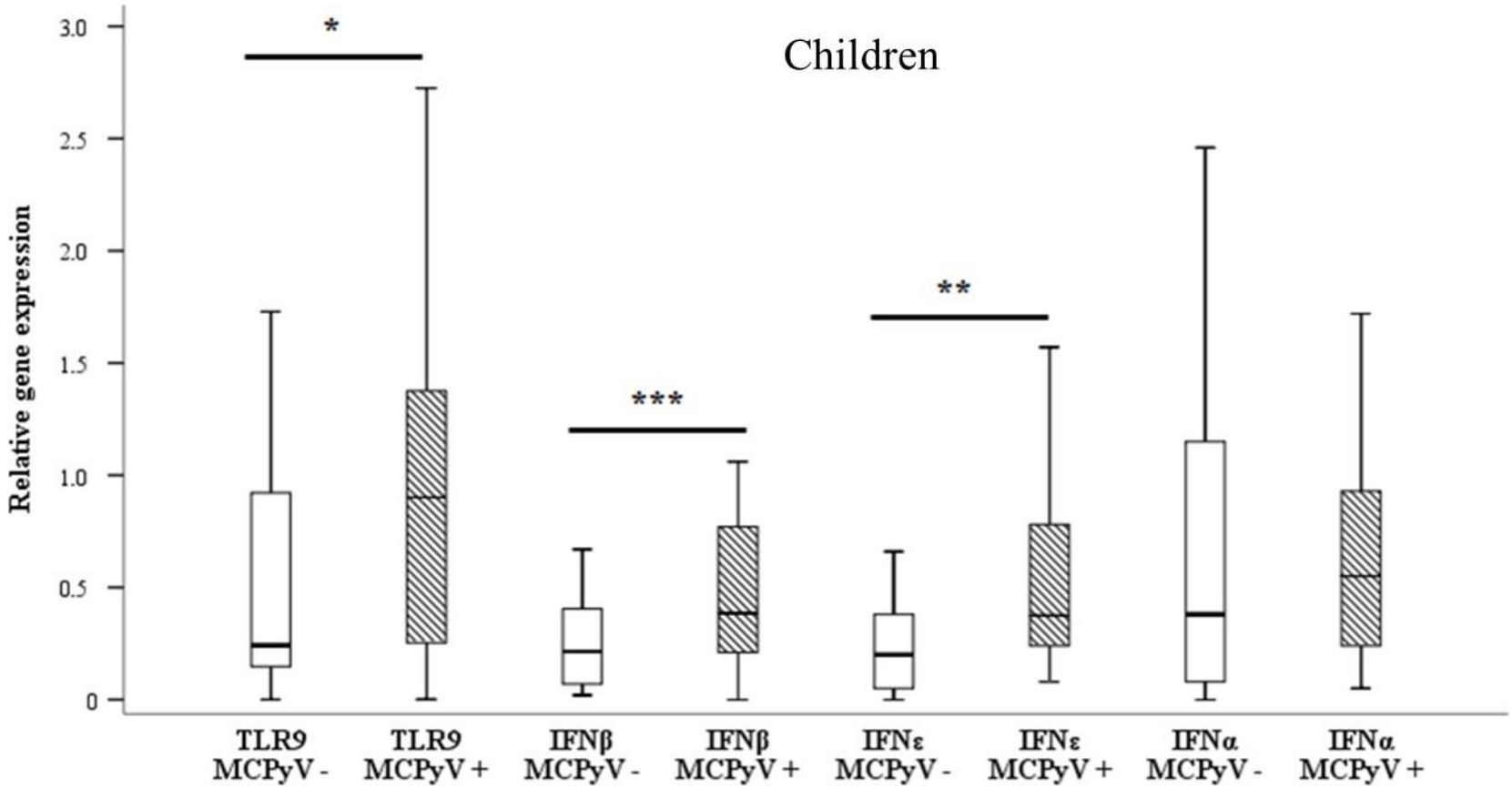

Figure 2 Panel B

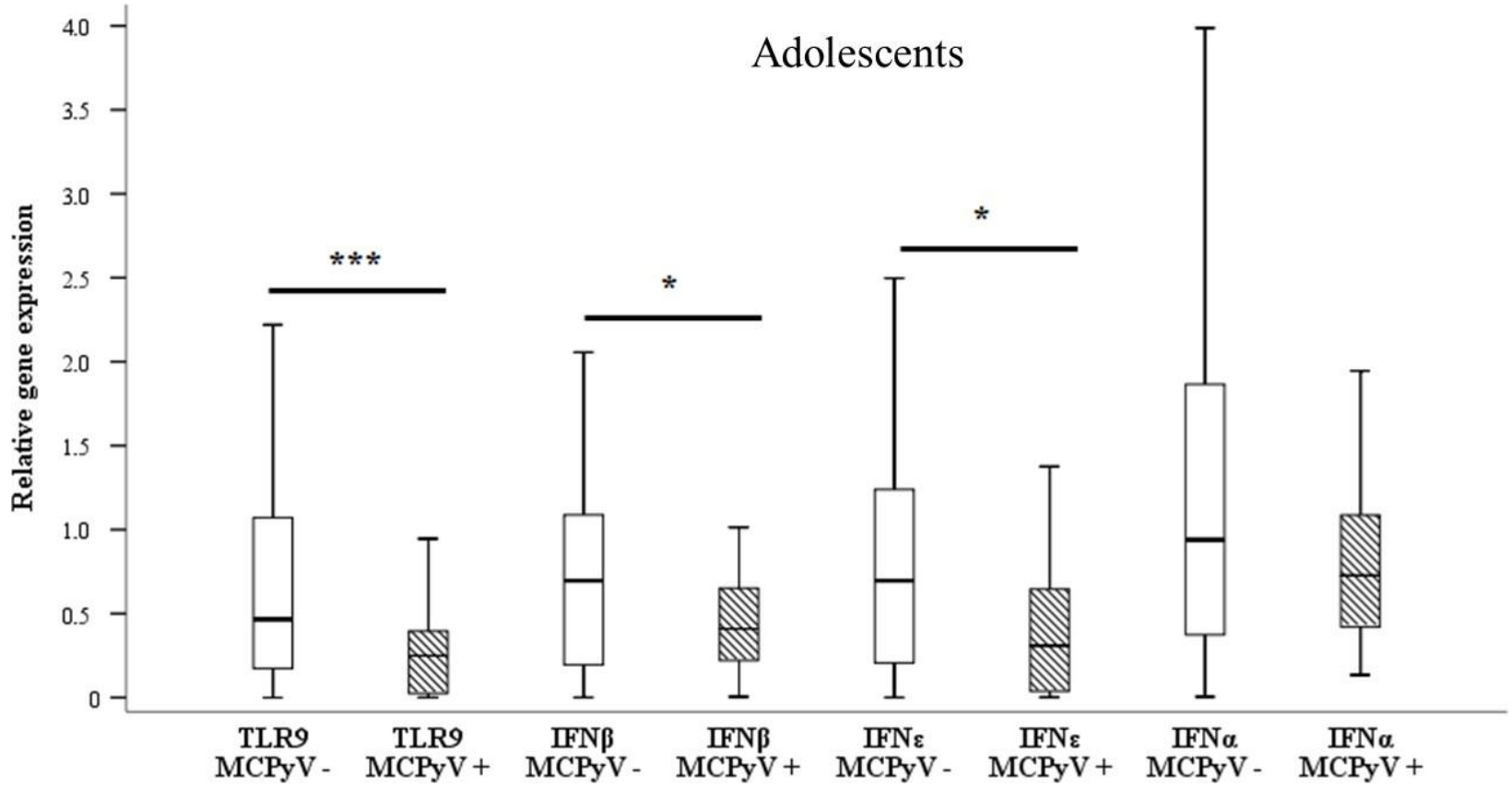

Figure 2 Panel C

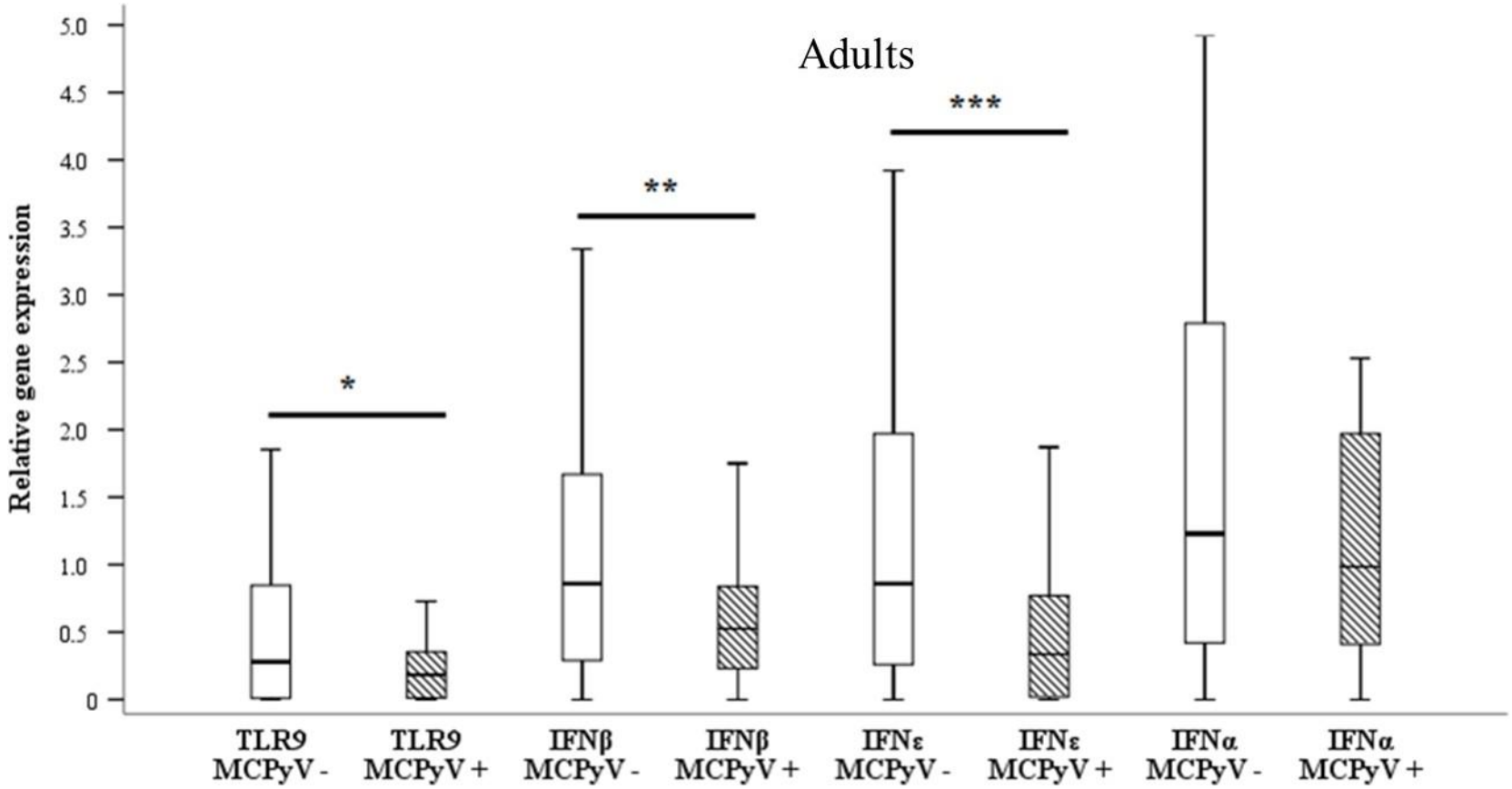

Figure 3 Panel A

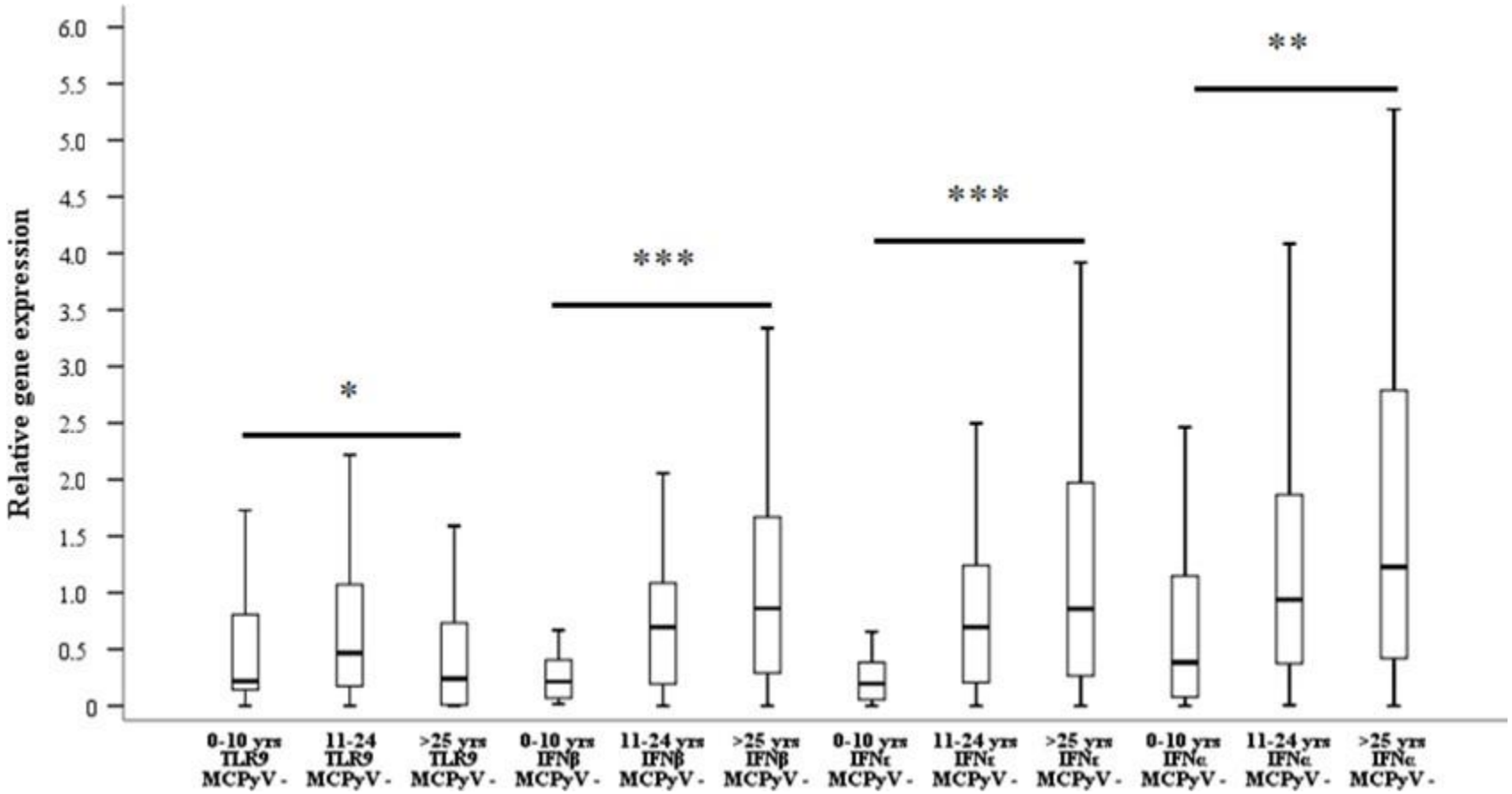

Figure 3 Panel B

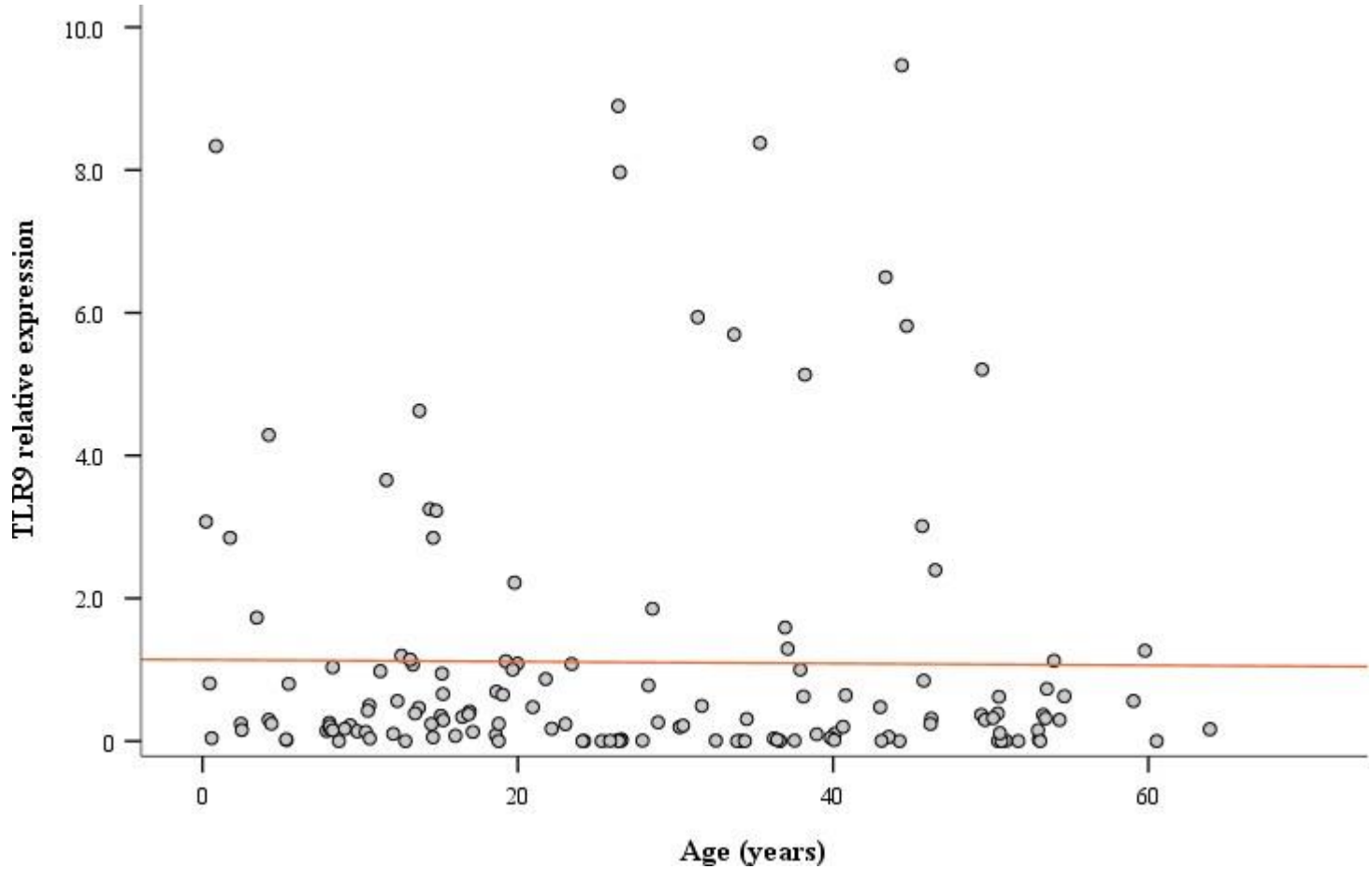

Figure 3 Panel C

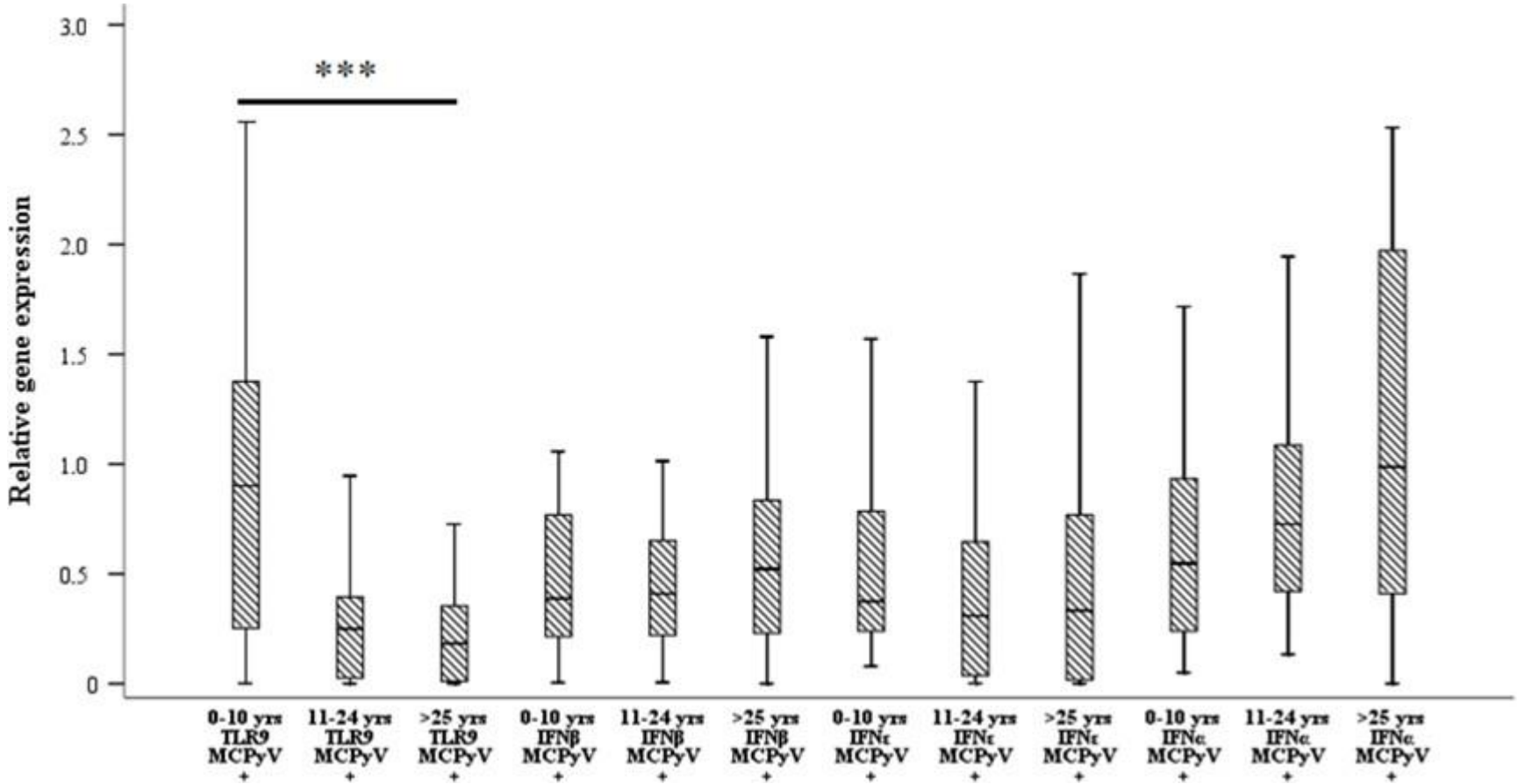

