Revisiting the tuberculosis and leprosy cross-immunity hypothesis: expanding the dialogue between immunology and paleopathology.

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ABSTRACT

Objective: Our primary objective is to re-visit the tuberculosis and leprosy cross-immunity hypothesis through the careful integration of immunology and paleopathology. **Methods**: Using an integrated theoretical analysis that evaluates clinical literature on human innate immunological responses, paleomicrobiology, bioarchaeology, and paleopathology, we develop a multifactorial model. **Results:** Past populations do not represent homogeneous immunological landscapes, and therefore it is likely that leprosy in Medieval Europe did not uniformly decline due to cross-immunity. **Conclusions**: We recommend that bioarchaeological reconstructions of past disease experience take into consideration models that include variation in immune function based on past environments and social contexts. This provides a unique opportunity to conduct comprehensive analyses on complex immunological processes. **Significance:** Extrapolating results from experimental immunology to larger populations elucidates complexities of disease cross immunity and highlights the importance of synthesizing archaeological, social, paleopathological and biological data as a means of understanding disease in the past. **Limitations:** All extrapolations from data produced from *in vitro* studies to past populations, using living donors, pose significant limitations where, among other factors, the full reconstruction of past environmental and social contexts can frequently be sparse or incomplete. **Suggestions for Future Research:** To reduce the limitations of integrating experimental immunology with bioarchaeological reconstructions (i.e. how to use skeletal samples to reconstruct inflammatory phenotypes), we propose that osteoimmunology, or the study of the interplay between immune cells and bone cells, should be considered a vital discipline and perhaps the foundation for the expansion of paleoimmunology.

1. INTRODUCTION

Leprosy and tuberculosis (TB) are close related infectious diseases that can be associated with similar social, ecological, and biological factors such as poverty, poor access to health care, malnourishment, urban living, and compromised immune systems. Leprosy, also known as Hansen disease, is a chronic infectious disease that, depending on the host immunological response and if left untreated, can slowly progress and cause debilitating impairments and disabilities associated with peripheral nerve damage and cutaneous lesions. Of all mycobacterial species, *Mycobacterium leprae* has humans as its natural host, although leprosy-like disease has been described in non-human primates and armadillos (introduced via humans in the second) (Monot et al 2005; Truman 2005; Walsh et al.1988), occasionally in other animals, and most recently in red squirrels in the UK (Avanzi et al. 2016). Leprosy, in regard to host immune competence, can show a broad spectrum of clinical signs and symptoms, where at one end patients with tuberculoid leprosy (TL) express an extensive cell mediated immune response, and at the other patients with lepromatous leprosy (LL) have a cell mediated response or innate response significantly reduced (Kapplan 1994). However, it is recognized that a continuous spectrum exists between the polar states (Ridley and Jopling 1966).The exact mechanism of transmission of leprosy is still not clear but household and other prolonged close contact could be involved in the spread of leprosy where the nose and the skin are the most common microbe entrance points (Hussain 2007; WHO 2016). The TB pathogens, or organisms within the *Mycobacterium tuberculosis* complex, will infect the host through the lungs or the gastrointestinal tract, and the symptoms and progression of the disease vary according to where the infection first expresses itself (WHO 2017b). Pulmonary infection commonly causes shortness of breath, chest pain, cough, loss of blood and weight. Gastrointestinal infection also includes loss of blood and weight as well as abdominal pain

(Holgate and Frew, 2002). TB and leprosy have been known to be human pathogens for millennia (Spigelman and Donoghue 2003; Larsen 2015), where some researchers propose that TB is a younger disease, having evolved in the Neolithic (Bos et al. 2014; Kay et al. 2015), whereas others believe it could be older (Comas et al. 2013). Skin testing with tuberculin (extract of the tubercle bacillus) shows that up to one third of the world's population has been infected with *M. tuberculosis* and recently, due to immuno-compromised individuals and multi-resistant strains, TB has re-emerged in some regions (WHO 2017b). Leprosy is still endemic in some regions such as Southeast Asia and South America but it has shown a significant decline due to a combination of factors such as improved standards of living, relatively efficacy of multi-drug therapies, erosion of social stigma, albeit slow, and to a certain extent the protective capacity of the TB vaccine bacillus Calmette-Guérin-BCG (Stanford and Stanford 2002). However, social stigma still persists and can affect whether a person accesses diagnosis and receives care and treatment, apart from the fact that a diagnosis affect their lives in considerable ways (Kaehler et al. 2015). In terms of vaccine protection, the efficacy of the BCG vaccine can be variable. For example, a meta-analysis of the role of the BCG vaccine in the prevention of leprosy used seven experimental and 19 observational studies; where the latter showed an overall protective effect of 26%, and the former 61% (Setia et al. 2006). Recent findings challenged the idea that only acquired immune responses can develop immunological memory, proposing that innate responses can also develop immunological memory, especially non-specific cross protection against re-infection (Netea et al. 2016; Quintin et al. 2014). Furthermore, an emerging discipline such as ecological immunology is teaching us how multiple ecological and social factors can affect innate or acquired immune responses (McDade 2003; McDade 2005a).

Therefore, we consider that re-visiting the TB and leprosy cross-immunity hypothesis is now necessary, and particularly exploring how an emerging discipline such as ecological immunology and new findings on innate immune memory can expand our understanding on TB-leprosy cross immunity. Ultimately, we propose a multifactorial model that addresses how ecological immunology can help to contribute more comprehensive paleopathological reconstructions. In addition, this will help to build a model where (depending on different biological and ecological factors) individuals who are able to develop protective cross immunity could be recognized.

1.1 Cellular immune responses to leprosy and tuberculosis.

The immune system reacts to infection via two mechanisms: the cellular response, which is commonly associated with the innate immune response and involves white blood cells where some cells (called phagocytes) recognize, engulf, and destroy pathogens; and the humoral response, which is commonly associated with the acquired immune response, and involves the secretion of antibodies. These two responses work in concert and effectively protect us from infection through a complex multilayered network of cooperation that blurs the distinction between innate and acquired responses (Danilova 2008). While both mechanisms are involved against TB and leprosy (Britton and Lockwood 2004; North and Jung 2004; Sansonetti and Lagrange 1981), in this article, we focus our attention on the cellular response when reconsidering the cross immunity hypothesis. The innate response plays a crucial role in leprosy infection and, depending on its strength and efficacy, leprosy will progress into different clinical stages (Cooper et al. 2011; Modlin 2010; Montoya and Modlin 2010). In the final discussion we propose to incorporate new information on innate immune memory and the influence of environmental factors that could change our understanding of leprosy-TB cross immunity, and contribute to debates about the relationship between these two infectious diseases in the past.

