Universidade do Minho
Escola de Medicina

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Risk factors associated with multidrug-resistant tuberculosis transmission in Portugal

maio de 2021

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Tese de Doutoramento
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Trabalho efetuado sob a orientação da
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da
Doutora Raquel Duarte
e da
Doutora Margarida Correia-Neves

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DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

TITULO: FATORES DE RISCO ASSOCIADOS À TRANSMISSÃO DA TUBERCULOSE MULTIRRESISTENTE EM PORTUGAL

RESUMO

Apesar dos grandes avanços e conquistas no controlo da tuberculose (TB), a doença continua a ser um importante problema de saúde pública, agravado pelo surgimento de resistência aos fármacos antituberculosos. Embora a carga da doença esteja a diminuir, é necessário acelerar este processo para atingir a meta ambiciosa de erradicação da TB até 2035, nomeadamente em Portugal. Para isso, é fundamental reavaliar e ajustar as medidas a nível nacional e local. Esta tese teve como objetivo fornecer conhecimentos sobre a emergência e transmissão da TB multirresistente (TBMR) em Portugal, visando o ajustamento das estratégias locais, de forma a prevenir e reduzir a incidência da TBMR no país.

Com este propósito, e em primeiro lugar, analisamos a distribuição espacial da TBMR e TB não MR entre os municípios de Portugal, utilizando modelos espaciais Bayesianos; em segundo lugar, avaliamos a dinâmica da emergência e transmissão da TBMR, incluindo a identificação dos fatores de risco associados e estabelecemos a taxa de transmissão recente, combinando assim dados epidemiológicos e genéticos; por último, avaliamos os resultados do tratamento e identificamos os fatores associados à morte entre pacientes com TBMR e TBXDR (TB extensivamente resistente). Foram utilizados dados epidemiológicos do Sistema Nacional de Vigilância de TB (período 2000-2017) e de serviços de Saúde Pública.

Encontramos heterogeneidade significativa na distribuição espacial de TBMR e TB não MR, identificamos 36 áreas de alto risco para TB não MR e 8 áreas de alto risco para TBMR. Estimamos que pelo menos 14,9% dos casos de TBMR foram atribuíveis à transmissão recente, que foi associada a indivíduos nascidos em Portugal. A taxa de sucesso do tratamento para TBMR no período em estudo foi de 77,9%. Observamos ainda que 18,4% dos pacientes com TBMR morreram durante o tratamento e, destes, 40,7% morreram nos primeiros 6 meses. A infeção pelo vírus da imunodeficiência humana foi independentemente associada à morte durante o tratamento.

Desta forma, é necessário revisar e ajustar as estratégias de controlo da TB, focado na procura ativa de casos de doença e infeção latente e na deteção precoce de resistência aos fármacos, por meio de rastreio extensivo de contatos, investigação de clusters e rastreio sistemático entre grupos vulneráveis em áreas de alto risco identificadas.

Palavras-chave: tuberculose, tuberculose multirresistente, transmissão, fatores associados

TITLE: RISK FACTORS ASSOCIATED WITH MULTIDRUG-RESISTANT TUBERCULOSIS TRANSMISSION IN PORTUGAL

ABSTRACT

Despite great advances and achievements in the control of tuberculosis (TB), this disease remains an important public health problem aggravated by the emergence of resistance to anti-tuberculosis drugs. Although the disease burden is decreasing, it is necessary to accelerate this process to reach the ambitious target of eradicating TB by 2035, namely in Portugal. For this, it is essential to reassess and adjust the measures at national and local level. This thesis sought to provide knowledge on the emergency and transmission of multidrug-resistant TB (MDR-TB) in Portugal aiming at the adjustment of local strategies in order to prevent and reduce MDR-TB incidence in the country.

For this purpose, firstly, we analyzed the spatial distribution of the MDR- and non-MDR-TB across municipalities in Portugal, using Bayesian spatial models; secondly, we assessed the dynamics of MDR-TB emergence and transmission, including the identification of associated risk factors and established the rate of recent transmission, combining epidemiological and genetic data; and finally, we evaluated treatment outcomes and identified the factors associated with death among patients with MDR and XDR-TB (extensively drug-resistant TB). We used epidemiological data from the national TB Surveillance System (regarding 2000-2017 period) and from Public Health services.

We found significant heterogeneity in the spatial distribution of MDR- and non-MDR-TB and we identified 36 high-risk areas for non-MDR-TB and eight high-risk areas for MDR-TB. We estimated that at least 14.9% of MDR-TB cases were attributable to recent transmission, further associated with Portugal-born individuals. The estimated treatment success rate for MDR-TB was 77.9%. We observed that 18.4% of MDR-TB patients died during treatment, and, within these, 40.7% died in the first six months. Human immunodeficiency virus infection was independently associated with death during treatment.

Therefore, it is necessary to review and adjust strategies for TB control, focusing on active search for cases of TB disease and latent infection, and early detection of drug-resistance, through extensive contact tracing, cluster investigations and systematic screening among vulnerable groups in identified high-risk areas.

Keywords: tuberculosis, multidrug-resistant tuberculosis, transmission, factors associated

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ATS/CDC/ERS/IDSA	The American Thoracic Society, the European Respiratory Society, the Infectious Diseases Society of America and the United States Centers for Disease Control and Prevention
DGS	Direção-Geral da Saúde
DST	Drug Susceptibility Testing
ECDC	European Centre for Disease Prevention and Control
EU	European Union
HIV	Human Immunodeficiency Virus
I$	International Dollar
IUATLD	International Union Against Tuberculosis and Lung Disease
LTBI	Latent Tuberculosis Infection
LVT	Lisbon and Tagus Valley region
MDGs	Millennium Development Goals
MDR LTBI	Multidrug-Resistant Latent Tuberculosis Infection
MDR/RR-TB	Multidrug- or Rifampicin-Resistant Tuberculosis
MDR/XDR-TB	Multidrug-Resistant or Extensively Drug-Resistant Tuberculosis
MDR-TB	Multidrug-Resistant Tuberculosis
MIRU-VNTR	Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat
Mtb	*Mycobacterium tuberculosis*
NTP	National Tuberculosis Programme
RFLP	Restriction Fragment Length Polymorphism
RR-TB	Rifampicin-Resistant Tuberculosis
SDGs	Sustainable Development Goals
TB	Tuberculosis
US$	United States Dollar
WGS	Whole-Genome Sequencing
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis

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CHAPTER I

INTRODUCTION AND THESIS AIMS

1. Multidrug-resistant tuberculosis: worldwide relevance

Tuberculosis (TB), one of the oldest diseases of mankind, continues to kill people and represents a public health problem in the twenty-first century. Undoubtedly, thanks to advances in diagnosis, the existence of effective treatment and measures taken in the two past decades, the global disease burden has continuously decreased, but we are still far from achieving the ambitious goal of TB eradication by 2035. The emergence of resistance to anti-tuberculosis drugs assumed particular relevance and made it even more difficult to achieve this task, delaying the goal of "End the global TB epidemic".

In addition to already existing problems and challenges, a new obstacle for TB control has emerged in early 2020: the COVID-19 pandemic. The disruption of essential health services, the reallocation of human and financial resources from TB to the COVID-19 response resulted in 21% drop in TB case notifications worldwide in 2020 (vs. 2019) [1]. The COVID-19 pandemic threatens to unwind the gains made over recent years. According to recent projections, global TB incidence and deaths in 2021 would increase to levels last seen in 2013 and 2016, respectively – implying a setback of at least 5 to 8 years in the fight against TB. Additional 6.3 million cases of TB and 1.4 million TB deaths are expected by 2025 [2].

1.1 Tuberculosis: historical background

TB has plagued humankind throughout known history and human prehistory and there are evidence through the analysis of human remains derived from archaeological sites around the world [3, 4]. It has assumed particular importance in great epidemics during the 18th and 19th centuries and may have killed more persons than any other microbial pathogen [5, 6]. At those time the decimated populations thought that TB was hereditary or caused by “bad air” [7].

The history of TB was changed definitely on March 24, 1882, when Robert Koch announced the discovery of the microbial cause of TB, *Mycobacterium tuberculosis* (Mtb) [5, 7]. Robert Koch’s work over the next 20 years led to correct assertions that pulmonary TB is infectious, tubercle bacilli are killed by light, and he suggested that cases should be notified. He discovered tuberculin in 1890, thinking that it could cure TB. This rise a hope throughout the world, but the product turned out to be ineffective as a treatment. Later, tuberculin proved to be valuable to use for TB diagnostic [7].

With no effective treatment, approximately 50% of TB patients died within 5 years after the onset of disease [8, 9]. This situation has improved a lot after 1944, when streptomycin revealed its potential as

an anti-TB drug [7, 10]. In the following decades, all the currently used first-line anti-TB drugs were discovered and put into practice [11]. Consequently, in the late 1970s and early 1980s, with the availability of effective treatment regimens, the incidence of TB began to decline [7, 11]. By then, it was generally considered that one of the most serious infectious diseases ever, was apparently under control [7].

However, in the late 1980s and early 1990s, this trend reversed, with a further increase in the global TB burden. Decreased attention to TB control, poor public health infrastructure [12]; developing world on the one hand, but increased poverty and situations of social exclusion on the other hand; collapse of health services in Eastern Europe and migratory movements from regions with very high TB rates; the spread of the human immunodeficiency virus (HIV) and emergence of anti-TB drugs resistance were the causes that led to this resurgence of TB [7, 13]. Hence, World Health Organization (WHO) took an unprecedented step and declared TB a global emergency in 1993 [14].

1.2 Anti-tuberculosis treatment and the emergence of drug-resistance

Even after the discovery of the cause of TB, the disease remained with no effective treatment for over half a century. The identification of streptomycin in 1944, second therapeutically useful antibiotic after penicillin, started a path in TB treatment [15]. Streptomycin was the first effective anti-TB drug to be demonstrated and used [16].

However, already in the first clinical trials, the emergence of resistance to streptomycin has been observed when it was used in a monotherapy regimen against pulmonary TB. [17]. Later, the use of para-aminosalicylic acid in combination therapy with streptomycin allowed to prevent the development of resistance [18].

