Assessment of an ELISA for serodiagnosis of active pulmonary tuberculosis in a Cuban population

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ARTICLE INFO

Abstract

Objective: To explore the serodiagnostic potential of the five recombinant Mycobacterium tuberculosis antigens CFP-10 (Rv3874), ESAT-6 (Rv3875), APA (Rv1860), PstS-1 (Rv0934), Ag85A (Rv3804c) and their combination in a Cuban population with active pulmonary tuberculosis.

Methods: The serodiagnostic potential of the recombinant antigens rESAT-6, rCFP-10, rAPA, rPstS-1 produced in Escherichia coli, rAg85A produced in Streptomyces lividans and the combination of the five proteins was evaluated by an indirect ELISA. Humoral immune response was analysed in a group of 140 patients with active pulmonary tuberculosis (smear-, Mantoux- and culture-positive) and in a control group consisting of 34 bacillus Calmette-Guerin vaccinated, Mantoux-negative, healthy subjects.

Results: With the exception of CFP-10, the use of the separate recombinant antigens or the antigenic cocktail in ELISA-based serodiagnosis resulted in a significant difference in the mean optical density values between sera of patients and healthy subjects. The highest sensitivity of the assay using single antigens, being 58.57%, was achieved with rPstS-1 compared to 27.14% with rCFP-10, 31.65% with Ag85A, 42.86% with rAPA and 44.29% with rESAT-6. Single antigen ELISAs provided high specificity values ranging from 94.12% to 97.06%. A cocktail of the aforementioned antigens increased the sensitivity to 87.14% and the specificity to 97.06%.

Conclusions: An ELISA using a multi-antigen mix containing recombinant immuno-dominant antigens of Mycobacterium tuberculosis, namely, rCFP-10, rESAT-6, rAPA, rPstS-1 and rAg85, increases the sensitivity and specificity compared with that using the single antigens and shows potential as a complementary tool for the diagnosis of active pulmonary tuberculosis in Cuba.

1. Introduction

Tuberculosis (TB) is an airborne infectious disease caused by bacteria of the Mycobacterium tuberculosis (M. tuberculosis) complex. It ranks as the second leading cause of death from a single infectious agent. An estimated 1.1 million (13%) of the 9 million people who developed TB in 2013 were HIV-positive. Of the estimated 9 million people who developed TB in 2013, more than half (56%) were in the South-East Asia and Western Pacific regions and 1.5 million people died as a consequence of the disease[1,2].

Presently the diagnosis of TB is a worldwide problem due to the absence of a rapid, sensitive, specific and cost-effective test[3,4]. In Cuba, despite the low incidence of TB compared to other countries, finding and developing a diagnostic method with the aforementioned characteristics is a necessity to improve current methods in use (sputum smear, sputum culture, chest X-ray and tuberculin skin test).

Although the detection of acid-fast bacilli (AFB) by microscopic examination of sputum smears is a simple and relatively quick means, approximately 105 bacilli/mL of sputum are required for reliable detection[5]. The sensitivity of the smear test for detection
of pulmonary TB reaches values greater than 80%, but lower percentages in the range between 20% and 80% have also been reported[6,7]. Moreover, the sensitivity of this method is low for the detection of extrapulmonary forms of the disease[6,9]. The culture of *M. tuberculosis* *in vitro* remains the golden standard for the diagnosis of TB, but it is not commonly available in low income countries. Moreover, the growth of the tubercle bacilli takes about 4–6 weeks and has still other shortcomings[10].

In asymptomatic (latently infected) and in symptomatic individuals, TB infection can be diagnosed with the help of the intradermal injection of tuberculin, the Mantoux test, using a purified protein derivative (PPD)[11]. Most proteins found in PPD, are not specific to *M. tuberculosis* but are also found in environmental mycobacteria. Thus, individuals infected with mycobacteria other than *M. tuberculosis*, or vaccinated with *Mycobacterium bovis* (*M. bovis*) bacillus Calmette-Guerin (BCG) strain, generally react to PPD, albeit in general with smaller skin indurations[12].