As mentioned above, leprosy can produce a broad spectrum of clinical symptoms: patients with TL express an extensive cell mediated immune response, and in patients with LL, the cell mediated response or innate response is significantly reduced (Kaplan 1994). Higher expression of Toll-like receptor 2/1 heterodimer have been detected in TL, and both ends of the leprosy spectrum (TL and LL) differentially stimulate the production of pro-inflammatory cytokines such as tumor necrosis factor-TNFα (Modlin 1994). However, a significantly higher expression of interleukin-2 (IL-2) and interferon gamma (IFNγ) associated with a Th1 inflammatory response was detected in tuberculoid lesions commonly present in TL. This elevated expression of pro-inflammatory cytokines could be associated with an extensive cellular response and resistance to growth of *M. leprae* in TL. We must consider that the differential cell mediated and cytokine responses between TL and LL indicate that leprosy is not a static disease but a dynamic disease in which immune shifts alter the disease progression and clinical symptoms (Modlin 1994). While, overall, the human immune response to leprosy is mostly based on cellular immunity and not humoral (acquired) immunity (Hunter and Thomas 1984), in some patients (LL) a polyclonal lymphocyte B response has also been detected, leading to an acquired or humoral reaction (Nath et al. 2015).

TB commonly infects the host through the lungs or the gastrointestinal tract, and the innate response is crucial in containing the infection, where (as in leprosy) IFNγ and TNFα are two of the most important cytokines involved in the inflammatory response (Cavalcanti et al. 2012; van Crevel et al. 2002). In individuals with latent TB, TNFα plays a crucial role and acts on a variety of different immune cells (Cavalcanti et al. 2012; Lin and Flynn 2010). Interestingly, increased levels of TNFα have been detected when *in vitro* cultures of peripheral blood mononuclear cells (PBMCs) from patients with pulmonary TB were exposed to mycobacterial antigens (Al-Attiyah et al. 2012; Dlugovitzky et al. 2000). Both cytokines, IFNγ and TNFα, act in the early stages of infection to conduct the innate response and at the later stage to sustain and regulate it (Cooper et al. 2011). Interestingly, in a recent study, it was shown that different mycobacterial strains will affect the interferon response (Wiens and Ernst 2016). Ultimately, a constant battle will unfold between the host and the pathogen that will recruit innate and acquired immune mechanisms. It has been suggested that the exacerbated inflammatory response (innate response) present in psoriasis could have also reduced the clinical progression of leprosy, leading to the argument that the "psoriasis genotype" expanded in different human populations under the selection pressure of historical leprosy epidemics (Bassukas et al. 2012). Moreover, it has been proposed that latent infections can create a polarized cytokine environment and develop a prolonged state of cross protection when the host faces different pathogens (Barton et al. 2007). Relevant for our analysis, in cases of latent or dormant TB, most patients mount a strong immune response, and contain but do not eliminate the infection (Ferraz et al. 2006; Flynn and Chan 2001).

1.2 Why propose cross-immunity between leprosy and tuberculosis?

The family *Mycobacteriaceae* contains more than 200 species, where some species are pathogenic to humans and other animals (Stone et al. 2009). It is known that different species of the genus mycobacteria share many antigens, and therefore it can be expected that there would be a complex pattern of immunological interaction between concomitant infections (or casual exposure to) different mycobacterial species (Fine 1984). While the similarities and potential antagonism of TB and leprosy infections have previously been identified (Rogers 1924), Roland Chaussinand is mostly credited with proposing the hypothesis on cross-immunity between TB and leprosy. He proposed a theory of disease antagonism, and suggested that this interaction could partially explain the decline of leprosy in areas where TB had become prevalent (Chaussinand 1953; Chaussinand

1955). Lowe and McNulty also highlighted that for more than 20 years different researchers had discussed the idea that individuals that are immune to TB may show some degree of immunity to leprosy (Lowe and McNulty 1953). These authors pointed out, as Chaussinand did, that the advent of the use of the BCG vaccination (derived from *Mycobacterium bovis*) as a prophylactic measure against TB showed that immunity to leprosy developed in some individuals (Chaussinand 1955; Lowe and McNulty 1953). Perhaps the most conclusive scientific evidence was based on people who were not exposed to the leprosy pathogen but were infected with *M. tuberculosis*, or received the BCG vaccine and showed a later positive reaction to the lepromin test (based on the injection of inactivated *M. leprae* pathogen) (Fernandez 1939; Fernandez 1957). Simply put, the cross reactions could be explained by early immunological exposure to one pathogen that facilitated the immune recognition of a secondary infection produced by a pathogen antigenically related to the first one.

During the 20th century, while still recognizing the potential role of cross-immunity, other factors were considered as playing a role in TB and leprosy interaction. It was proposed, from a social and sanitary approach, that as communications improve in human societies but sanitation lags behind, leprosy can affect the population, and as TB penetrates the community, leprosy tends to decline (Muir 1957). Interestingly, because in some areas where humans showed some type of cross-sensitivity but where leprosy was not present, it was suggested that the presence of non-pathogenic mycobacteria could be also involved, inducing some form of priming of the immune system (Muir 1957). In the case of the lepromin test, a curious observation is that this reaction gives positive results mainly for the resistant form of leprosy (TL) but it also shows positivity in individuals that have had no contact with leprosy, and perhaps may be regarded as an allergic non-specific reaction (Fernandez 1939; Lowe and McNulty 1953; Muir 1957). Moreover, as discussed above, some differences in responses to tuberculin or lepromin tests were due to exposure to mycobacteria other than *M. tuberculosis* or *M. leprae* as well as other co-infections (i.e. *Candida albicans*), and also due to the clinical status (healthy vs. non-healthy) of the individuals considered in those studies (Sartwell 1968). Many clinical trials during the last decades have shown a positive correlation between BCG vaccination and later protection against leprosy (Kinnear Brown and Sutherland 1968; Ohara et al. 2000; Rahete et al. 2007; Roche et al. 2001; Rodrigues et al. 2007; Shepard 1966; Zodpey et al. 2005), and in the last decades, two meta-analyses provided more support for the correlation (Merle et al. 2010; Setia et al. 2006).

Not all studies have shown BCG protection against leprosy, but we should consider that different factors such as *in vivo* models (mouse or human), the human population chosen for study, BCG dose quantity, and the timing and age of the vaccination can affect the outcome of this interaction, thus revealing the complex and heterogeneous interaction between pathogen and host. It was suggested that the lack of experimental and clinical conclusiveness regarding the protective effect of BCG vaccination against leprosy was due in part to the slow-progressing nature of leprosy, its long silent incubation period, and relatively low infection rate (Hunter and Thomas 1984). In addition to the use of the BCG vaccination as potential proof of cross-immunity between TB and leprosy, antigens ESAT-6 and CFP-10 expressed in most pathogenic strains from the *M. tuberculosis* complex have homologous proteins in *M. leprae* that show significant cross reactivity in human immune cells (Geluk et al. 2002; Geluk et al. 2004). Recently, new candidate vaccines for TB, ID83/GLA-SE and ID93/GLA-SE, generated inflammatory responses in both TB and leprosy (TL) patients, suggesting that due to this cross-reactivity these vaccines could be also used as an additional control for leprosy (Duthie et al. 2014).