Figure 3 Panel D

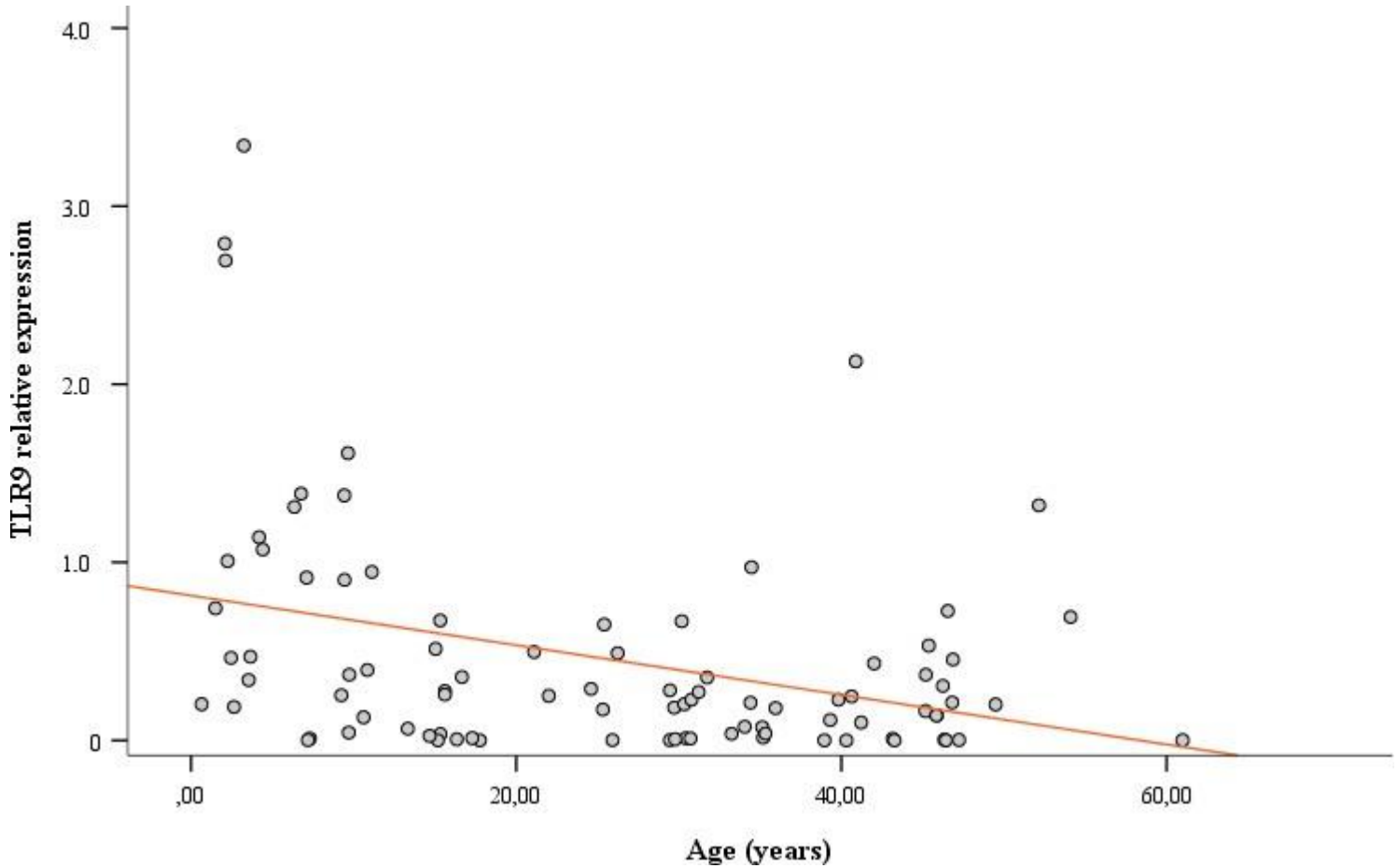

Figure 4

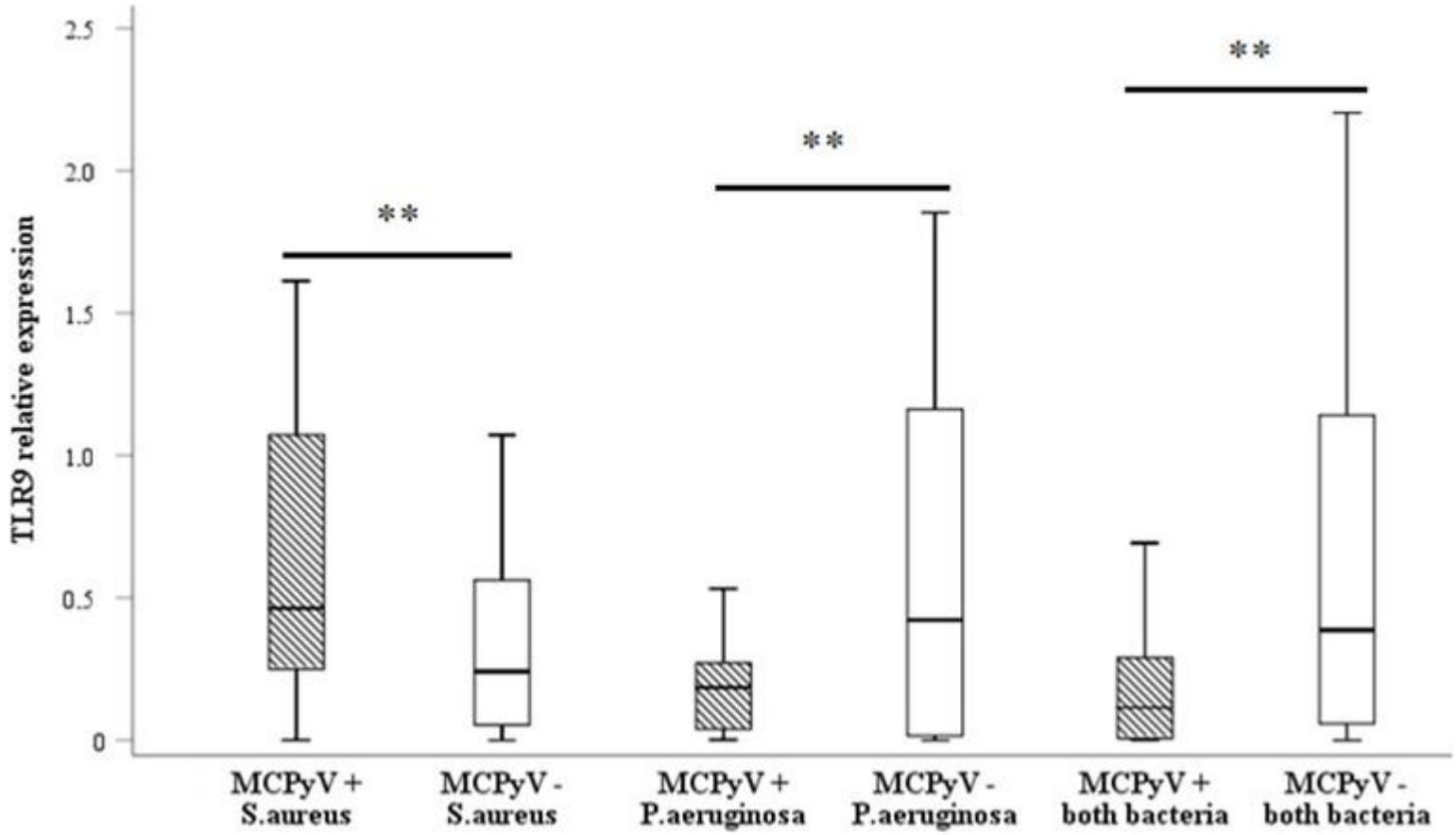
TLR9 relative expression
2.5
2.0
1.5
1.0
0.5
0
**
**
**
MCPyV + S.aureus
MCPyV - S.aureus
MCPyV + P.aeruginosa
MCPyV - P.aeruginosa
MCPyV + both bacteria
MCPyV - both bacteria