Many alternative methodologies have been applied in TB diagnosis, such as PCR and cell-mediated immune response reactions such as QuantiFERON and enzyme-linked immunospot assay. These methods require trained personnel and specific laboratory conditions, which hinder their implementation in many areas with high TB incidence. Immunological tests based on specific humoral responses of the host, in particular, ELISA, are simple and do not require expensive reagents or equipment. Previous TB serodiagnostic studies using immuno-based tests have revealed some major antigens of *M. tuberculosis* including ESAT-6, CFP-10, the 38-kDa glycolipoprotein PstS-1, the A.g85 complex, lipoparabinomannan, and the 19-kDa and 16-kDa antigens[13,14]. However, those studies have indicated that the antigenic reaction pattern of antibodies in serum varies greatly from patient to patient and no antigen alone is sufficient to perform a sensitive and specific TB diagnosis[15,16].

The aim of the present study was to explore the potential of an indirect ELISA using five recombinant antigens from *M. tuberculosis* (ESAT-6, CFP-10, APA, PstS-1 and A.g85A), individually or mixed together, for the diagnosis of active pulmonary TB in a Cuban population.

2. Materials and methods

2.1. Study population (serum samples and patients)

Serum samples of 140 confirmed active TB patients were collected from National Hospital of Pneumology “Benéfico Jurídico” of Havana, Cuba. Active TB patients were diagnosed by the isolation and identification of *M. tuberculosis*, as well as clinical and radiological findings. All patients were classified as smear-positive for AFB and culture-positive pulmonary TB patients. All patients had undergone the Mantoux test at the time of blood sampling. All patients were in the first two weeks of the antituberculous therapy before sera were collected.

Control sera (*n* = 34) included in this study were obtained from healthy subjects without previous history of TB. All subjects had been previously vaccinated with *M. bovis* BCG and were negative in the Mantoux test, with a diameter of induration smaller than 10 mm. Sera were obtained by centrifugation of clotted blood samples at 4 °C and the respective supernatants were stored at -20 °C.

This research was in compliance with the Helsinki Declaration and was approved by the National Hospital of Pneumology “Benéfico Jurídico” Ethics Committee. Written informed consent was obtained from all patients and healthy donors.

2.2. Cloning of *M. tuberculosis* genes

Escherichia coli (*E. coli*) strain JM109 carrying the aps gene (gene ID Rv1860) from *M. tuberculosis* H37Rv under the regulation of the bacteriophage T7 promoter and the gene is N-terminally tagged with a hexahistidine sequence[21]. To express the ESAT-6, PstS-1 and CFP-10 proteins the open reading frames of the genes were amplified by PCR using *M. tuberculosis* strain H37Rv genomic DNA as template and the appropriate primers (Table 1). The amplified fragments were ligated into the pGEM®-T Easy Vector (Promega) and the sequences were confirmed by DNA sequencing. The gene-containing fragments were extracted from the recombinant pGEM-T Easy Vector derivatives by digestion with the appropriate enzymes shown in Table 1 and were cloned into similarly digested pET-3a (Stratagene) for *exsa* (Rv3875 encoding ESAT-6) and *psts-1* (Rv3904) and pET-23d for *exsb* gene (Rv3874 encoding CFP-10) following the Stratagene’s recommendations. These genes were then expressed from the T7 promoter in *E. coli* BL21(DE3)/pLYS5 cells with an N-terminal HisTag fusion in the case of ESAT-6 and PstS-1 proteins and a C-terminal HisTag fusion in CFP-10. The *fhpA* gene (Rv3804c), encoding antigen 85A, was equipped with a 3′-Strep-tagII-encoding sequence and placed under control of the *Streptomyces venezuelae* CBS762.70 subtilisin inhibitor (svi) transcriptional, translational and signal sequences as described earlier[22].

Table 1

| Gene | Primers (restriction recognition sites) |
|------|---------------------------------------|
| esaA | TAGAATATGCATCACCATCACCACATCAAACTGAAAGAGCACAGCACG (<NM>) |
| esaB | GGGCGGCCATGGCAGAGATGAAGAC (<NdeI) |
| psts-1 | GGATATTCATATGTCAATCACCACATCAAACTGAAAGAGCACAGCACG (<BamHI) |

Restriction recognition sequences are underlined and the corresponding restriction enzyme is mentioned between brackets.