For example, in India in 2011, it was reported as 0.02% of all concomitant infections (TB and leprosy) per 100,000 population (Rawson et al. 2014), and it was suggested that one potential explanation for this apparent decline in concomitant infection that should not be ruled out could be the protective effects of cross immunity (Rawson et al. 2014). Furthermore, in contemporary Brazil, one possible factor for the recent decline in co-infected people, as well as the decline in the detection rate of leprosy, is BCG vaccination (Trindade et al. 2013).

1.3 Leprosy decline in Western Medieval Europe and the case for disease interaction.

When formalizing the cross immunity hypothesis between TB and leprosy, Roland Chaussinand in his seminal paper called not only for more experimental work but also finding evidence in human populations were we can study the time of exposure, in the lifetime of an individual or population, of both diseases (Chaussinand 1953). In Western Europe, while TB has remained at epidemic proportions, leprosy is rarely found today (Hussain 2007). The reason for the significant decline of leprosy as an endemic infectious disease in Western Europe after the 13th century is still unclear. Some bioarchaeologists have tried to reconstruct the interaction of both infectious diseases in antiquity (Manchester 1984; Manchester and Roberts 1989; Roberts 2002). While considering the limitations of using the evidence from skeletal remains to achieve this task, but also the presence of ecological and social factors that could have been responsible for the decline of leprosy in Medieval period in Europe (12th-16th centuries), it was proposed that it could be possible to extrapolate contemporary clinical findings to Medieval populations and argue that cross-immunity did also play a role in the leprosy decline associated with a rise in TB in Medieval period in Europe (Manchester 1984). Social factors will also have played a role, such as disparities in the presence of leprosy in the Medieval period, inferred from the rise and decline of leprosy hospitals (Blondiaux et al. 2015), but it could also reflect changes in beneficence of the wealthy (Manchester and Roberts 1989).

It is likely, when considering the continental decline of a long lasting chronic infection such as leprosy, that more than one factor should be considered, and perhaps multiple factors played a significant role at different times and in different regions. Hunter and Thomas (1984) summarized most factors that should be considered: *1.* Antigenic shift resulting in loss of pathogenicity; *2.* Attenuating effects of isolation and quarantine; *3.* Direct or indirect mortality caused by acute infections such as Medieval plague epidemics; *4.* Dietary changes; *5.* Clothing habits; *6.* Changes in housing and sanitation; *7.* Interaction with other chronic infections such as TB (Hunter and Thomas 1984).

When modeling transmission in TB and leprosy, Lietman and colleagues (1997) tried to assess the degree of cross-immunity that would be necessary for *M. tuberculosis* to compete and eliminate *M. leprae*, revealing that (while centuries will be required) if the reproductive rate of leprosy was relatively slow, TB could have played a significant role in the disappearance of leprosy from western Europe (Lietman et al. 1997). Interestingly, while still controversial due to some doubts about using only the IS6110 DNA marker to detect ancient *M. tuberculosis* (Muller et al 2016), a study conducted to evaluate the existence and relationship of both pathogens in archaeological human remains showed that several individuals with skeletal signs of leprosy were found to contain DNA from both pathogens (Donoghue et al. 2005). The authors of this study concluded that the impairment in cell mediated immune response found in LL (commonly identified in archaeological remains), coupled with social factors, would lead to a re-activation of latent TB and perhaps induce a speedier death and decline in the number of individuals with leprosy (Donoghue et al. 2005). Recently, another study using a mathematical approach for modeling the

epidemiological consequences of the co-infection hypothesis supported by Donoghue and colleagues, concluded that the co-infection hypothesis should be considered a significant alternative to the cross-immunity hypothesis proposed by Chaussinand (Hohmann and Voss-Bohme 2013). Other researchers have studied the incidence of both infections and searched for evidence of cross-immunity in populations from Texas, USA, dating from the last two centuries. They found few significant negative correlations in the data (TB increase and leprosy decrease); on the contrary, some data showed an inverse correlation, or in some cases both diseases were declining at the same time (Wilbur et al. 2002). However, debates on the interaction of these two diseases in Medieval Europe is far from closed, as demonstrated in a recent symposium on past, current, and future research on leprosy, organized during an Annual Meeting of the American Association of Physical Anthropologists (2014), where three different presentations explored and re-analyzed the TB-leprosy cross immunity hypothesis with partial antagonizing conclusions (cross immunity did or did not play a role in leprosy decline in Medieval Europe) (Donoghue et al. 2014; Roberts 2014; Wilson et al. 2014).

Whilst not ruling out any hypothesis for the significant decline of leprosy at the end of the Medieval period in Europe, in this paper we will focus our attention on the cross-immunity TB-leprosy hypothesis. In so doing, we will propose a novel multifactorial model that includes social and ecological elements that address most of the factors proposed by Hunter and Thomas (1984). This ultimately affects the reconstruction of immune competence of each individual when considering novel findings in innate immune memory and plasticity.

1.4 Innate immune memory: trained cell mediated immunity?

Immune responses in humans are usually divided into innate responses and acquired or specific responses. While it has been recognized that these two responses work in concert and effectively protect us from infection through a complex multilayered network of cooperation (Danilova 2008), both responses are commonly associated with different cell components and mechanisms. The innate response usually presents a quick cellular and phagocytic response, and the acquired response shows a more delayed cellular and humoral mechanism that can present a specific immunological memory to future infections. However, lately, the statement that the innate cellular mediated responses lack memory has been reconsidered (Quintin et al. 2014).

In recent years, it has been proposed that the term “trained immunity” should be used to describe enhanced innate host defense mechanisms in those organisms that lack acquired immune responses, but mounting evidence is showing that similar innate memory can be found in mammals (Netea 2013; Netea et al. 2011). It is important to point out that this “trained immunity” against reinfection can be applied to the same or different pathogen.

Interestingly, it is well recognized that infection by *M. tuberculosis* can impact subsequent infections such as HIV and malaria (Berry et al. 2010; Havlir and Barnes 1999; Whalen et al. 1995). Therefore, depending on different cofounding factors, the impact of TB infection can benefit or harm the host (Stelekati and Wherry 2012). During TB infection an elevated expression of pro-inflammatory cytokine IFNγ may protect a person from subsequent *Plasmodium* infection (Page et al. 2005). Additionally, commensal bacteria can modulate host innate immune responses to unrelated pathogens and help in mounting optimal antiviral immune responses (Abt et al. 2012). Detecting microbial patterns by different immune cells involved in the innate responses not only leads to cell activation but also can lead to reshaping their response to a subsequent microbial

insult (Quintin et al. 2014). For example, the non-specific BCG protective effect can also be attributable to activated macrophages (Van't Wout et al. 1992).