The following decade was very successful regarding the discovery of new anti-TB drugs: isoniazid (in 1951), pyrazinamide (in 1952), cycloserine, (in 1952), ethionamide (in 1956), rifampin (in 1957) [19]. Resistance was still being reported whenever a new drug was included in the treatment regimen, including isoniazid [20] and rifampicin [21]. Nevertheless, the use of multidrug combination therapy resulted in significant improvements in TB treatment. Even today, isoniazid, with its potent early bactericidal activity and low cost, and rifampicin, with its sterilizing activity, continue to be the most effective and preferred anti-TB drugs and it still constitutes the basis of the combination therapy against TB with a 6-month duration for pulmonary disease. [22].

It was only in the late 1980s and early 1990s, more than 40 years after the first description of resistance, that the first outbreaks of TB simultaneously resistant to isoniazid and rifampicin, defined as multidrug-resistant TB (MDR-TB), have been reported in the United States of America and Europe [23-27]. These outbreaks, which were associated with nosocomial transmission of MDR-TB among patients infected with the human immunodeficiency virus (HIV) and with high case-fatality rates (72 and 91%) [24, 27], drew international attention to the scale of the problem. At the same time, high rates of MDR-TB among immunocompetent patients have been reported in India, Korea, Nepal and Bolivia [28].

In view of this problem and lack of new anti-TB drugs, it was necessary to find alternative treatments. Several classes of antimicrobial drugs (injectable aminoglycosides, thioamides, fluoroquinolone, cycloserine and para-aminosalicylic acid), which were developed for other bacterial infections, were used in the MDR-TB treatment regimens as second-line reserve drugs extended to 18-20 months [29-31].

However, as early as 2006, reports raised attention to the emergence of Mtb strains resistant not only to isoniazid and rifampin but also resistant to second-line drugs [32]. Thus, MDR-TB cases presenting additional resistance to at least one of the fluoroquinolones and one of the injectable drugs were defined as extensively drug-resistant TB (XDR-TB) [33] and considered as a serious and emerging public health threat. This definition was revised very recently, in January 2021 (after the publication of our paper presented in Chapter IV). The current definition takes into account the permanent key role of fluoroquinolones, introduction of new drugs and disintegration of second-line injectable drugs in the treatment of MDR-TB. Likewise, XDR-TB is defined as MDR-TB with additional resistance to any fluoroquinolone (levofloxacin or moxifloxacin) and at least one additional Group A drug (bedaquiline or linezolid) [34].

New anti-TB drugs, bedaquiline and delamanid, have been assessed and recommended by WHO for use in MDR-TB treatment in 2013 [34] and 2014 respectively [35]. However, although rare, there is already evidence reporting Mtb resistance to these newly approved drugs [36-41].

Linezolid is an antibiotic that also demonstrated its effectiveness in the treatment of MDR-TB [42, 43], but it was not initially recommended for routine use, due to insufficient evidence of its safety or efficacy [30]. In 2018, linezolid was recognized as one of “Medicines to be prioritised" and recommended for use in longer MDR-TB regimens [44]. Again, the large-scale use of the drug is being accompanied with reports of observed resistance [45-47]. Likewise, the treatment of MDR/XDR-TB remains a challenge for clinicians, public health and the community in general.

In addition to the bacterial determinants, host conditions and behavioral factors are considered as risk factors for MDR-TB and whom can also compromise treatment success. One of the most important factors is previous exposure to anti-TB drugs that is known as strongest risk factor for MDR-TB [48-51]. Another important risk factor is co-infection with HIV [48-50, 52] that is recognized as factor associated with primary but not acquired drug resistance [53] and as a factor associated with death during treatment [54-56]. Young age (less than 40 years) [48], being foreign-born [48, 57], homelessness [57], having history of imprisonment [57], consumption of alcohol [50, 57] and injectable drug use [57] were also identified as risk factors for MDR-TB.

1.3 Current state of multidrug-resistant tuberculosis

1.3.1 Multidrug-resistant tuberculosis in the world

Globally, an estimated 10 million people developed TB in 2019 [58]. However, the distribution of disease across the regions is not homogeneous: most TB cases occurred in South-East Asia (44%), Africa (25%) and regions of the Western Pacific (18%), with smaller percentages in the Eastern Mediterranean (8.2%), the Americas (2.9%) and Europe (2.5%). Countries like India (26%), Indonesia (8.5%), China (8.4%), the Philippines (6.0%), Pakistan (5.7%), Nigeria (4.4%), Bangladesh (3.6%) and South Africa (3.6%) accounted for two thirds of the global total TB cases (Figure 1A) [58].

The national TB incidence rates in different countries varies from less than 5 to more than 500 cases per 100 000 population per year. Globally, the reduction of TB incidence rate was 9% since 2015, at about 2% per year. However, this is still not enough to reach the first milestone of the “End TB Strategy” of 20% reduction until 2020 [58, 59].

TB was estimated to cause 1.4 million deaths in 2019. The reduction in the total number of TB deaths between 2015 and 2019 was only 14% [58], which is less than halfway towards the milestone of 35% reduction between 2015 and 2020 [59].

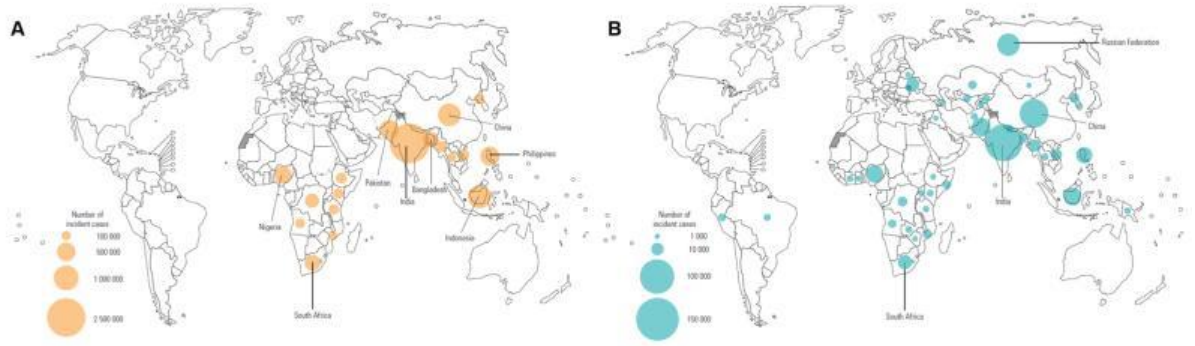

Figure 1. Estimated TB (A) and MDR/RR-TB[a] (B) incidence in 2019, for countries with at least 100 000 incident cases [a] MDR-TB is a subset of RR-TB (From: *WHO. Global tuberculosis report 2020. Geneva, Switzerland, 2020*)

Unfortunately, the COVID-19 pandemic has further aggravated the situation. Their negative impact in TB incidence and deaths can lead a setback of at least 5 to 8 years in the fight against TB [2].

In 2019, among 465 000 incident cases of rifampicin-resistant TB (RR-TB), 362 700 (78%) cases had MDR-TB. Countries like India, China and the Russian Federation had the largest share of the global burden: 27%, 14% and 8% of total RR-TB cases respectively (Figure 1B) [58]. However, only 206 030 cases of MDR/RR-TB (44% of the estimated 465 000 incident cases) were detected and notified in 2019, which represented a 10% increase from 186 883 in 2018. Among MDR/RR-TB notified cases, 12 350 (6%) cases had XDR-TB [58].

Regarding the total estimated TB cases: 3.3% of new and 18% of previously treated TB cases had MDR/RR-TB [58]. The percentage of MDR-TB among TB cases remains stable since 2012, while the effective number of MDR-TB cases somewhat reduced since 2017 (Figure 2).

The latest data show global treatment success (i.e. cured or treatment completed) rates of 85% for TB and 57% for MDR/RR-TB [58], which remains below 90%, a combined target for drug-susceptible and drug-resistant TB set for 2025 [59].

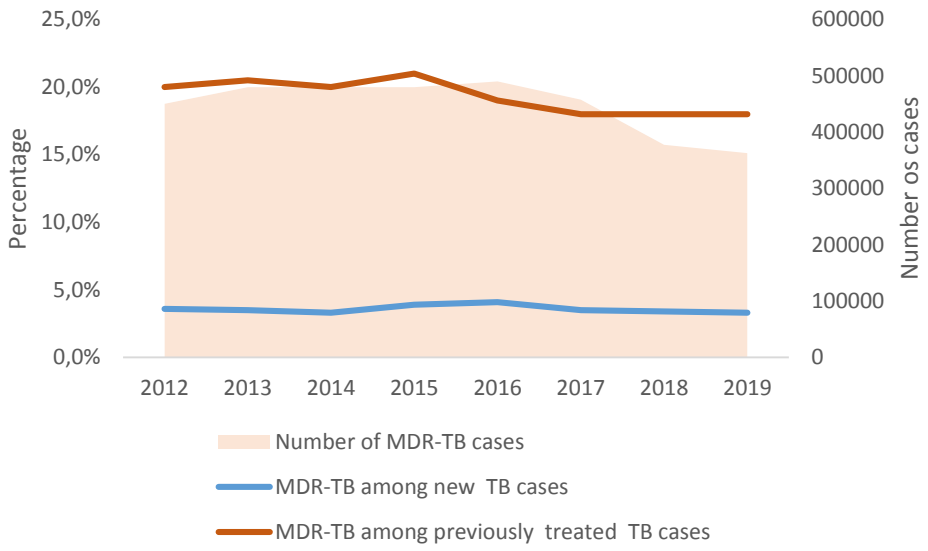

Figure 2. Number and percentage of MDR-TB cases among globally estimated new and previously treated TB cases, 2012-2019 (Adapted from: *WHO. Global tuberculosis report 2013. Geneva, Switzerland, 2013; WHO. Global tuberculosis report 2014. Geneva, Switzerland, 2014; WHO. Global tuberculosis report 2015. Geneva, Switzerland, 2015; WHO. Global tuberculosis report 2016. Geneva, Switzerland, 2016; WHO. Global tuberculosis report 2017. Geneva, Switzerland, 2017; WHO. Global tuberculosis report 2018. Geneva, Switzerland, 2018; WHO. Global tuberculosis report 2019. Geneva, Switzerland, 2019 and WHO. Global tuberculosis report 2020. Geneva, Switzerland, 2020)*

1.3.2 Multidrug-resistant tuberculosis in the European Union

Assessing the epidemiological situation in the European Union (EU), according to the latest available data, 52 862 of TB cases were reported in 2018. Countries like France, Germany, Poland, Romania and the United Kingdom reported more than 5 000 cases, accounting for 63.0% of all reported cases, while Romania alone accounted for 23.0% [60]. Portugal accounted for 4.0% of all reported cases Portugal accounted for 4.0% of all reported cases [60] (more details about TB in Portugal are described in "3.2 Current epidemiological overview", p. 23).