Table 1. Merkel cell polyomavirus (MCPyV) prevalence in cystic fibrosis (CF) patients (n=539). Demographic, clinical characteristics of Merkel cell polyomavirus (MCPyV) positive (n=137) and negative (n=402) cystic fibrosis patients.

Items	Total patients n=539	MCPyV-DNA (+) patients n= 137*	MCPyV-DNA (-) patients n=402
Respiratory samples			
One sample (%)	173 (32.1)	17 (12.4)	156 (38.8)
Two samples (%)	197 (36.5)	45 (32.8)	152 (37.8)
Three samples (%)	105 (19.5)	40 (29.2)	65 (16.2)
Four samples (%)	64 (11.9)	35 (25.6)	29 (7.2)
At least one positive sample (%)	137 (25.4)	137 (100)	NA
At least two positive samples (%)	82 (15.2)	82 (59.9)	NA
At least three positive samples (%)	36 (6.7)	36 (26.3)	NA
Four positive samples (%)	13 (2.4)	13 (9.5)	NA
Two consecutive positive samples (%)	50 (9.3)	50 (36.5)	NA
Three consecutive positive samples (%)	19 (3.5)	19 (13.9)	NA
Four consecutive positive samples (%)	13 (2.4)	13 (9.5)	NA
Male patients, n (%)	273 (50.6)	68 (49.6)	205 (50.9)
Age, mean (SD)	25.9 (16.5)	23.7 (15.4)	26.5 (16.8)
0-10 years	114 (21.1)	33 (24.1)	81 (20.2)
11-25 years	167 (31.0)	43 (31.4)	124 (30.8)
>25 years	258 (47.9)	61 (44.5)	197 (49.0)
ΔF508 homozygous/ heterozygous/others patients, n (%)	82 (17.6) / 211 (45.3) / 173 (37.1)	21 (16.7) / 51 (40.5) / 54 (42.8)	61 (17.9) / 160 (47.1) / 119 (35.0)
BMI, mean (SD)	20.5 (4.1)	19.9 (3.2)	20.7 (4.4)

*Patients positive for MCPyV-DNA detection in respiratory samples at least one time. Data are presented as number or mean. N.A.: not applicable.

Table 2. Demographic, microbiological and clinical features of Merkel cell polyomavirus (MCPyV) positive (n=268) and negative (n=870) cystic fibrosis (CF) samples divided by three age groups (0-10 years, 11-24 years, >25 years).