While both immune responses, innate and acquired, are involved in TB and leprosy (Flynn and Chan 2001; Modlin 1994; Nath et al. 2015), perhaps we can incorporate the "trained immunity" of the innate responses as another factor when exploring the potential cross-protection between TB and leprosy. Ultimately, such a proposal calls for more exploration of how exposure to one pathogen can affect or shift the inflammatory responses (i.e.: pro-inflammatory cytokine expression) when the same cells are subsequently exposed to another pathogen, meaning: can we "train" immune cells by early exposure to *M. tuberculosis*, and later modify the cell response to *M. leprae*?. Clearly, the results of such *in vitro* analysis should be carefully applied when extrapolated to real humans with complex life histories. However, we consider that such extrapolation and discussion can benefit disciplines such as bioarchaeology and its subdiscipline, paleopathology, especially when proposing a multifactorial model to explore TB-leprosy cross immunity. Our ultimate goal is to explore a model where early and chronic exposure to one (or more) mycobacterial species can produce an inflammatory shift that either can generate protection to later leprosy infection, or push leprosy infection towards the TL end of its spectrum where a heightened cellular immunity is required (Figure 1). However, the final outcome of "training" of the innate immune responses to generate cross immunity will also be influenced by ecological and social factors.

2. BUILDING A MULTIFACTORIAL MODEL

The proposed model considers and discusses the existence of heterogeneous biosocial immunological landscapes, and takes special consideration of the following factors: the immunological spectrum of the innate response to TB; the role of other mycobacterial species; and the complexity of the paleopathological record as potential evidence.

2.1 Consideration of the broad immunological spectrum of tuberculosis and the potential shift of the innate response against leprosy

In TB, the type of immune response produced against *M. tuberculosis* will significantly influence the course of the disease, where the majority of infected immunocompetent individuals will remain healthy and asymptomatic. Usually these individuals mount a strong immune response but the bacteria persist in the host (Ferraz et al. 2006). During TB latent infection, the immune system controls the pathogen by means of "balanced inflammation" and generally causes minimal collateral damage, but in some patients TB can be characterized by non-resolved inflammation during latency and active phases (Kaufmann and Dorhoi 2013). Interestingly, immune function at the site of the infection may evolve differently than at the systemic level (Wallis and Ellner 1994). Ultimately, a "delicate" local and systemic cytokine equilibrium will be generated when controlling the progress and growth of *M. tuberculosis*. In murine models, it was shown that two antagonistic "protective" mechanisms are involved: first, the initial Th1 "protective" inflammatory response against the pathogen (where cytokines such as IFNγ and TNFα are involved); and second, a Th2 "protective" anti-inflammatory response to minimize tissue damage at the site of the infection (Wallis and Ellner 1994).

Epidemiologically, *M. tuberculosis* is commonly characterized by long periods of persistence where the bacteria have developed the capacity to live in balance with the immune response. However, such a balance (especially on the host side), including that of Th1/Th2, can be impaired or compromised by different factors involving not only biological but ecological and behavioral

causes (Huynh et al. 2011). As proposed by one of the authors of this study (FC) we should consider how latent TB can systemically affect immune responses to other pathogens (Crespo et al. 2017). Ultimately, we must consider how heightened systemic inflammatory responses (i.e. increased expression of TNFα) can affect subsequent infections (such as leprosy) not only due to acquired immune memory but because of over-reactive innate responses. Clearly, the potential spectrum of immunological responses observed in TB infection should be considered when analyzing its impact on a subsequent leprosy infection in a person.

As mentioned above, in leprosy the elevated expression of pro-inflammatory cytokines, such as TNFα and IFNγ, is linked to extensive cellular response and resistance to growth of *M. leprae* leading to TL (Modlin 1994; Modlin 2010). While it was observed that TB infection can occur in people across the entire leprosy spectrum (Kumar et al. 1982), it can be suggested that TL through an enhanced innate or cellular response, could offer the best immunological context for cross immunity. However, it has been argued that LL is far from representing a generalized immune deficient system; on the contrary, some individuals could develop a hyper-immune state (Ell 1987).

Therefore, an enhanced inflammatory response due to active or latent TB, could have an impact on leprosy, but we should be careful when considering a particular stage of the clinical spectrum in leprosy. As expanded below (2.3), we must consider all leprosy stages, and if TB infection is active or latent (Figure 2).

2.2 A consideration that more than one mycobacterial species can train the innate immune system.

Usually, an infection generates a priming ("activation") of innate immunity that normally declines after the infection is resolved. However, if we consider a chronic infection such as TB, there is an *in vitro* shift in the expression of different inflammatory proteins (Crespo et al. 2017). More than temporarily primed, the system can end in a steady-state level that remains enhanced and ultimately reprograms the innate response (Netea 2013).

Exposure to *M. tuberculosis* and related environmental mycobacterial species (EM), could also generate a quasi-permanent shift of systemic immune responses and "train" the innate response. EM are not obligate pathogens but exhibit great variation in growth rates and virulence (Primm et al. 2004). The majority of human-EM interactions are transient, where the immune responses in most members of the population clear the bacteria from the body but this involves the release of potent immunomodulators (Primm et al. 2004). Interestingly, it has recently been proposed that chronic exposure to EM can result in systemic tolerance toward these EM; moreover, it has been shown that the variable protective efficacy of the BCG vaccine is partially due to exposure to EM (Price et al. 2016). Perhaps, one of the most compelling pieces of evidence for "trained immunity" is developed by immunization of mice with the BCG vaccine where immunization also induces a T-cell independent protection against secondary infections with *C. albicans* or *Schistosoma mansoni (Netea et al. 2016).* Both, human immune cells infected or exposed to *M. bovis* can stimulate inflammatory cytokine expression (Atkinson et al. 2000). Recent findings from clinical investigation involving BCG vaccination and inflammatory responses suggest that BCG vaccination of leprosy patients (especially those with LL) induces immune cell activation, likely through "trained immunity", that works as an additional protective mechanism (de Carvalho et al. 2017; Kleinnijenhuis et al. 2014).