The TB notification rate in the EU in 2018 was 10.2 per 100 000 population, continuing the downward trend observed since 2002. Since 2014, the average annual decline was 4.0% (Figure 3). Though the continuous decline is encouraging, the downward curve should be more pronounced in order to achieve the target of an 80% reduction in the TB incidence rate until 2030 [60].

The percentage of TB patients (from a 2017 cohort, i.e. who started treatment in 2017) who successfully completed treatment was 67.6% [60], which is lower compared to the previous year's result 70.1% (from a 2016 cohort) [61] and is clearly below global treatment success rate (85% from a 2018 cohort) [58].

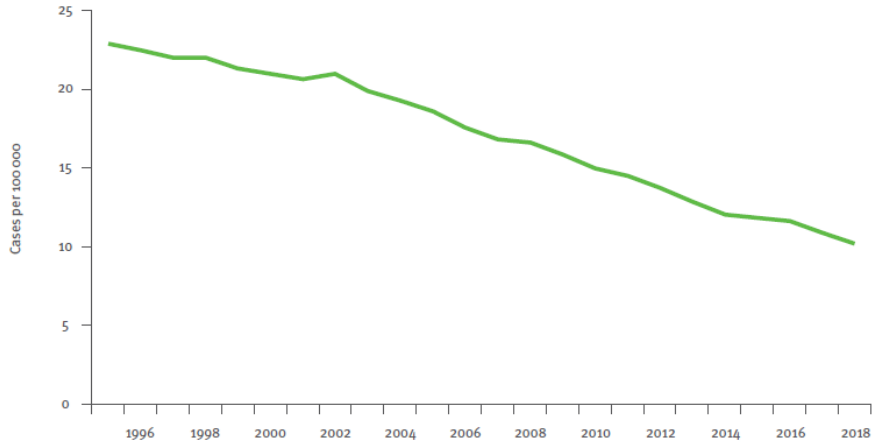

Figure 3. TB notification rate per 100 000 population by year of reporting, EU, 1995–2018 (From *ECDC/WHO. Tuberculosis surveillance and monitoring in Europe 2020-2018 date. Stockholm, Sweden: 2020*)

The number of MDR-TB cases show a tendency to decrease since 2012 (Figure 4). In 2018, the rate of notified MDR-TB cases remained the same as 2017 at 0.2 per 100 000 population, showing a reduction after remaining at 0.3 per 100 000 population from 2013 to 2016 [60]. MDR-TB was reported for 999 (3.7%) of 26 881 TB cases with results for first-line drug susceptibility testing (DST). XDR-TB was reported for 158 (19.6%) of 808 MDR-TB case with results for second-line DST, 66.5% of which were reported in Lithuania and Romania [60].

In particular, among pulmonary TB cases, MDR-TB accounted for 2.3% of new and 13.1% of previously treated cases [60]. The percentage of previously treated pulmonary MDR-TB cases decreased compared to 2012, but it has remained stable since 2016, moreover the percentage of new pulmonary TB cases remains stable since 2012 (Figure 4).

The percentage of patients with MDR-TB (2016 cohort) who successfully completed treatment was 48.1%[60], which is higher than treatment success in the previous year 44.8% (cohort from 2015) [61], but it is far below the 2020 target of the action plan for the WHO European Region (75%) [62].

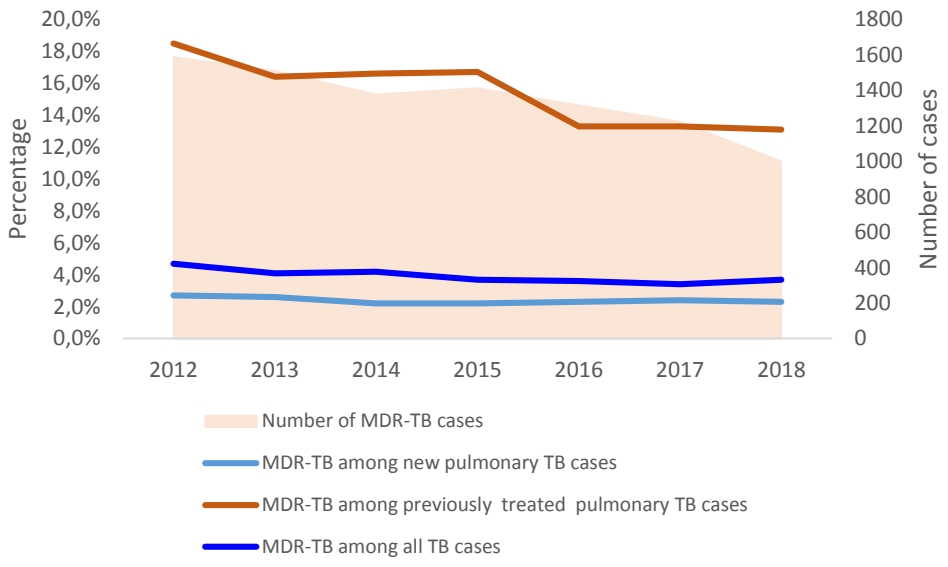

Figure 4. Number and percentage of MDR-TB cases among all TB cases, among new and previously treated pulmonary TB cases, EU, 2012-2018 (Adapted from: *ECDC/WHO. Tuberculosis surveillance and monitoring in Europe 2018-2016 date. Stockholm, Sweden: 2018 and ECDC/WHO. Tuberculosis surveillance and monitoring in Europe 2020-2018 date. Stockholm, Sweden: 2020)*

1.4 Economic, social and psychological impacts of multidrug-resistant tuberculosis

1.4.1 Economic burden

TB brings direct and indirect costs both to the community and at the individual level for the patient.

The costs of TB prevention measures, diagnosis and treatments are mostly supported through national efforts, denominated as domestic funding sources. These include both funding for TB-specific budgets and funding for inpatient and outpatient care [58]. Therefore, international donor funding is crucial for low- and middle-income countries with high TB burdens.

The Global Plan 2018–2022 estimated that around 15 billion US$ (United States dollar) were required in these countries in the coming years. The US$ 8.3 billion (64%) were destined for diagnosis and treatment of drug-susceptible TB and the US$ 4.3 billion (33%) for diagnosis and treatment of MDR-TB. The estimated average cost per drug-susceptible TB patient was about US$ 1,050, while the average cost per MDR-TB patient was about US$ 15,500 [63].

However, according to reports of national TB programmes, the total funding in 2020 was only half of the estimated in the Global Plan, despite its increase since 2006. The US$ 4.2 billion funding (65% of the

total) was available for diagnosis and treatment of drug-susceptible TB, while funding for MDR-TB reached US$ 2.26 billion (22% of the total), increasing from the US$ 1.4 billion available in 2015 (Figure 5) [63].

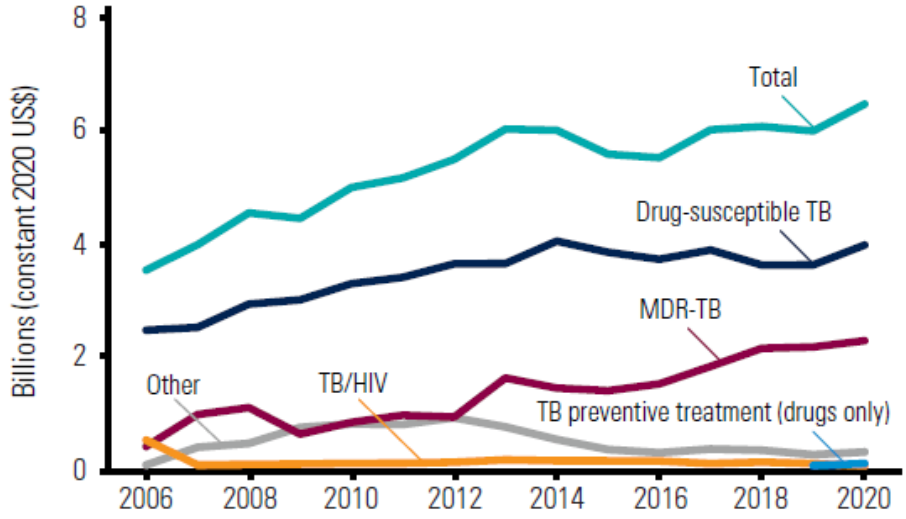

Figure 5. Funding for TB prevention, diagnosis and treatment in total and by category of expenditure, 121 countries with 98% of reported cases, 2006–2020 (From: *WHO. Global tuberculosis report 2020. Geneva, Switzerland, 2020*)

In 2019, the average cost per patient treated for drug-susceptible TB was US$ 860 and US$ 5,659 per patient treated for MDR-TB, which was lower than in 2018 (US$ 6,400) [58].

Evidently, provider treatment costs vary between countries with different level of income. Average drug-susceptible TB treatment costs per patient can reach to US$ 14,659 in high-income countries, to US$ 840 in upper middle-income countries, to US$ 273 in lower middle-income, and to US$ 258 in low-income countries. The respective costs for treating MDR-TB can reach to US$ 83,365, US$ 5284, US$ 6313 and US$ 1218 [64].

Expenditure associated with care and drugs are responsible for a large share of provider treatment costs. In lower-middle income countries, decentralized ambulatory model of treatment and care is often used for being more economic. High-income countries choose hospitalization, whose duration for MDR-TB can reach up e.g. to 192 days in Estonia and 321 days in Russia, that represents more than half of provider treatment costs in these countries [64].

In lower-middle income countries expenditures on drugs correspond to 14% of total costs in drug-susceptible TB treatment and to 46% in MDR-TB treatment. In high-income countries, although they do not benefit from lower drug prices, which pharmaceutical companies provide to low-income countries, and have much higher drug expenditure, these expenditure correspond to 10% of total costs in drug-susceptible TB treatment and only to 24% in MDR-TB treatment [64].