Items	MCPyV-DNA (+) samples (n=268)			MCPyV-DNA (-) samples (n=870)		
	0-10 years (n=74)	11-24 years (n=75)	>24 years (n=119)	0-10 years (n=182)	11-24 years (n=271)	>24 years (n=417)
Aspirate samples, n (%)	64 (86.5)	44 (58.7)	54 (45.4)	165 (90.7)	177 (65.3)	115 (27.6)
Sputum samples, n (%)	10 (13.5)	31 (41.3)	65 (54.6)	17 (9.3)	94 (34.7)	302 (72.4)
MCPyV titer, mean	$7.3*10^3$	$6.9*10^3$	$9.5*10^3$			
P. aeruginosa, n (%)	2 (2.7)	7 (9.3)	52 (43.7)	13 (7.1)	23 (8.5)	171 (41.0)
S. aureus, n (%)	41 (55.4)	42 (56.0)	20 (16.8)	84 (46.1)	137 (50.5)	73 (17.5)
P. aeruginosa and *S. aureus*, n (%)	7 (9.45)	9 (12)	32 (26.9)	23 (12.6)	61 (22.5)	95 (22.8)
P. aeruginosa mucoid, n (%)	2 (2.7)	7 (9.3)	62 (52.1)	4 (2.2)	42 (15.5)	170 (40.8)

Data are presented as number or mean. MCPyV titer is expressed as number of copies/ml.

Table 3. Demographic and clinical characteristics of Merkel cell polyomavirus (MCPyV) positive (n=95) and negative (n=147) samples selected for TLR9 and IFN-I genes (IFNα, IFNβ and IFNε) analysis from the cystic fibrosis (CF) cohort patients.

Items	Total n=242	MCPyV + samples n= 95*	MCPyV - samples n=147*
Males, n (%)	122 (50.4)	44 (46.3)	78 (53.1)
Age, mean (SD)	26.4 (16.4)	25.1 (15.8)	27.3 (16.8)
ΔF508 homozygous/ heterozygous/others, n	110/58/62	43/24/25	67/34/37
BMI, mean (SD)	20.2 (3.7)	19.8 (3.5)	20.5 (3.9)
Exacerbation events, n (%)	20 (8.3)	10 (10.5)	10 (6.8)
Aspirate samples, n (%)	139 (57.4)	57 (60)	82 (55.8)
Sputum samples, n (%)	103 (42.6)	38 (40)	65 (44.2)
P. aeruginosa presence, n (%)	59 (24.4)	26 (27.4)	33 (22.5)
S. aureus presence, n (%)	77 (31.8)	29 (30.5)	48 (32.6)
P. aeruginosa and *S.aureus* presence, n (%)	54 (22.3)	17 (17.9)	37 (25.1)
Mixed microbial flora, n (%)	52 (21.5)	23 (24.2)	29 (19.8)
TLR9 (CV%)	0.27 (36.7)	0.23 (72.3)	0.31 (40.1)
IFNα (CV%)	0.92 (30.7)	0.72 (29.8)	0.98 (31.4)
IFNβ (CV%)	0.53 (37.1)	0.46 (46.6)	0.62 (37.9)
IFNε (CV%)	0.41 (48.8)	0.32 (45.0)	0.64 (52.3)

*Gene expression was performed in 95 MCPyV positive respiratory samples collected from 72 CF patients and 147 negative respiratory samples collected from115 patients. Data are presented as number or mean. N.A.: not applicable. Transcript levels of TLR9, IFNβ, IFNε and IFNα related to β-glucoronidase mRNA were calculated using 2^-ΔCt, and indicated as median. CV: coefficient of variation.

Table 4. Demographic and microbiological characteristics of Merkel cell polyomavirus (MCPyV) positive (n=266) and negative (n=842) cystic fibrosis (CF) samples.

Items	MCPyV-DNA (+) samples (n=266)		MCPyV-DNA (-) samples (n=842)	
	Exacerbated (n=27)□	Non exacerbated (n=239)	Exacerbated (n=62)□	Non exacerbated (n=780)
0-10 years, n (%)	6 (22.2)	67 (28.0)	11 (17.7)	168 (21.5)
11-25 years, n (%)	4 (14.9)	71 (29.7)	12 (19.3)	248 (31.8)
>25 years, n (%)	17 (62.9)⧫	101 (42.3)	39 (63.0)●	364 (46.7)
P. aeruginosa, n (%)	9 (33.3)	52 (21.7)	26 (41.9)	175 (22.5)
S. aureus, n (%)	10 (37.0)	93 (38.9)	12 (19.3)	274 (35.3)
P. aeruginosa and *S. aureus*, n (%)	6 (22.2)	41 (17.1)	15 (24.2)	161 (20.7)
Mixed microbial flora, n (%)	2 (7.4)	53 (22.2)	9 (14.5)	167 (21.5)

Data are presented as number. □Respiratory exacerbation rate was similar between those positive or negative for MCPyV (p=0.16 by Chi-square test); ●Exacerbation events were higher in the adult group compared to adolescents and children in MCPyV negative patients (p=0.04 by Chi-square test); ⧫Exacerbation events did not differ according to the age of MCPyV positive patients (p=0.12 by by Chi-square test).

Declaration of interests

☐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.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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