M. bovis infection in cattle and other animals represents a public health concern (O'Reilly and Daborn 1995), especially in populations where raw unpasteurized milk is still consumed. In past populations, *M. bovis* was probably a major source of TB infection in humans through the consumption of untreated dairy and other products, leading primarily to extra pulmonary or intestinal lesions (Manchester 1991; Smith et al. 2004), and increased cattle trade (and other animals) could have contributed to the spread of natural vaccination against leprosy (Boldsen and Mollerup 2006; Dangvard Pedersen et al 2018). While *M. bovis* is very rare in the paleomicrobiological record, this species was found in a group of Iron Age Siberian pastoralists associated with skeletal lesions typical for TB (Taylor et al. 2009). It was suggested that an unequal distribution of *M. tuberculosis* and *M. bovis* should be considered when exploring different communities, where crowded urban centers favored *M. tuberculosis*, and small agrarian communities favored *M. bovis* (Mays et al. 2001).

Mycobacterium smegmatis is a saprophytic usually non-pathogenic EM (Tyagi and Sharma 2002) that can induce cytokine expression, mostly by macrophages, presumably being one the mechanisms by which these species are eliminated from the host (Beltan et al. 2000). Perhaps, chronic exposure to this species can also contribute to generate an enhanced quasi-permanent hyper-inflammatory phenotype. Recently it has been shown that some of its proteins can modify the mammalian host immune response (Sweeney et al. 2011) and in different animal models has been illustrated to induce immunomodulation as well as generate opportunistic infections (Bercovier and Vincent 2001). Remarkably, this mycobacterial species has been isolated from diseased animals, but more frequently from cattle (Bercovier and Vincent 2001).

A recent study of ancient DNA of the *M. tuberculosis* complex, showed the complications ("interference") generated by the presence of EM in skeletal remains that colonized the individual either pre- or post mortem (Müller et al. 2016). This line of evidence suggests that, as shown by different authors, we should take into consideration that previous exposure to EM might have played a natural vaccination role or a confounding role in the development of cross protection (Fine 1995; Lietman et al. 1997; Wilbur et al. 2002). Therefore, it cannot be ruled out the potential role of other mycobacterial species as contributory factors in "training" or at least priming the innate responses. Moreover, in the last decades, the risk of human *M. bovis* infection is increasing in populations with a high prevalence of HIV infection (Mfinanga et al. 2004; O'Reilly and Daborn 1995; Thoen and LoBue 2007).

2.3 Limitations of paleomicrobiological and bioarchaeological studies.

When considering the impact of TB and leprosy on past populations, paleomicrobiological and bioarchaeological studies are showing that past populations faced biological and ecological heterogeneous landscapes. This makes it difficult to accept that a uniform (constantly present) cross protection or immunity was present between TB and leprosy.

As mentioned above, more than one mycobacterial species can cause TB in humans where *M. tuberculosis* and *M. bovis* are the most common cause of illness (Stone et al. 2009); and within the same species we can also have genetic pools leading to different bacterial strains. It is crucial to know the pathogen strain affecting people, especially for TB, because the immunological response induced by *M. tuberculosis* is bacterial strain-dependent (Wiens and Ernst 2016). Recently, two separate paleomicrobiological studies (from England and Hungary), show that different TB strains were present at varying periods of time (Kay et al. 2015; Muller et al. 2014; Roberts 2016). Clearly, as cited by other authors, changes in pathogen biology as well as in host immune

competence may modify TB morbidity and virulence (Sparacello et al. 2016) . As opposed to TB pathogens, different studies on ancient *M. leprae* genomes have shown that the DNA of the leprosy pathogen did not change significantly in Medieval Europe and therefore pathogen virulence cannot explain leprosy's decline (Donoghue et al. 2015; Mendum et al. 2014; Roffey et al. 2017; Schuenemann et al. 2013; Schuenemann et al. 2018; Taylor et al. 2013). In addition, genetic studies in contemporary populations have shown that the immune genetic variance of the host is a key factor for the progress and outcome of leprosy infection, where candidate genes (alleles) such as human leukocyte antigen (HLA) and Toll-like receptors (TLR) show significant correlations with increased susceptibility to leprosy (Alcais et al, 2005; Mira 2006; Wong et al 2010). Moreover, a recent study on ancient DNA from skeletal samples from a Medieval cemetery in Denmark also demonstrated a significant association between the HLA class II allele DRB1*15:01 and LL (Krause-Kyora et al. 2018). Interestingly, when correlating the immune genetic variance observed, in past and contemporary populations, with the presence of paleopathological evidence for leprosy, a complex scenario unfolds suggesting that we should incorporate the immune competence of each individual (with or without prior exposure to other mycobacterial species) to explain individual differences observed in different studies (Inskip et al. 2015; Mendum et al. 2014).

The skeletal data also show a complex landscape when considering the impact of TB and leprosy on past populations. The first problem is that around 5% of patients with leprosy today exhibit skeletal signs of the disease (Resnick and Niwayama 1995), and in TB, bone changes are described only in 3-5% of individuals with known diagnosis (Resnick and Niwayama 1995; Roberts and Buikstra 2003). Most skeletal markers or lesions (bilateral and symmetrical) are found in LL, and can be minimal or non-existent in TL (Roberts 2011) but in a study of records from leprosy hospital patients, bilateral or unilateral hand and foot bone involvement and no rhinomaxillary syndrome have been identified for a diagnosis of TL (Matos 2009). In the case of TL, where an extensive cell mediated immune response is present, many skeletons may not show any bone changes (Roberts 2011). Clearly, a skeleton without signs of infection could have experienced the infection but died before developing bone lesions; this suggests that skeletal remains with signs of TB or leprosy infection indicate that these people must have had a long lasting chronic infection (Roberts 2015). Here, we must consider different risk factors such as living conditions and diet that may affect the extent of skeletal involvement in both diseases (Dixon and Roberts 2001; Roberts 2015), and where the immune competence of an individual will also influence whether skeletal lesions will occur (Roberts 2015).

We must reassess the situation that most archaeological skeletons are identified in LL. Therefore, a problem emerges here: we should predict a higher incidence of cross immunity for individuals with TL (with extensive cellular immune response) but most of the skeletal evidence is absent for this disease state, or at least (so far) cannot be recognized as such. Consequently, we propose that when studying the cross-immunity hypothesis using skeletal remains, we should carefully explore the immunological differences between TL and LL but, as pointed out by Wilbur and colleagues, more problems arise when most studies do not distinguish between these two disease states (Wilbur et al. 2002). Evidently, a complex and heterogeneous landscape emerges when contemporary epidemiological data show significant variation among populations for LL/TL ratios in the occurrence of leprosy (Hunter and Thomas 1984). Recognizing LL and TL (especially in paleopathological analysis) could be crucial when expecting (or not) cross immunity between TB and leprosy, where the presence of TL or LL can potentially inform us about the immunological status of each individual. For example, it was proposed that TB infection in living populations

could confer some protection to TL but not to LL (Leiker et al. 1968), and it was suggested that in the late Medieval period, leprosy did not disappear but just shifted to a higher frequency of TL. Perhaps also the number of people who experienced TB in the past has been significantly underestimated (probably more than leprosy) because a skeleton with no bone changes of TB (or leprosy) could have experienced the infection but died before expressing any skeletal marker for the disease (Roberts 2015; Wood et al. 1992). Interestingly, it was proposed that impaired cellular immunity could have led to a re-activation of an underlying latent TB infection, or to superinfection with *M. tuberculosis*, and to a speedier death. This would lead to a decline in the number of individuals experiencing leprosy and end with the observed phenomenon of its decline (Donoghue et al. 2005). Molecular evidence also supports the case for the underestimation of TB when using osteological data, where some individuals have tested positive for ancient DNA from *M. tuberculosis* but have no skeletal signs of the infection (Cooper et al. 2016; Donoghue et al. 2005), and a similar case must be considered for leprosy.