In Europe, drugs prices are generally higher in North-West European countries than in Eastern or Southern European countries. The cost of a standardized 6-month treatment regimen for drug-susceptible TB range from I$ 94 (international dollar, which equals one US$) in the Republic of Moldova to I$ 1 165 in Switzerland. The price of a full 20-months MDR-TB regimen is on average I$ 32 000, but is as low as I$ 4 600 in the Republic of Moldova, and as high as I$ 79 300 in Finland [65].

The introduction of new drugs as bedaquilin and delamanid in the MDR-TB treatment regimens allowed to cut the treatment time [66]. However, this can bring a 24.5% increase of weighted average cost of combination treatment regimens [67].

1.4.2 Individual and social costs

Even though in most countries, the access to TB care is free, many patients and their families are still facing high costs related to transport, food and income foregone due to their temporary disability to work or possible loss of employment. This situation is even more worrisome for MDR-TB patients that more often suffer a reduction in salary due to work absenteeism or even unemployment associated with to the long duration and complexity of treatment [68, 69]. For example, in Ethiopia, 76% of drug-susceptible TB patients and 72% of MDR-TB patients lost their job, and 92% of drug-susceptible TB patients and 79%of MDR-TB patients reported income loss due to TB [70].

Costs of patients and their households due to TB care, called catastrophic costs, are defined as total costs equivalent to more than 20% of annual household income. The elimination of these costs by 2020 was third high-level impact target of the WHO End TB Strategy [59]. However, still in 2019, globally 49% of people with TB faced catastrophic costs, ranging from 19 to 83% at country level [58].

Costs incurred by patients depends on the economic conditions of countries leading often to catastrophic social consequences [71]. In low-income countries, direct costs incurred by patients range, on average, from US$155 in drug-susceptible TB to US$406 in MDR-TB. In lower-middle income countries, these costs range from US$84 to US$1 616, respectively. However, similar values, US$603 for drug-susceptible

TB and US$660 for MDR-TB, were observed in upper-middle income countries [64]. Although in absolute numbers these values may not seem very high, they are very significant for families from low-income countries. In order to no TB patients and affected families face catastrophic costs is a need to ensure that they receive appropriate income replacement and other social protection interventions (e.g. provision of transport vouchers and food assistance) [71, 72]. It is impossible to guarantee this support in low-income countries.

However, it is expected that the scenario is going to get even worse due to an economic recession caused by COVID-19 pandemic. Unemployment and Income loss consequently will also have their negative impact on catastrophic costs due to TB [73].

1.4.3 Psychological impact

Economic and financial challenges in conjunction with feelings of hopelessness and fear, alongside the distress of living an "isolated life" related to MDR-TB diagnosis aggravated by the rigorous drug treatment and its side effects have an important psychological impact on patients and their families throughout the disease course [68]. MDR-TB patients are considered to have lower quality of life in terms of their psychological, social and environmental domains when compared to drug-susceptible TB patients [74].

Regarding social issues, stigma continues to be a major concern faced by MDR-TB patients. There are reports stating experiences of social seclusion or rejection from family members, friends, neighbors, and/or health providers; internalized shame; financial instability; discrimination [68, 75]. Besides these, stigma associated with MDR-TB TB has been described to impact in family life causing a range of scenarios going from divorce, cancellation of impending marriages, breakdown of family relationships and even isolation within the family [68, 76].

2. Achievements and challenges in multidrug-resistant tuberculosis in the stage of eliminating the global TB epidemic

2.1 The "End TB Strategy" for multidrug-resistant tuberculosis

From 1993, year when TB was declared a global public health emergency, the world has changed its position of neglect regarding the disease. Subsequently, important strategies were launched and

implemented. The DOTS (directly observed treatment, short-course) strategy (1994-2005), with a close supervision and monitoring of the medicine intake and patient compliance of the therapeutic regimen during a relatively short period [77], and the "Stop TB Strategy" (2006-2015), aiming at reducing inequalities and achieving universal access to high-quality care for all people with TB [78] were very important. These two strategies were essential to meet the TB-related target of the Millennium Development Goals (MDGs), to halt and start reversing the TB incidence [79].

As a reflection of the measures implemented, TB mortality has fallen 47% from 1990 to 2014 and an estimated 43 million lives were saved between 2000 and 2014 due to effective diagnosis and treatment [80]. These achievements provided the basis for further targets and progress. Thus, global strategy and targets for TB prevention, care and control after 2015, called the "End TB Strategy", aims at ending the global TB epidemic and includes three high-level, overarching indicators, with corresponding global targets and milestones set by WHO [59, 72] and by the United Nations in the third Sustainable Development Goal (SDG) (Table1) [81].

Table 1. The End TB Strategy's three high-level global indicators and associated targets* and milestones (From: *WHO. The End TB Strategy. Global strategy and targets for tuberculosis prevention, care and control after 2015. Geneva, Switzerland, 2014*)

INDICATORS	MILESTONE		TARGETS	
	2020	2025	**2030***	**2035**
Reduction in number of TB deaths compared with 2015	35%	75%	**90%**	**95%**
Reduction in TB incidence rate compared with 2015	20% (<85/100 000)	50% (<55/100 000)	**80%** (<20/100 000)	**90%** (<10/100 000)

* The targets are for 2030, marking the end of the SDGs, and for 2035, marking the end of the period covered by the WHO Strategy.

Implementing the "End TB Strategy", requires multisectoral collaborations between biomedical, public health and socioeconomic entities, combined with research and innovation mechanisms. Interventions need to be supported on these three pillars: (1) integrated, patient-centred care and prevention; (2) bold policies and supportive systems; (3) intensified research and innovation [59]. The whole effort is aimed at improving and strengthening TB control, focusing on disease prevention, which is a global health priority. Each of the three pillars and their components applies to the control and prevention of MDR-TB

(Figure 6) [82], where early diagnosis, effective therapy and preventive therapy for contacts are crucial issues.

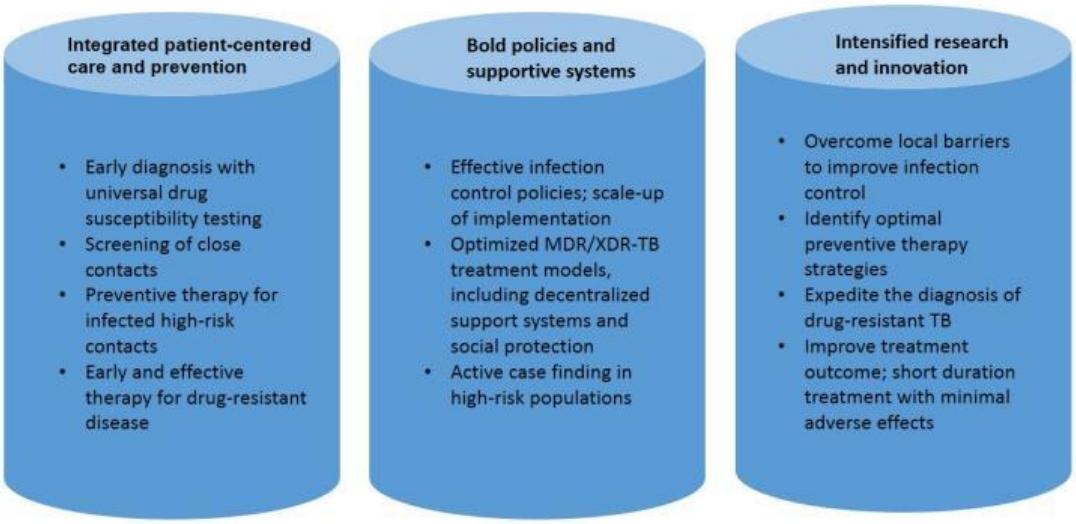

Figure 6. **Strategies to prevent MDR-TB within the End TB Strategy framework** (Adapted from: *Fox, G. J. et al. Preventing the spread of multidrug-resistant tuberculosis and protecting contacts of infectious cases. Clinical Microbiology and Infection 2017; 23(3):147-53*)

2.1.1 Early diagnosis

The gold standard for TB diagnosis is identifying the presence of Mtb from a clinical sample. Specific diagnosis of MDR-TB is based on testing the susceptibility of Mtb to anti-TB drugs, which reveals resistance to isoniazid and rifampicin with or without additional resistance. It must be done urgently to correctly decide on individualized and optimized treatment regimen [57].

Conventionally, culture-based phenotypic DST methods are used to measure the susceptibility of bacteria to a drug, based on qualitative methods that assess in-vitro growth using a single critical concentration of the drug in liquid or solid media. Although these methods continue to be the gold standard for drug resistance detection, they are time-consuming (results are only obtained after weeks of incubation), require sophisticated laboratory infrastructures, qualified staff and strict quality control [83, 84].

Currently, molecular and genotypic techniques, are the preferred methodologies to detect resistance-conferring mutations in specific gene. They have the advantage of speed, with results becoming available

in a few hours or few days, allowing earlier initiation of appropriate treatment for drug-resistant TB [58]. These techniques are considered safer, they remove the need to perform microbiological culture and to manipulate large numbers of highly infectious bacteria. However these techniques can be costly, require a certain degree of technology and of team expertise. In addition, there is a limited number of loci that molecular tests can analyze at one time and this can represent a disadvantage. Although sequential testing of genes is possible, it will increase costs and might delay the final identification of drug-resistance [57].

A technique that recently began to be used widely, DNA sequencing, provides detailed information on resistance across multiple gene regions and whole-genome sequencing (WGS), providing resistance profiles for all drugs within a single analysis [57]. WGS-based approaches are quickly moving from research laboratories to clinical care and are becoming a fast, affordable and increasingly accessible alternative [85]. WHO recognizes the added value offered by WGS and supports the work for the development and validation of novel molecular diagnostic tool whose use for routine genotypic DST is being evaluated [58].

However, to test for the potential resistance to some important second-line drugs including bedaquiline, linezolid, and others, culture-based DST are still needed since knowledge of the molecular basis of resistance to these drugs is still limited [84].