2.3 Recombinant protein expression and purification

The recombinant antigens APA, ESAT-6, CFP-10 and PstS-1 were purified as His-tagged proteins by means of immobilized metal ion affinity chromatography using HisTrap™ HP columns (GE Healthcare) according to the manufacturer’s recommendations. Briefly, *E. coli* cells carrying the expression plasmids described above were grown in Luria-Bertani broth supplemented with ampicillin at 37 °C and 200 rpm to an optical density (OD) of 0.4–0.8 at 600 nm. At this point expression of the antigens was induced with isopropyl-β-D-thiogalactoside (1 mmol/L) and cultures were further incubated for 4 h at 30 °C. The cells were collected by centrifugation, resuspended in 20 mmol/L phosphate buffer (pH 7.4), 10 mmol/L imidazole and disrupted by sonication. The cell debris...
was removed by centrifugation and clarified lysates were loaded onto the columns. Then, recombinant antigens were eluted with a step gradient of 100, 200 and 500 mmol/L imidazole. rAg85A antigen was purified from S. lividans supernatant by affinity chromatography using the Strep-Tactin® Superflow™ matrix (IBA Gmbh BioTAGnology) as reported before(22). Finally, the protein eluates were subjected to buffer exchange using PD-10 columns (Amersham Biosciences) and phosphate-buffered saline (PBS) as replacement buffer. Purity of the protein preparations was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by gel scanning densitometry analysis using the GeneGenius Bio Imaging System (Syngene). Protein concentrations were determined by Bradford method using the Bio-Rad protein assay following manufacturer’s instructions. Proteins were stored at -20 °C until use.

2.4. ELISA

Polystyrene flat-bottomed microtiter 96-well plates (MaxiSorp, NUNC™ Serving Life Science) were coated overnight at 4 °C with 100 µL of antigen solution in coating buffer (15 mmol/L Na2CO3, 28.5 mmol/L NaHCO3, pH 9.6) containing ESAT-6 (2.5 µg/mL), CFP-10 (0.5 µg/mL), Ag85A (3.0 µg/mL), APA (1.5 µg/mL), PstS-1 (5.0 µg/mL) and a cocktail of five antigens at the same concentrations as single antigen solutions except for Ag85A (1.0 µg/mL) and PstS-1 (1.0 µg/mL). Wells were washed once with PBS and subsequently blocked with 200 µL PBS containing 5% (w/v) skimmed milk. The plates were incubated for 1 h at 37 °C in humid environment. Afterwards, wells were washed twice with PBS and incubated with 100 µL of serum samples at different dilutions [1:50 for antigens ESAT-6, CFP-10, Ag85A and the antigenic cocktail, and 1:200 for APA and PstS-1 in PBS TWEEN 20 (0.05%, v/v) (PBST) containing 3% skimmed milk (incubation buffer)]. Samples were added in duplicate and plates were incubated for 1 h at 37 °C. Next, wells were washed 6 times with PBST, incubated for 1 h at 37 °C with 100 µL of antihuman-immunoglobulin G (IgG) (H+L) horseradish peroxidase conjugate (Promega) diluted 1:10 000 in incubation buffer and washed again 8 more times with PBST. Finally colour development was done via incubation with 100 µL of 5.5 mmol/L substrate o-phenylenediamine (Sigma) and 5.3 mmol/L H2O2 (Sigma) in buffer (24.3 mmol/L citrate, 51.4 mmol/L Na2HPO4). The OD at 492 nm of the plates was read immediately with a Titertek Multiskan® PLUS reader.

2.5. Statistical analysis

Differences in the means of ELISA-obtained OD values from TB patients and healthy subjects were evaluated with a two-tailed student’s t-test by using the statistical package GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). A receiver-operator characteristic (ROC) curve analysis of the ELISA results was performed for each antigen to determine the cut-off point for distinguishing between a positive and a negative result. The area under the ROC curve (AUC) was also calculated for each antigen and the antigenic cocktail with the aid of the GraphPad Prism 5.