We must recognize that skeletal data should ideally be used to reconstruct the immune phenotype of each individual, ultimately helping to understand the clinical progression of each infection and their potential interaction. As mentioned above, in leprosy the immune competence of each individual plays a significant role in the infection's progression and clinical manifestation (more than the direct interaction with TB). It has been proposed that a population with many generations of exposure to TB may produce stronger immune responses, greater survival, and a higher occurrence of bone changes in affected individuals (Roberts and Buikstra 2003). This would have an impact on leprosy progression if such individuals where later exposed to *M. leprae*. Therefore, when exploring the cross-immunity hypothesis using archaeological skeletal markers of these infections, we must consider a model where consideration is made regarding the expected immunological shift generated by early exposure to TB, taking into account different levels of cellular immunity ultimately affecting the progress (or not) of leprosy infection. This model should include the immunological differences (and potential interplay) observed in latent and active TB, and in TL or LL in leprosy (Figure 2), along with the bio-social landscape.

2.4 Heterogeneous biosocial landscapes and differential cross immunity: searching for a multifactorial model.

While biology dictates a great proportion of the immune responses of an individual, we cannot rule out the role of environmental and social factors in such responses, especially when considering long lasting chronic infections, and this could be crucial, for example, when predicting or testing the potential role of TB in leprosy's decline. Populations and individuals, today and in the past, are and were immersed within specific environments and social contexts that generated heterogeneous biological (immunological) landscapes, and we cannot consider that all populations in time and space represent identical immunological units (Crespo and Lawrenz 2014).

Perhaps leprosy could be considered as one of the chronic infectious disease with the heaviest social burden, where susceptibility to and the clinical manifestation of leprosy have roots in social and cultural factors that affect overall health and immunity (White and Franco-Paredes 2015). While skeletal remains from cemeteries associated with hospitals, infirmaries, or poor houses could be strongly biased as a result of selective or differential mortality (Connell et al. 2012), bioarchaeological studies can teach us how heterogeneous social and ecological contexts could ultimately have impacted on the immune competence of different individuals or even whole populations. One of the main lessons from bioarchaeological reconstructions is to show, especially with leprosy, how different people and populations evoked (in time and space) different

experiences according to different variables such as sex, age, social status, and religion (Roberts 2011).

In Medieval Europe leprosy was not an isolated regional phenomenon, and large-scale institutional care in the form of leprosy hospitals or leprosaria were introduced, although the status and organization of such institutions varied in time and space. Moreover, unlike monastic institutions, there was not a regular plan or predefined lay out for leprosy hospitals (Roffey 2012). The organizational heterogeneity probably impacted on the diet, sanitation, care, treatment, and daily life of those who lived in these institutions (Roffey 2012). Not only have institutional differences been observed during the peak of leprosy in Medieval Europe, but regional differences in attitudes towards people with leprosy were also common (Brenner 2010; Dematrie 2007; Rawcliffe 2006; Roberts 2011). Recent bioarchaeological analyses are also showing that, in some regions, segregation of those with leprosy is a more recent behavior than previously thought (Baker and Bolhofner 2014). Interestingly, as observed in past populations in South Asia, even within the same region, complex societal changes can also affect the social perception of an infectious disease, whose related pathogen perhaps, did not change at all (Robbins Schug 2016). Moreover, in India, one of the three areas of the world today with the highest rate of "new cases" of leprosy (WHO 2016), people in leprosy hospitals can represent an element of bias due to a higher risk of exposure to and developing TB (Rawson et al. 2014). Such social and institutional disparities, in time and space must have had a significant impact on disease progress and the overall health of people with leprosy (including immune competence).

It is well recognized that the immune system presents a high degree of plasticity and requires a delicate energetic balance, and marked fluctuations in the immune response occur as a reaction to environmental and social factors during an individual's lifetime (French et al. 2009). Ecological immunology is an emerging discipline that helps us to understand how the immune response is both plastic and dynamic (McDade 2003; McDade 2005a), and we cannot extrapolate experimental data without carefully considering social and ecological factors. The main objective of ecological immunology (or ecoimmunology) is to study and explain natural variation in immune function, especially considering how biotic and abiotic factors contribute to such variation in immunity in free-living organisms (Martin et al. 2011). While the first studies were focused on non-human organisms, the discipline started expanding and including humans, with special attention on how individual life history can affect immune responses (McDade, 2003). Immunity can be costly, and defense mechanisms are regulated within the context of costs, ecological influences and constraints (Brock, 2014). For example, and important for the multifactorial model proposed in this study (Figure 3), early studies explored how chronic social stressors in childhood (i.e. family environment, caretaking attention) are associated with a higher average level of cortisol, which is found correlated with alterations of the immune function and increased frequency of infections (Flinn and England, 1995; Flinn and England, 2003). Status-related stress (also in part through the effects of cortisol), recently shown in forager-horticulturalists populations in Bolivia, can affect inflammatory responses, ultimately playing a role on the progress of different infectious diseases (VonRueden et al, 2014). It has also been proposed that immune function and sickness responses vary seasonally, where in some populations with TB the highest incidence occurs in winter (Nelson, 2004), and where one mechanism could be associated with the lowest bactericidal activity observed in some immune cells (neutrophils) during winter (Klink et al, 2012). Interestingly, the season of birth might be another important factor that could influence susceptibility to TB because it could affect in utero nutrients, fetal/neonatal exposure to infection, or possible neonatal immunological resistance to TB (Miura et al, 1992). Thomas McDade

proposed the "eco-logics" of inflammation and the importance of early environments shaping the development and function of the human immune system, suggesting that comparative studies across different ecological settings are needed (McDade, 2012).