The “End TB Strategy” calls for universal access to DST with 100% coverage for TB patients [59]. However, although the coverage of DST increased since 2012 (7%), in 2019, only 61% of the bacteriologically confirmed pulmonary TB cases notified globally were tested for first-line drugs [58]. In the EU, 80.2% of all bacteriologically confirmed TB cases, diagnosed in 2018, had DST results for rifampicin and isoniazid [60].

2.1.2. Effective and successful treatment

In addition to the importance of MDR-TB diagnosis, it is also very important to start and complete a full course of anti-TB treatment. Appropriate treatment should be available and accessible to all. Globally, despite some improvements, the number of people enrolled in treatment in 2019 was equivalent to only 38% of the estimated number of people who developed MDR/RR-TB. China and India accounted for 41% of this global gap [58]. In the EU, this indicator reached 98.4% in 2018 (according to the latest available data) [60].

Regarding MDR-TB treatment strategies, standard regimens for new and retreatment cases (i.e. previously treated for TB) were used since the 1990s [29]. Posteriorly, in addition to the standardized, empirical regimens, which take into account prevalence of resistance to first-line and second-line drugs in the country, were recommended. These regimes were adjusted when DST results become available [30]. Currently, due to advances in diagnosis and the availability of new drugs, it became possible to know drug resistance patterns of the Mtb strain right at the beginning of treatment and to design individualized patient-tailored treatment regimen taking into account patient preference and clinical judgment. The use of this strategy can improve adherence to treatment and consequently treatment outcome [66, 84]. However, this depends to a great extent on the technical and financial capacity for drug-susceptibility testing, availability of high-quality drugs and the capacity of health care in each country.

MDR-TB treatment duration is often 20 months or longer. Since 2016, a standardized shorter treatment regimen (9–12 months) can be applied to MDR/RR-TB patients who did not present additional resistances to fluoroquinolones or second-line injectable agents [86]. The last WHO consolidated guidelines on TB proposed new shorter and fully oral regimens for patients with MDR-TB, replacing the previous recommendation that included an injectable agent[66].

Treatment success depends not only on the choice of an effective treatment regimen but also on patient adherence, monitoring patient response and the management of therapeutics adverse events and comorbidities. All of these aspects can be improved through patient-centred approach that was recommended on the first pillar of the “End TB Strategy” [72]. This approach considers the needs and circumstances of each patient and will need to be devised and customized to diverse settings and contexts. This intention relies on a more active patient involvement in treatment and this good treatment adherence can only be achieved through patient and staff education, material and psychological support for the patient [57, 72].

The clinical management of MDR-TB patients is very complex and requires a balanced management model between available resources and prevalence of disease, that are specific for each country. It should be ideally carried out in MDR-TB reference centers, where skilled clinicians can operate with adequate infrastructure. Currently, this is only possible in countries with low prevalence and high resources [84, 87]. A decentralized model of care has proven to be effective, and it is advisable in settings with limited resources and high prevalence of MDR-TB [88, 89].

TB treatment success is one of the indicators for monitoring implementation of the “End TB Strategy”. Globally, the recommended target level for 2025 is at least 90% for drug-susceptible and drug-resistant

TB [59]. But, if treatment success in drug-susceptible TB is approaching the target level, it is far below in drug-resistant TB (85% and 57% respectively)[58].

2.1.3 Preventive therapy

The screening of close contacts of MDR-TB patients is a priority to prevent further transmission. These contacts, similarly to what happens with drug-sensitive TB, should be identified according to national guidelines. First, active TB must be excluded and then these individuals should be screened for Mtb infection (through tuberculin skin test and/or an interferon-ɣ release assay) [48]. A meta-analysis showed that 6.5% of household contacts with MDR-TB source cases had active TB disease and 50.7% of them had LTBI [90].

For decades, there was no consensus or sufficient evidence to provide preventive treatment to contacts with presumed MDR LTBI, neither about their optimal duration. According to recent recommendations by WHO, preventive treatment may be considered in selected high-risk contacts of patients with MDR-TB, based on "individualized risk" and "sound clinical justification". With very low certainty, children, people on immunosuppressive therapy and people living with HIV infection were considered as household contacts at high risk [91]. A recent systematic review also conclude that the chemoprophylaxis for child contacts of MDR-TB patients is beneficial, although the available evidence is of moderate quality [92]. However, the new Clinical Practice Guideline, approved by the American Thoracic Society, the European Respiratory Society, the Infectious Diseases Society of America and the United States Centers for Disease Control and Prevention (ATS/CDC/ERS/IDSA) suggests offering treatment for contacts with presumed MDR LTBI for 6 to 12 months with fluoroquinolone or with a second drug, on the basis of source-case isolate DST [93].

There are three ongoing randomized controlled trials currently evaluating a single-drug preventive treatment regimen for MDR-TB contacts, which should start reporting around 2022 [84, 94]. Until these results are known, preventive treatment should be determined on the basis of the DST results of the source-case's Mtb isolate [84].

2.2 Molecular epidemiology and transmission dynamics of multidrug-resistant tuberculosis

2.2.1 At patient level

The phenomenon of drug resistance of Mtb is cause by spontaneous mutations particular genes. This is a complex phenomenon and its underlying biological mechanisms continues to be studied with the continuous development of new molecular genetic tools and approaches [95].

For many years, primary drug resistance, which indicated transmission of drug-resistant strains in the community, was regarded only in case of the isolation of resistant Mtb strains from patients who have not previously been treated with anti-TB drugs. Inversely, the isolation of resistant Mtb strains from a patient that was previously infected with Mtb and undergone treatment, was considered that drug resistance emerged during treatment (the so called acquired resistance) [96-98]. This assumption was changed with the development of molecular genetic tools and the possibility to compare the genetic characteristics of the Mtb strains in each infection episode [57].

According to the last worldwide data, 3.3% of new versus 18% of previously treated TB cases had MDR-TB [58]. This can lead to the assumption that the majority of MDR-TB cases arise from acquisition of resistance during treatment rather than the active transmission of resistant strains. However, a population-based transmission model suggests that, in some epidemic settings of the world, more than 80% of incident MDR-TB cases result from transmission rather than selection of *de-novo* resistance during previous treatment (95.9% of all incident and 61.3% of incident MDR-TB cases among previously treated) (Figure 7) [57, 99].

MDR-TB can also result from reinfection with a drug-resistant strain during or after successful treatment for drug-susceptible TB, or mixed infection with a susceptible and resistant strains [57, 100-103].

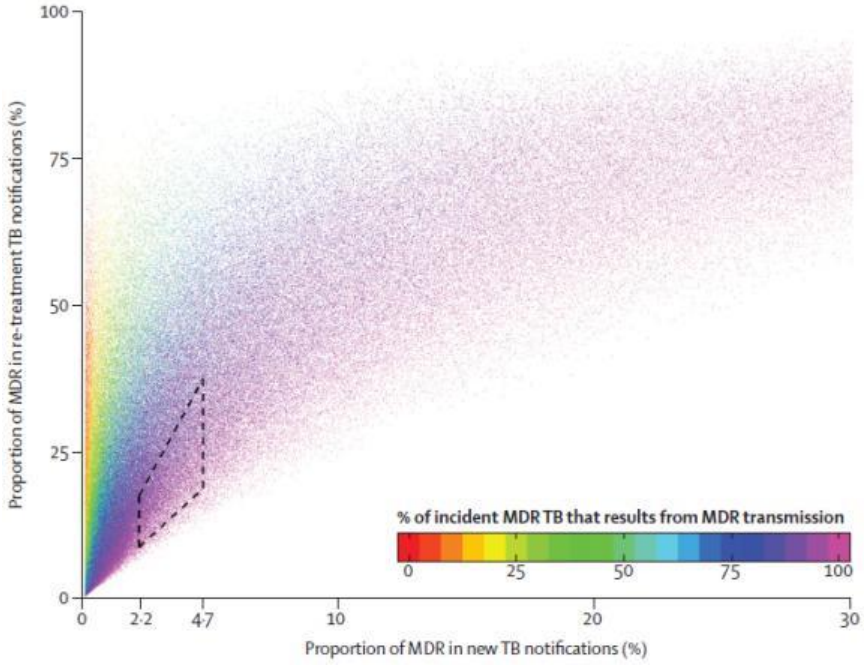

Figure 7. Converting MDR-TB notifications into estimated proportion of incident MDR-TB due to MDR transmission. Estimated proportion of incident MDR-TB cases resulting from MDR transmission (rather than acquisition during previous treatment by the same person) and within the ranges defined by WHO's estimates and confidence intervals for worldwide prevalence of MDR among TB notifications (namely, MDR prevalence of 2.2–4.7% among new TB notifications and a four to eight times higher MDR prevalence among re-treatment notifications. Current global notifications were consistent with a vast majority of MDR-TB cases resulting from transmission (median 96%, 95% uncertainty range 68–100). Treatment-related acquisition of resistance was high only when the ratio of MDR prevalence in re-treatment versus new notifications was extremely high (red and yellow dots) (Adapted from *Kendall EA et al. Burden of transmitted multidrug resistance in epidemics of tuberculosis: a transmission modelling analysis. The Lancet Respiratory Medicine. 2015; 3(12):963-72*)

2.2.2 In the population

Besides the important knowledge of drug resistance at a patient level, genotyping techniques allow to understand the chains and dynamics of transmission of TB in the population, comparing the Mtb strains isolated from TB or MDR TB cases. The first technique applied for this, IS6110 restriction fragment length polymorphism (RFLP), has been widely used since the early 1990s [104]. Subsequently, other techniques like spoligotyping [105] and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) [106] were developed, each time increasing its discriminatory power (ability to differentiate between two unrelated strains). [57, 107]. Finally, the last breakthrough of molecular epidemiology, the use of Whole-Genome Sequencing (WGS) through Next-Generation Sequencing (NGS) technology, have the ability to determine the sequence variation at a maximum possible scale of discrimination, identifying the exact source(s) of infection and to determine in detail the evolutionary relationship between isolates [107, 108].