3. Results

3.1. ELISA assays for IgG antibodies against a single antigen

In the present study, the serum reactivity against recombinant antigens ESAT-6, CFP-10, APA, PstS-1 and Ag85A, alone or in combination, were measured by an indirect ELISA in 140 AFB-positive, active TB patients and 34 healthy BCG vaccinees. The mean of OD values obtained in the ELISA tests for recombinant CFP-10, ESAT-6, APA, PstS-1, Ag85A and the cocktail of them, using sera from patients and healthy subjects are shown in Table 2.

| Antigens | Healthy controls | TB patients | S/N ratio |
|----------|------------------|-------------|-----------|
|          | Mean OD | SD | Mean OD | SD |          |          |
| CFP-10   | 0.429 | 0.089 | 0.548 | 0.375 | 1.277 |
| ESAT-6   | 0.221 | 0.052 | 0.314 | 0.133 | 1.421 |
| APA      | 0.307 | 0.108 | 0.545 | 0.384 | 1.775 |
| Ag85A    | 0.226 | 0.132 | 0.524 | 0.341 | 2.319 |
| PstS-1   | 0.091 | 0.037 | 0.414 | 0.537 | 4.549 |
| Antigenic cocktail | 0.181 | 0.052 | 0.802 | 0.522 | 4.431 |

*: Significantly different results between controls and TB patients (two-tail, P < 0.001); S/N ratio: The signal-to-noise ratio, the ratio of mean OD value of TB patients to mean OD value of healthy donors.

With the exception of CFP-10, the remaining antigens showed a substantially higher reactivity with sera samples from active TB patients compared to sera from healthy donors, with the mean ODs of the groups statistically different at P < 0.001. PstS-1 and the antigenic cocktail based ELISAs had the highest S/N ratio (mean OD value from TB patients/mean OD value from healthy controls) compared to single antigens Ag85A, APA, ESAT-6 and CFP-10 (Table 2). The S/N ratio was used as a standard for the overall comparison of the antibody response. Scatter plot diagrams for individual antibody responses to each antigen are shown in Figure 1.

Table 3

| Antigens | Cut-off value | Healthy controls | TB patients | Highest likelihood point | AUC± (95% confidence interval) |
|----------|--------------|------------------|-------------|--------------------------|-------------------------------|
|          | % Specificity | % Sensitivity    |             |                          |                               |
| CFP-10   | 0.598 | 97.06 (33) | 27.14 (38) | 9.23 | 0.551 (0.465-0.636) |
| APA      | 0.485 | 97.06 (33) | 42.86 (60) | 14.57 | 0.697 (0.616-0.778) |
| ESAT-6   | 0.311 | 97.06 (33) | 44.29 (62) | 15.06 | 0.753 (0.680-0.826) |
| PstS-1   | 0.139 | 94.12 (32) | 58.57 (82) | 9.96 | 0.800 (0.732-0.868) |
| Ag85A    | 0.548 | 97.06 (33) | 31.65 (44) | 10.76 | 0.853 (0.775-0.931) |
| Antigenic cocktail | 0.268 | 97.06 (33) | 87.14 (122) | 29.63 | 0.959 (0.933-0.985) |

*: The OD value defining a threshold for positive and negative ELISA test results. It is estimated by the ROC analysis; ±: The fraction of healthy donors who are correctly identified with a negative test; ±: The fraction of people who have the disease that are correctly identified with a positive test; ±: The highest ratio between the true positive rate/true positive rate = % Sensitivity/ (100% - % Specificity); ±: It is defined as the area under the ROC curve, and represents the probability that a randomly selected patient will have a higher test result than a randomly selected control.

We performed ROC analyses to quantify the overall ability of the ELISAs to discriminate between those individuals with the disease and those without the disease, and to determine the cut-off OD values between negative and positive test results(21). ROC analysis of ELISAs using single antigens revealed that the greatest diagnostic accuracy (AUC = 0.853) was obtained using rAg85A, while the lowest value was obtained for rCFP-10 (AUC = 0.551) (Table 3 and Figure 1). These results suggest that an ELISA assay using rCFP-10 as a capture antigen does not discriminate properly the active TB patients from the healthy subjects vaccinated with BCG. ROC analysis allows computing the sensitivity/specificity pairs.
corresponding to a particular decision threshold (cut-off) of OD values[23]. For this analysis we chose the highest likelihood ratio, which is the maximum point for the ratio of true positive rate/false positive rate (% sensitivity/(100% - % specificity)). At this point the sensitivity of the ELISAs based on the single antigens (CFP-10, ESAT-6, APA, Ag85A and PstS-1) ranged modestly from 27.14% to 58.57%. Overall, the specificities of the tests were high, ranging from 94.12% to 97.06% (Table 3).