The integration of ecological immunological and human health studies in living populations has developed extensively over the last 20 years, but more consistent integration of ecological immunology into paleopathological research is still pending and should be considered one of the next frontiers in bioarchaeological studies. When studying TB in skeletal samples, Wilbur and colleagues (2008) proposed that immunological, epidemiological, and archaeological data can be integrated to generate predictions for tuberculosis in skeletal samples, where the contributions of dietary proteins and iron can affect immune function and determine the course and outcome of infection (Wilbur et al 2008). We propose that this should also be a factor when determining the individual capacity to develop TB-leprosy cross immunity. Interestingly, Klaus and Tam (2009) studied the bioarchaeology of systemic stress in colonial Peru, proposing a complex model of systemic biological stress in Morrope, Peru, as shaped by postcontact population aggregation, where the interplay of different cultural and environmental stressors (i.e. increased proximity to waste, poor sanitation, contaminated water supplies) can ultimately affect the immune competence of individuals and intensify host-pathogen relationship (Klaus and Tam, 2009). While not formalizing integration of paleopathology with ecological immunology, but applicable to the objective of this manuscript, a recent article studying TB in medieval to early modern Denmark suggested that ecological and social factors explained changing patterns in TB prevalence (Dangavrd Pedersen et al 2018), where we propose that those ecological and social factors should be factored into studies when trying to understand the heterogeneous immune competence of populations in time and space. A recent paleopathological study on leprosy explored the association between childhood non-specific markers of stress and leprosy immunity in Medieval England, finding that immune processes were likely more influenced by maternal and early physiological stress than environmental factors later in life (Filipek-Ogden 2014). This is also relevant to the Developmental Origins Hypothesis of Health and Disease (Barker 1992). If early physiological stress leads to constant frailty, then the general level of functioning of the immune system can have a significant impact on the progress of leprosy in any one person (Boldsen 2005). Therefore, we can argue that early stress or constant frailty could also affect an individual's capacity to develop cross immunity or not.

As we previously discussed (Crespo et al. 2017), experimental protocols do not consider at all the systemic or whole organism immune competence, whereas skeletal analysis deals with pathological markers that reflect not only a clinical snapshot of skeletal health at death but the interaction of different cofounding risk factors for disease over a life time. Cross sectional studies in modern populations can also offer a biological and clinical foundation that could help an often sparse archaeological record when testing the model proposed in this manuscript. As explained earlier, LL patients have been shown to mount a lower cellular immunity and inflammatory response (i.e. lower TNFα response) and this could explain increased TB reactivation or dissemination (Rawson et al 2014), but most studies of modern populations that consider concomitant infection with TB and leprosy are case reports for one or few individuals (Ayra et al 2016; Mangum et al 2018; Sendrasoa et al 2015; Verma et al 2015). However, some modern populations offer a unique opportunity to study disease interaction and concomitant infection, as recently analyzed in Marshallese, Arkansas, US, where high rates of TB and leprosy are present. The authors of this study concluded that while a study on a larger scale is needed, preliminary data are supportive of the existence of cross-immunity (Cardenas et al, 2016).

As recently proposed by one of the authors of this study (FC), understating the heterogeneous immunological landscapes detected in different populations in time and space requires a new dialogue to develop between scientific and social disciplines (Crespo and Lawrenz 2014). A comprehensive bioarchaeological analysis of the interaction of TB and leprosy in past populations can help with such complex reconstructions. Heightened or weakened innate or cellular immune responses influenced by ecological factors can ultimately play a role in the progression (or not) of cross protection. Ecological immunology is reminding us that shaping the immunological phenotype of an individual starts early in life (McDade 2005b; McDade et al. 2010). Therefore, in an ideal world we should also consider (and reconstruct) early ecological and social contexts for the life of each individual studied.

In our final multifactorial model, we consider different scenarios when predicting cross-immunity, or not (Figure 3). For example, during latent infection, a potential balance of both inflammatory and anti-inflammatory factors can be generated. Further, in addition to biological factors, social and ecological factors can also affect this balance to produce the final impact on the immune competence of individuals, and ultimately the development of cross-immunity. When diagnosing leprosy in paleopathology, it has been proposed that there is a need to think about the epidemiological process, and develop a better understanding of the dynamic relationship between pathogens and human populations (Boldsen 2001). Perhaps it is time to suggest that the complex nature of immunological variance among human populations should be considered in interpretations of past health, especially when considering the complex interaction of TB and leprosy. As reiterated recently by Spigelman and Rubini, the origin of TB and leprosy cross immunity is still controversial, but perhaps the most conclusive clinical fact is that, with some degree of variability, leprosy may be prevented following BCG vaccination (Spigelman and Rubini 2016).

3. CONCLUSIONS

When exploring or testing the TB-leprosy cross immunity temporally and geographically, we propose that different inherent factors should be considered, such as novel findings in innate immunity memory and ecological immunology. When studying the individual capacity to develop (or not) cross immunity, biological, ecological, and social factors should not be considered to work in isolation but synergistically. Such a complex and multifactorial approach presented in the current study shows that past populations do not represent homogeneous immunological landscapes, and therefore it is likely that leprosy in Medieval Europe did not show a uniform decline, especially when considering disease interactions, and especially TB. We propose that bioarchaeological reconstructions that take into consideration experimental immunological data to explain underlying cellular and molecular mechanisms present a unique opportunity to develop a more comprehensive analysis of the complex multifactorial process involved in TB-leprosy cross immunity.

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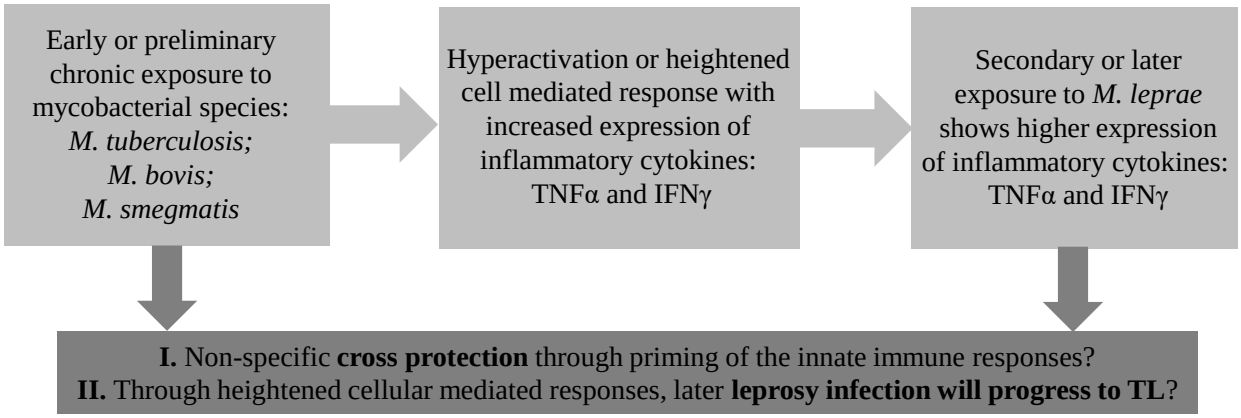

Figure 1: Summarized rationale for linking a chronic exposure to different mycobacterial species (except *M. leprae*) and a systemic inflammatory shift that either can generate protection to later leprosy (*M. leprae*); or predispose leprosy clinical phenotype towards tuberculoid leprosy (TL).