In an epidemiological scenario, the identification of the same strain types of Mtb in more than one case, forming a cluster [57], might indicate ongoing transmission of TB. It means that when two patients are infected with bacteria isolates with matching genotypes, they could be likely involved in the same chain of recent transmission (i.e., within the previous 2 years) [109, 110]. The proportion of cases attributable to recent transmission is estimated through the proportion of clustered Mtb strains [107, 110], and a high number in this parameter indicates high levels of transmission [57]. But one should not overvalue genotyping identification, since the genetic link between Mtb isolates from different patients need to be evaluated in terms of epidemiologic links, that would explain where and how they might have transmitted TB among themselves, and this data is essential to identify chains of recent transmission [109, 111]. Therefore, it is essential to combine genotyping data with epidemiological data to determine the correct proportion of cases that are attributable to recent transmission [110, 112].

In that regard, a good correlation between genetic and epidemiological data could be achieved using WGS technology of Mtb isolates [113, 114]. Due to its higher discriminatory power this technique increases the confidence on previously established epidemiologically linked cases, reducing the number of matches within an epidemiological scenario (comparing with genotyping techniques with lower resolution) [113].

Thus, WGS has the potential to become an important tool for early detection and tracing of TB outbreaks and mapping transmission routes [115]. Nevertheless, the successful use of WGS requires high-quality in-country laboratory capacity, with the capacity for direct sequencing from clinical samples for shorten the time to results, a centralized TB surveillance system that allows linking of clinical, epidemiological and laboratory data to be able to have a “real-time surveillance” and fully interpret transmission dynamics inside each region [116]. Definition of specific criteria, such as resistance to second-line and new anti-TB drugs or high likelihood of recent transmission, and standardization of sequence data and metadata, could be important for the identification of cross-border clusters to justify an international epidemiological investigation and to use the available resources most effectively [116, 117].

Identification of phylogenetic diversity of Mtb strains, which can be detected through genotyping and genomic approaches, helps understanding the spread of drug-resistant strains within a country, between countries, and even across continents [118]. Beijing strains of Mtb (lineage 2) is the most common genotypic lineage associated with drug-resistance and transmission of TB worldwide (Figure 8) [118, 119] and in the EU in particular [120].

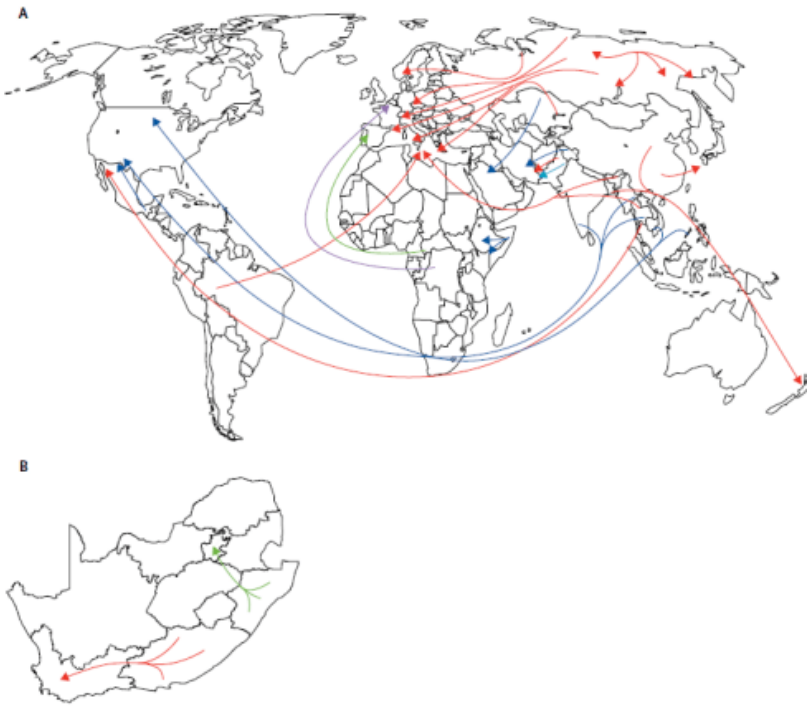

Figure 8. Inter-country and intra-country spread of drug-resistant tuberculosis according to *Mycobacterium tuberculosis* genotype. (A) Worldwide spread of drug-resistant strains of *Mycobacterium tuberculosis*. Red=Beijing strain. Green=LAM9 strain. Light blue=Haarlem1 strain. Purple=T1 strain. Dark blue=untyped strains. (B) Ongoing intra-country spread of extensively drug-resistant tuberculosis strains in South Africa. Red=atypical Beijing strains. Green=LAM4 strain. *(From Dheda, K. et al. The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. The Lancet Respiratory Medicine. 2017; 5(4):291-360.)*

3. Multidrug-resistant tuberculosis in Portugal

3.1 Strategies and measures taken in response to multidrug-resistant tuberculosis

The resurgence of TB, the emergence of HIV and anti-TB drugs resistance in the early 1990s in the world also had its impact in Portugal. In 1995, to resolve the TB situation, which presented an important public health problem, the *"Programa Nacional de Luta contra a Tuberculose"* (NTP) was reorganized and restructured [121].

At the time, drug resistance surveillance was sporadic and concerned only chronic or unresponsive patients [17]. The first survey, which was part of the Global Project on Anti-tuberculosis Drug Resistance Surveillance of the WHO and the IUATLD [122], analyzed isolates from TB patients treated from 1995 to 1998 and concluded that MDR-TB occurred in 4.3% of total tested cases and 29.2% of MDR-TB patients were HIV positive [123]. This survey highlighted the need for continuous monitoring of TB drug resistance and better programme management, particularly in high-risk settings [123].

In response to this, several measures were taken. Since 2000, the performance of first-line TSA was required for all Mtb isolates, both in new cases and in retreatments [124]. In 2001, the information system of NTP that fed a national database since 1992 was replaced by the actual national TB Surveillance System (SVIG-TB) [125]. This system contains clinical and laboratory data from the mandatory registrations of all TB patients who underwent treatment, compiling knowledge about disease control [126].

In the 2002-2006 period, the proportion of MDR-TB cases was 1.9%, of which 22% were XDR-TB [127]. That is, the situation with MDR-TB remained worrisome and further measures would be needed.

In 2007, a new rule was implemented, requiring second-line anti-TB drugs tests for all Mtb strains resistant to isoniazid and rifampicin. The TB National Reference Laboratory of the National Institute of Health Doctor Ricardo Jorge (INSA) in Porto, which is also a WHO Supranational Reference Laboratory, was responsible for carrying out these tests [128]. For the purpose of early diagnosis of drug resistance among specific patient groups (patients with previous TB treatment, vulnerable populations or health professionals), molecular testing for detection of isoniazid and rifampicin resistance directly on clinical samples started to be used since 2008 [129].

Additionally, in 2007, the National Reference Center for MDR-TB was created [127]. This Center aimed to reducing the prevalence of MDR-TB and preventing its transmission in the country through monitor and support the treatment of MDR-TB cases and elaboration the national guidelines and recommendations [130-132]. Later, to support the implementation of these standard procedures, to decentralize this approach and to facilitate accessibility, the Regional Reference Centers were created in each of the seven health regions of the country [133] (the first center opened in the Northern Region, in 2009 [87, 134, 135] and the last one in Lisbon and Tagus Valley Region, in 2013 [136]).

Each Regional Reference Center is responsible for the clinical management of MDR-TB patients, including the choice and adjustment of treatment regimen according to the adverse effects, the determination of hospitalization requirement and it is also responsible for providing treatment and accomplishment of contact tracing. Its team is composed by a pulmonologist (group coordinator), an infectious disease specialist, a public health physician, a microbiologist, a pharmacist, a pediatrician and a thoracic surgeon [134, 135]. This approach made it possible to achieve a treatment success rate of 73.2% in MDR-TB patients followed by the Northern Region Reference Centre between 2009 and 2015 [87]. While the overall national treatment success rate was 62% between 2000 and 2008 [137].

3.2 Current epidemiological overview

In Portugal, the TB notification rate decreased about 61% since 2000 (Figure 8) [138], similarly to the downward trend observed in Europe (Figure 3) [60]. The notification rate in 2018 was 16.6 cases per 100 000 population (Figure 9). However, the districts of Porto and Lisbon stood out with the highest notification rates, which remain above 20 cases per 100 000 population, 25.3 and 23.7 cases per 100 000 population, respectively [138].

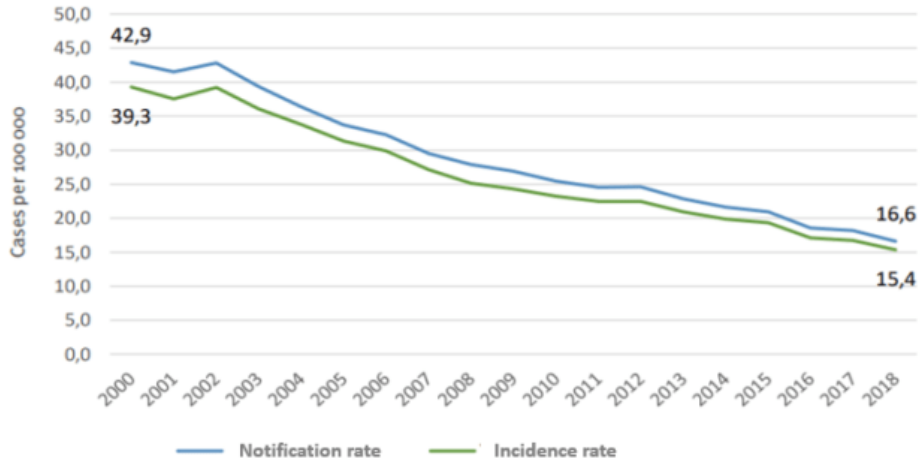

Figure 9. Evolution of notification and incidence rates, Portugal, 2000-2018. (From *DGS. Tuberculose em Portugal 2018 (dados provisórios). Programa Nacional para a Tuberculose. Lisboa, DGS, 2018)*

The majority of TB cases in Portugal occurs in the native population (79.8 % in 2018), in contrast to what occurs in the majority of the Western European countries [138].

TB mortality rate decreased by about 3.2% per year since 2014 and it was 1.9 cases per 100 000 population in 2018 [60].

The percentage of TB cases co-infected with HIV in Portugal was 9.6% in 2018. Although it has been slowly decreasing since 2014, when the percentage was 11.5%, it was in 2018 still the third highest in the EU (only under 12.5% in Iceland and 11.2% in Spain) [60].