3.2 Improved diagnostic sensitivity using an antigenic cocktail ELISA

Pair values of 87.14% for the sensitivity and 97.06% for the specificity were obtained by the combination of the 5 antigens. These values were higher than the results of ELISAs based on the single antigens (Table 3). In addition, the highest AUC value, 0.959, was obtained from the antigenic cocktail ELISA, showing the best diagnostic performance (Table 3 and Figure 1).

4. Discussion

Recently, advances in molecular techniques have accelerated the identification of novel immunodominant M. tuberculosis antigens, which have been used for the diagnosis of TB. Novel antigens of M. tuberculosis have been tested individually or in combination to obtain a higher sensitivity and specificity of the serological diagnostic methods[24-26]. In this work, we improved the diagnostic potential of an antibody-capture ELISA assay by using a cocktail of five M. tuberculosis antigens: CFP-10, ESAT-6, APA, PstS-1 and Ag85A. The antigens used in this study are well-known and have been frequently used in previous studies of serodiagnosis[26-29].

Our results revealed a significantly stronger IgG antibody response to the cocktail of the five antigens and for single antigens, except for CFP-10, in the TB group as compared to the healthy BCG vaccinees. A combination of antigens resulted in higher ELISA S/N ratio and AUC parameters than the antigens alone. The levels of IgG antibodies reacting to the mixture of five antigens were higher...
in comparison with the single antigens in the group of patients with active pulmonary TB, where sensitivity rose to 87%. For a single antigen, PstS-1 and A g85A showed the strongest seroreactivity (S/N ratio) and diagnostic potential (AUC = 0.800 and 0.853 respectively) among the five antigens, while CFP-10 showed the lowest performance (AUC, 0.532). Although native CFP-10 antigen elicits potent T-cell responses[30], rCFP-10 produced in E. coli, as a stand-alone antigen does not seem to be a major target for the humoral immune response in TB patients[31]. In our study, few sera from TB patients reacted to CFP-10 with high OD values.

ESAT-6 protein is, as CFP-10 antigen, a major T-cell antigen found in M. tuberculosis short-term culture filtrates[32]. Renshaw et al. showed that CFP-10 and ESAT-6 form a tight complex[33] which is actively secreted from M. tuberculosis and M. bovis[34,35]. Different studies showed that ESAT-6 is a good marker for progressive TB[28,29], while other reports have proved that ESAT-6 is not related to active disease, but is associated with risk factors for future active disease, suggesting the potential of this antigen as a marker for latent TB[36,37]. Taking into account that the sera evaluated in our approach corresponded to patients with active pulmonary TB, could explain that only a cohort of patients showed a considerable response to ESAT-6 antigen. As in other reports, this antigen demonstrated not to be a good marker for active TB in the tested population.

The APA has been described as a 45/47 kDa antigen complex secreted by M. tuberculosis, M. bovis and M. bovis BCG and it seems to be implicated in host cell attachment, entry, and immune evasion[38]. APA has also been identified as a highly immunodominant antigen, with the presence of mannose and arabinose residues in the N-terminal and the C-terminal domains of the mature protein appearing to play a determinant role in its B cell immunogenicity[39,40]. In our approach, the reactivity of sera from TB patients using this antigen was moderate, whereas sensitivity was 42.86%. Espitia et al. reported that antibodies to native M. tuberculosis APA antigen were found in a large proportion (70%) of sera from pulmonary tuberculosis patients[41], whereas Mori et al. reported that only 36% of sera from TB patients reacted to rAPA also purified from E. coli[42]. The difference in seroreactivity using native and rAPA could be explained by the lack of glycosylation as a result of using E. coli as a host for heterologous expression[43].