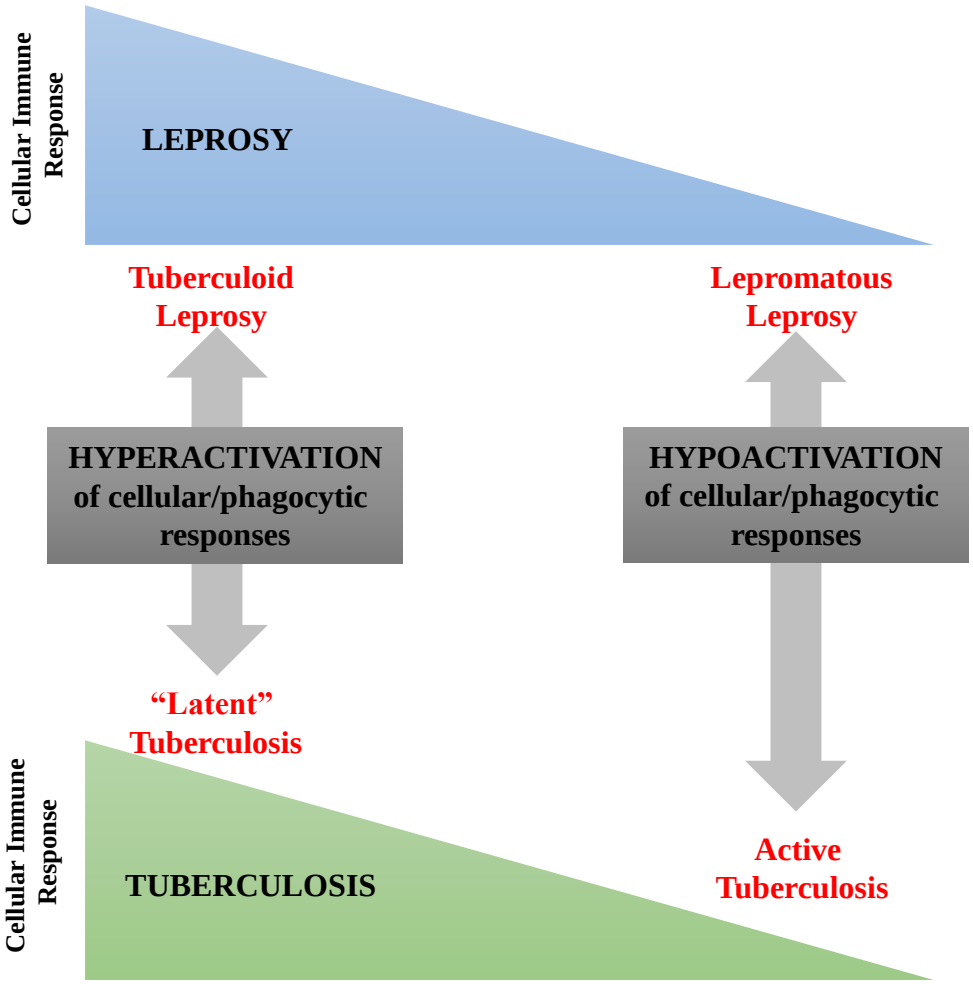

Figure 2: Proposed model to integrate the expected immunological shift generated by an early exposure to tuberculosis, taking into consideration different levels of cellular immunity (hyper- or hypoactivation) and the clinical progress expected for leprosy (TL or LL)

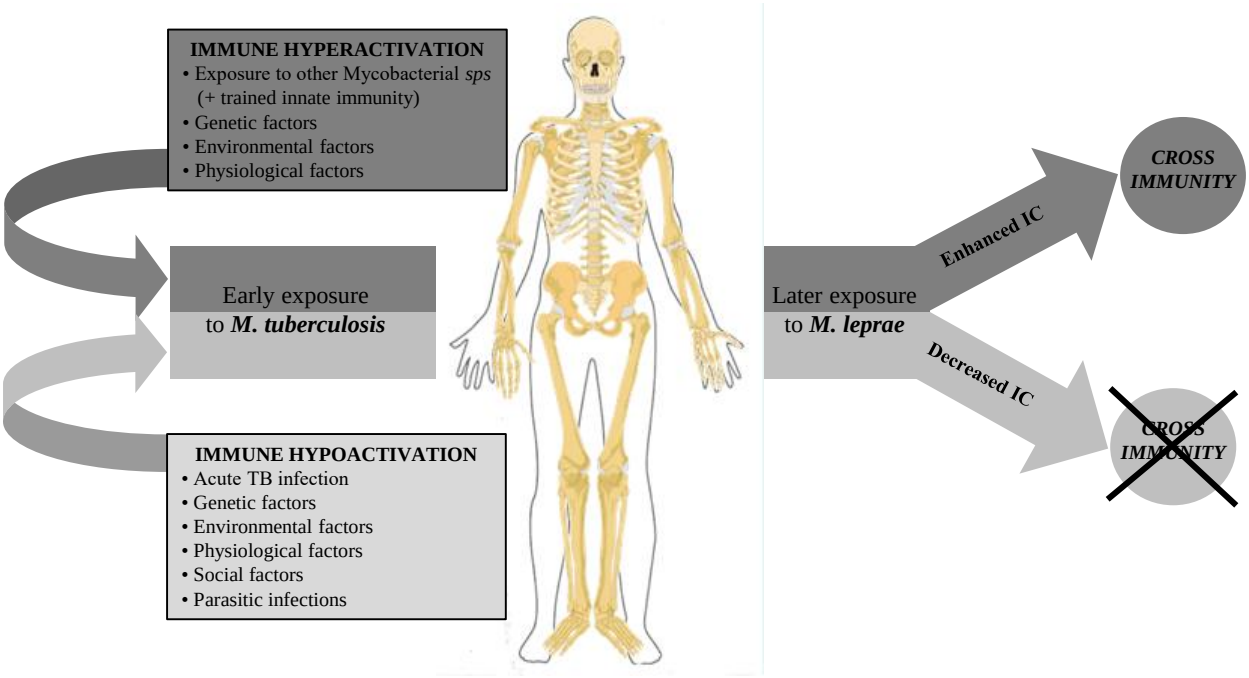

Figure 3: Proposed multifactorial model to predict the progress of cross-immunity between tuberculosis and leprosy when taking into consideration the regulation of immune activation due to different biological, ecological, and social factors.
Different grey colors help to follow the conditions and predict "enhanced or decreased immune competence-IC", and finally inducing (or not) cross immunity.