In 2018, 0.9% of all bacteriologically confirmed TB cases had MDR-TB, reducing from 2.0% in 2014. Among pulmonary TB cases, MDR-TB accounted for 0.7% of new and 2.6% of previously treated cases [60].

In 2014, 15.4% of MDR-TB cases had XDR-TB. In the meantime, no XDR-TB cases were reported in 2017 and 2018 [60].

3.3 Multidrug-resistant tuberculosis transmission: genetic evidence

In the 1990s, when MDR-TB outbreaks associated with nosocomial transmission of MDR-TB among HIV-infected patients and patients with Acquired Immunodeficiency Syndrome (AIDS) have been reported in the United States of America and in Europe [23-27], in Portugal, the incidence of MDR-TB cases also has risen dramatically in hospitalized HIV-infected patients. The first studies, which used RFLP genotyping technique and were performed in 1996 and 1999, demonstrated that most of the MDR-TB strains isolated in Lisbon hospitals belong to a genetic cluster (cluster A, consisting of subclusters A1, A2 and A3) [139-141]. It is accepted that the emergence of this cluster outbreak may have begun at least in 1992 [139, 141]. However, it has also been demonstrated that transmission of this strains occurred not only between patients with AIDS, but also in immunocompetent individuals. The knowledge generated by these studies provided evidence of MDR-TB transmission in the community in addition to the nosocomial transmission in hospitalized HIV-infected patients [141].

Later, in 2008, another study, which used 12-*loci* MIRU–VNTR genotyping technique, identified nine clusters. Five of these 9 clusters contained 55.2% of isolates that belonged to the same family, initially identified as Clusters A [141], which came to be named Lisboa family [142]. One of the clusters of this Lisboa family, named Lisboa 3, which included 20.7% isolates originating from 10 different hospital, was the most predominant cluster not only in Lisboa family, but also in the context of the Lisbon Health Region family of isolates. This led to the conclusion that strains belonging to this cluster continued to be responsible for more MDR-TB cases in the Region, and probably throughout the country [142].

In 2011, the genotyping analysis of MDR-TB isolates revealed six clusters, but two of them, Lisboa 3 and Q1, were most predominant. Three clusters (Lisboa3, Lisboa4 and Q1) were related to XDR-TB, with Lisboa3 having the highest number of XDR-TB cases. This analysis found a high prevalence of XDR-TB among MDR-TB isolates (between 44.3% and 66.1%) [143].

Three years later, a new research, which used 24-loci MIRU-VNTR genotyping technique and WGS, showed once again that the two previously described genetic clusters Lisboa3 (this time subdivided in two other clusters Lisboa3-A and Lisboa3-B) and Q1 were responsible for the great majority of MDR-TB in Portugal [144]. The Lisboa3 cluster was originally described in the 1990s and was highly associated with MDR-TB outbreak in Lisbon [141] and posteriorly with XDR-TB (Lisboa3-B) [143-145]. The Q1

spoligotyping data has revealed that this cluster is related with the B cluster identified in the 1990s outbreak [144].

Therefore, the epidemiological importance of Lisboa 3 and Q1 phylogenetic clades in MDR-TB strains circulating, particularly in the Lisbon Health Region, has been demonstrated over the past two decades [142-144, 146]. The most recent genetic study corroborated that active transmission of MDR-TB, due to the continued transmission of particular genetic clusters strains, is taking place outside and inside Lisbon Health Region. A possibility *de novo* emergence of MDR-TB has also been suggested in this study [147].

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4. Thesis aims

The resurgence of TB and the emergence anti-TB drugs resistance in the early 1990s worldwide also had its impact in Portugal. Since then, very important measures were taken that resulted in improved diagnosis and treatment of MDR-TB. However, throughout the years, genetic studies corroborated the existence of active transmission of MDR-TB, confirmed by the continued transmission of strains within particular genetic clusters, in the Portuguese population, particularly in the Lisbon Health Region. Hence it is crucial to understand MDR-TB transmission to adjust local control strategies in order to prevent and reduce MDR-TB incidence in Portugal ahead of the goal of TB eradication by 2035. We sought to provide this knowledge using genetic methods coupled with epidemiological data and spatial analysis and we conducted a set of studies with the following aims specified ahead.

Aim 1 - To characterize the spatial distribution of MDR-TB in Portugal. A detailed description was performed of the disease distribution across the different regions, along with an epidemiological characterization of the populations affected, paying special attention to the identification of geographical areas or subpopulations with especially high TB and MDR-TB burden.

Aim 2 - **To assess the dynamics of MDR-TB transmission, to establish the rate of recent transmissions and to identify associated risk factors.** High proportion of genetically clustered cases, supported by epidemiological links between them, indicates active transmission of TB within a population. Clustered cases are regarded as being in the same chain of transmission. Breaking these chains is essential for TB control.

Aim 3 - **To evaluate treatment outcomes and factors associated with poor treatment outcomes of MDR-TB patients.** Treatment of TB is focused on both curing the individual patient and minimizing the transmission of Mtb to others. Therefore, successful treatment of MDR-TB leads to clinical and public health benefits such as reduction of both transmission within the population and mortality among patients.

CHAPTER II

USING BAYESIAN SPATIAL MODELS TO MAP AND TO IDENTIFY GEOGRAPHICAL HOTSPOTS OF MULTIDRUG-RESISTANT TUBERCULOSIS IN PORTUGAL BETWEEN 2000 AND 2016

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Using Bayesian spatial models to map and to identify geographical hotspots of multidrug-resistant tuberculosis in Portugal between 2000 and 2016

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Multidrug-resistant tuberculosis (MDR-TB) is a major threat to the eradication of tuberculosis. TB control strategies need to be adapted to the necessities of different countries and adjusted in high-risk areas. In this study, we analysed the spatial distribution of the MDR- and non-MDR-TB cases across municipalities in Continental Portugal between 2000 and 2016. We used Bayesian spatial models to estimate age-standardized notification rates and standardized notification ratios in each area, and to delimitate high- and low-risk areas, those whose standardized notification ratio is significantly above or below the country's average, respectively. The spatial distribution of MDR- and non-MDR-TB was not homogeneous across the country. Age-standardized notification rates of MDR-TB ranged from 0.08 to 1.20 and of non-MDR-TB ranged from 7.73 to 83.03 notifications per 100,000 population across the municipalities. We identified 36 high-risk areas for non-MDR-TB and 8 high-risk areas for MDR-TB, which were simultaneously high-risk areas for non-MDR-TB. We found a moderate correlation (ρ = 0.653; 95% CI 0.457–0.728) between MDR- and non-MDR-TB standardized notification ratios. We found heterogeneity in the spatial distribution of MDR-TB across municipalities and we identified priority areas for intervention against TB. We recommend including geographical criteria in the application of molecular drug resistance to provide early MDR-TB diagnosis, in high-risk areas.

In Europe, the incidence of tuberculosis (TB) has been decreasing since 2008, at a rate of about 5% per year. In 2017 the incidence of TB was 30 new cases per 100,000 population. Although this rate of decline was higher than the global rate of decline of incidence (currently at 2%), it still needs to be improved to achieve the goals of the End TB Strategy[1].

Multidrug-resistant tuberculosis (MDR-TB) is defined as TB caused by strains of *Mycobacterium tuberculosis* resistant to the two most potent first-line anti-TB drugs, isoniazid and rifampicin. It contributes to the difficulty in achieving the goals of the End TB Strategy. MDR-TB treatment requires the use of second-line anti-TB drugs, which are less effective, more toxic and more costly[2], with a lower success rates than standard therapy[1]. Hence, prevention and control of MDR-TB are priorities for elimination of TB[3].

Since the 1990's, when MDR-TB was recognised as a potential threat to TB control, it was considered that in general, drug-resistance was acquired during treatment due to poor quality case management[4]. Currently, the premise that drug-resistant TB is predominantly acquired has changed. The use of new epidemiological tools, such as modelling and molecular techniques, demonstrates that the majority of MDR-TB cases result from transmission of MDR-TB strains rather than selection of de-novo resistance during previous treatment[5,6].

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In 2017, in Europe, MDR-TB was reported for 24% of all TB cases with first-line drug susceptibility testing (DST). This proportion was considerably lower among European Union (EU) countries (4%) compared with non-EU countries (28%). Among pulmonary TB cases, 18% of new and 48% of previously treated cases were MDR-TB[1]. In the same year, in Portugal, the incidence of TB was 16 cases per 100,000 population and 1% of all TB cases were MDR-TB. However, TB incidence was not homogeneous across the country, with 57% of cases in the two largest urban centers, Porto and Lisbon[7]. These cities were previously identified as the most critical regions for TB incidence[8], and pulmonary TB in particular[9,10]. Adaptation of strategies and interventions to national and local contexts is pivotal for effective TB control[11]. This can only be achieved with a detailed understanding of the disease distribution across the different regions, along with an epidemiological characterization of the populations affected, paying special attention to the identification of geographical areas or subpopulations with especially high TB burden[11]. Spatial statistics and disease mapping are effective approaches to investigate the detailed geographical variations in TB incidence[12], being particularly relevant in identifying high- and low-risk areas[8,13].

In the present study, we analysed the spatial distribution of notification of TB in municipalities in Continental Portugal to identify high-risk areas for MDR- and non-MDR-TB. We also assessed the correlation between the spatial distributions of MDR- and non-MDR-TB, highlighting populations that could be major targets for public health authorities to reduce and prevent the incidence of MDR-TB in Portugal.

Methods

Data collection. We used the national TB Surveillance System (SVIG-TB) as the source of data. We analysed all TB cases notified in Continental Portugal from January 2000 until December 2016. According to national regulations, 2 independent sputum samples are collected and tested. TB diagnosis is done either through positive identification using microscopy and nucleic acid amplification or positive *Mycobacterium tuberculosis* (Mtb) culture, followed by conventional first-line DST. All tests are performed in laboratories integrated in the national network, periodically certified and checked. All Mtb strains that have shown resistance to isoniazid and rifampicin at the same time should be tested for second-line anti-TB drugs in the TB National Reference Laboratory (Instituto Nacional de Saúde Ricardo Jorge: INSA). In the case of suspicion of MDR-TB (patients with previous TB treatment that report contact with MDR-TB patients, that belong to specific vulnerable populations, or that are health professionals), clinical samples are submitted to molecular testing for detection of isoniazid and rifampicin resistance.