PstS-1 antigen, a phosphate-specific ABC transporter, also identified as 38 kDa antigen, is a glycolipoprotein of M. tuberculosis partly present as a membrane associated protein and partially secreted in mycobacterial cultures[44]. PstS-1 is the most frequently studied serological antigen[25,45]. The sensitivity of the assays incorporating PstS-1 has been reported to range from 16% to 94%, depending on the smear status of the patients and the patient population[25,29,46]. In this study, the reactivity of sera from active TB patients to PstS-1 antigen was 58.57%, a little lower than the best reported results[25,47]. Those findings probably are related to different factors: the presence of anti-38 kDa antigen antibodies has been shown to correlate with the extent of pulmonary disease[48]. In addition, several studies on reactivity of human and murine sera to native M. tuberculosis antigens suggest that, in contrast to the murine antibodies, human antibodies produced during natural disease progression recognize glycosylated conformational epitopes on the native proteins[49,50]. As previously described, PstS-1 is a glycolipoprotein and the purification of this protein in a non-glycosylated form using E. coli as host could influence the antibody recognition and the sensitivity of this serodiagnostic assay[51].

Ag85 complex, a family of proteins (Ag85A, Ag85B, Ag85C), forms a major fraction of secreted proteins in the M. tuberculosis culture filtrate[52]. The three main members of the complex possess mycolyl-transferase enzymatic activity, required for biosynthesis of cord factor[53], and they share high sequence homology at the nucleotide and protein level both with each other and with Ag85 components from other mycobacterial species[54]. Antibodies to these proteins appear primarily in patients with extensive disease[14,55]. A g85A is a well-documented B-cell and T-cell immunogen and a promising vaccine candidate[53,56]. In the present study, Ag85A showed the second most prominent seroreactivity and the highest diagnostic performance of the ELISA method (AUC = 0.853) in relation to the other four single antigens. This work showed the potential of this antigen for the serodiagnosis of TB in a Cuban population. Further, we showed that the sensitivity (87.14%), specificity (97.06%) and diagnostic performance (AUC = 0.959) of ELISAs based on a cocktail of ESAT-6, CFP-10, APA, PstS-1 and Ag85 antigens are superior to ELISAs performed with single antigens. A additional analysis of the ELISA results showed that 53.57% of TB patients with negative test results to all five antigens in single antigen ELISA tests (15 out of 28), had positive test results with the antigenic cocktail, demonstrating the effectiveness of method that uses a combination of antigens.

In general, serodiagnostic tests for tuberculosis have never been very successful due to suboptimal sensitivity and specificity. To discourage the rampant use of TB commercial serodiagnostic tests, WHO has issued a policy note discouraging the use of current commercial serodiagnostic tests[57]. Heterogeneity in antigen recognition by serum antibodies during TB could explain the failure of specific antibody responses in a cohort of TB patients when single M. tuberculosis antigens, were used. This heterogeneity can be attributed to several factors. First, the immunogenetic background of the host has to be taken into account as some investigators have described an association between antibody titers against specific epitopes of M. tuberculosis and certain HLA alleles[58]. Second, the production of different antigens secreted by M. tuberculosis varies at different stages of disease[59,60]. A third determinant in heterogeneity of response resides in differential gene expression by different strains of M. tuberculosis as they cause disease in different patients[61]. Finally, the bacterial burden in the sputum may correlate with quantity of mycobacteria in the sites of infections, and obviously with the production of antigens[46]. Smear-positive TB patients usually show a more prominent antibody response than smear-negative TB patients[62,63]. However, as only sputum-positive patients transmit the infection, their diagnosis can have the strongest impact on the control of this disease[64].

In this work, we concluded that the use of a cocktail of five well-known antigens of M. tuberculosis: ESAT-6, CFP-10, APA, PstS-1 and Ag85A, yielded higher sensitivity, specificity and accuracy in comparison with individual antigens. These results indicate that a mixture of these antigens increases the diagnostic value of this serological test for TB. When used in combination with other methods, this ELISA assay would further enhance the specificity and sensitivity of TB diagnosis at a low cost. More important, it could be an early diagnostic tool targeting multibacillary patients and at risk populations to rapidly control the spread of disease. This work constitutes the first report of a serodiagnostic method, based on an ELISA test using recombinant M. tuberculosis antigens, for diagnosis of TB in a Cuban population.

Conflict of interest statement

We declare that we have no conflict of interest.
Acknowledgments

We express our appreciation to the staff of National Hospital of Pneumology “Benéfico Jurídico” for their help in this study. This work was supported by Research Project VLIR grant-ZEIN-2008PR346 of the “Vlaamse Interuniversitaire Raad” (University Development Cooperation) in collaboration with the KU Leuven, Rega Institute and the Scientific Institute of Public Health in Brussels, Belgium.

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