We selected MDR-TB cases (i.e., resistant to at least isoniazid and rifampicin) and divided all TB cases into two groups: MDR- and non-MDR-TB cases. We obtained notifications of MDR- and non-MDR-TB by municipality (n = 278), year of diagnosis, age (5-year age groups) and sex. Population counts by municipality, year, age (5-year age groups) and sex were obtained from Statistics Portugal (https://www.ine.pt/) for the study period.

Demographic and clinical characteristics of each patient, including age, sex, country of origin, health-related behaviours (e.g. drug or alcohol abuse), HIV status, reclusion (prison confinement), community residence (social housing for people with socio-economic vulnerabilities), homelessness, comorbidity (diabetes and silicosis), previous TB treatment and site of disease, were also collected from SVIG-TB.

Statistical analysis. Descriptive statistics [absolute and relative frequencies or median with interquartile range (IQR)], according to the nature of the variables, were used to describe patient characteristics. We compared these characteristics between patient groups using the Chi-squared test (or Fisher's test, if appropriate) for categorical variables and the Mann–Whitney U-test for continuous variables. In order to control for an effect of the different sample sizes of both groups (MDR and non-MDR, we selected two random samples with 583 cases of the non-MDR to compare with our MDR group).

To estimate age-standardized notification rates in each area and to delimitate high risk and low risk areas, we used hierarchical Bayesian spatial models. These models take into account the spatial autocorrelation and large variance of small areas. To minimize the effect of random fluctuations associated with small number of cases, and because we found no substantial differences in the geographical distribution of non-MDR and MDR TB across our study period, we considered the average rates of the 17-year study period. We assumed that the response variable, cases of TB (O_i) in each ith area, follows a Poisson distribution where E_i is the expected number of cases and θ_i the relative risk (RR), or Standardized Notification Rate (Eqs. 1 and 2). We used the Portuguese TB notification rates by sex and age group (5-year age groups) as a reference to compute the expected number of cases, according to the indirect method of standardization. The expected number of cases was obtained by summing the product of the age-sex specific notification rates of the standard population (in our study Portugal) by the population by age and sex of each Portuguese municipality.

$$O_i \sim Poisson(E_i, \theta_i), \tag{1}$$

$$log(\theta_i) = \alpha + s_i. \tag{2}$$

Here α is an intercept quantifying the average number of TB cases in the 278 areas. The area specific effect s_i was modelled considering a BYM model[14] with a parameterization suggested by Dean et al.[15] (Eq. 3)

$$s_i = \tau\left(\sqrt{\varphi} \times u_i + \sqrt{1-\varphi} \times v_i\right), \tag{3}$$

where u_i is the structured effect and v_i is the unstructured effect. The u_i effect was scaled in order to render the model more intuitive and interpretable[16], so that φ expresses the proportion of the spatial effect due to the structured part and $1/\tau$ is the marginal variance of s_i.

Additionally, we used the function 'excursions' to delimitate high risk and low risk areas[8,17,18]. High-risk areas are those whose standardized notification ratio is significantly above 1 (i.e., above the country's average) and low risk areas are those whose standardized notification ratio is significantly below 1 (i.e., below the country's average). This method uses the posterior joint distribution computed from the Integrated Nested Laplace Approximation (INLA) and takes into account the dependence structure, allowing to accurately identify areas where the notification ratio is greater than zero.

To analyse the correlation between MDR-TB and non-MDR-TB, the Pearson correlation coefficient (r and corresponding 95% Credible Intervals, 95% CrI) was computed based on the standardized notification ratios of MDR-TB and non-MDR-TB derived from the previously described models.

To facilitate interpretation, standardized notification ratios were converted into rates per 100,000 inhabitants by multiplying the standardized notification ratios by the crude national notification rates.

Statistical analyses were performed using SPSS version 18.0 (PASW Statistics 18), and p-values below 0.05 were considered statistically significant.

Posterior distributions were obtained using the INLA, which was implemented in the R INLA library[19].

Standardized notification rates and high and low risk areas were mapped using ArcMap release 10.5.1. (Environmental Systems Research Institute, Redlands, CA, USA).

Ethical considerations. Ethical approval and informed consent were not required, as the patient data, collected for an official national surveillance system, were anonymized in accordance with the research ethical guidelines in Portugal. Authorization for its use in the present manuscript was given by the National program for Tuberculosis.

Results

We evaluated 53,417 TB cases, notified in Continental Portugal during the study period (2000–2016) (Supplementary Table S1). We identified 583 (1.1%) cases of MDR-TB. We compared demographic and clinical characteristics between MDR- and non-MDR-TB patients. We observed that MDR-TB patients were younger (40.0 years vs. 42.0 years) and were more likely to be foreign-born (27.3% vs. 13.6%), infected with HIV (27.8% vs. 13.1%), alcohol abusers (24.5% vs. 15.0%), injectable drug users (20.3% vs. 10.3%), prisoners (6.2% vs. 2.3%), homeless (3.7% vs. 1.8%) and having a history of previous TB treatment (40.3% vs. 9.9%) than non-MDR-TB patients (Table 1). The same statistical differences were obtained with two randomized samples I and II of the non-MDR-TB with similar size as the MDR-TB group (Supplementary Table S2).

The crude non-MDR-TB notification rate was 31.19 notifications per 100,000 population (95% CrI 30.93–31.46) and the crude MDR-TB notification rate was 0.34 notifications per 100,000 population (95% CrI 0.32–0.37). Geographical differences in reporting were observed.

The spatial distribution of the age-standardised notification rates of non-MDR-TB is depicted in Fig. 1A with the delimitation of the high- and low-risk areas given in Fig. 1B. Age-standardized notification rates of non-MDR TB ranged from 7.73 to 83.03 notifications per 100,000 population. We identified 36 high-risk areas, mostly located in Porto and Lisbon metropolitan areas, and also in the southern regions of Alentejo and the Algarve (Fig. 1B). The spatial distribution of the age-standardized notification rates of MDR-TB is shown in Fig. 1C and the delimitation of the high- and low-risk areas is shown in Fig. 1D. Age-standardized notification rates ranged from 0.08 to 1.20 notifications per 100,000 population. Eight high-risk areas for MDR-TB were located mostly in the Lisbon metropolitan area (Fig. 1D). These 8 high-risk areas were also high-risk areas for non-MDR-TB. Only 22% (8/36) of the high-risk areas for non-MDR-TB were high-risk areas for MDR-TB (Supplementary Table S3). In order to confirm the stability of the inferred high-risk areas through the entire dataset, we performed the analysis on a time series across the 17 years. We obtained stable patterns for the geographical locations of risk areas (Supplementary Figs. S1 and S2).

We analysed the correlation between MDR- and non-MDR-TB standardized notification ratios and found a moderate correlation ($\rho = 0.653$; 95% CrI 0.457–0.728) between them.

Since only some areas with a high-risk for non-MDR-TB also have a high-risk for MDR-TB (Supplementary Table S3), we compared demographic and clinical characteristics of the non-MDR-TB patients from high-risk areas for only non-MDR-TB (28 areas) with patients from areas, which are also high-risk areas for MDR-TB (8 areas) to determine factors that could be associated with the risk for MDR-TB. We observed that the patients from high-risk areas for both MDR- and non-MDR-TB were younger (40.0 years vs. 42.0 years) than patients from areas with highest-risk for only non-MDR-TB. Among them, there was a higher proportion of females (34.6% vs. 31.3%), foreign-born patients (25.5% vs. 7.2%), HIV infection (20.9% vs. 12.2%), alcohol abusers (17.4% vs. 13.9%), injectable drugs users (16.3% vs. 10.2%), prisoners (4.0% vs. 1.5%), community residents (4.9% vs. 2.4%), homeless persons (3.3% vs. 1.1%), cases of extra-pulmonary disease (28.3% vs. 24.5%) and cases with a history of previous TB treatment (12.6% vs. 10.0%) (Table 2).

Discussion

In this study, we combined the epidemiological characteristics of MDR- (resistant to at least isoniazid and rifampicin) and non-MDR-TB (all other TB) patients, over a 17-year period, with a detailed spatial description to identify high- and low-risk areas, to obtain a systematic comparison between MDR- and non-MDR-TB high-risk areas across Portugal.

Patient's characteristics	Total n	MDR-TB		Non-MDR-TB		p-value
		n[a]	IQR or %	n[a]	IQR or %	
Age (years)						
Median (IQR)	53,417	40.0	19	42.0	26	0.002[b]
Gender						
Female	53,417	174	29.8	17,282	32.7	0.155
Male		409	70.7	35,552	67.3	
Country of origin						
Foreign-born	53338[g]	159	27.3	7184	13.7	< 0.001
Native		424	72.2	45,571	86.3	
HIV status						
Negative	53,417	421	72.2	45,929	86.9	< 0.001
Positive		162	27.8	6905	13.1	
Alcohol abuse[f]						
No	48775[g]	386	75.5	41,045	85.0	< 0.001
Yes		125	24.5	7219	15.0	
Injectable drug use[f]						
No	49337[g]	415	79.7	43,780	89.7	< 0.001
Yes		106	20.3	5036	10.3	
Reclusion[c]						
No	49482[g]	481	93.8	47,836	97.7	< 0.001
Yes		32	6.2	1133	2.3	
Community residence[d]						
No	49271[g]	480	94.6	47,011	96.4	0.084
Yes		26	5.1	1754	3.6	
Homelessness						
No	49436[g]	490	96.3	48,058	98.2	0.002
Yes		19	3.7	869	1.8	
Diabetes						
No	53,417	557	95.5	50,128	94.9	0.531
Yes		26	4.5	2706	5.1	
Silicosis						
No	53,417	578	99.1	52,414	99.2	0.812
Yes		5	0.9	420	0.8	
Previous TB treatment						
No	53,417	348	59.7	47,615	90.1	< 0.001
Yes		235	40.3	5219	9.9	
Site of disease						
Pulmonary	53263[g]	530	91.1	38,484	73.1	< 0.001
Extra-pulmonary		52	8.9	14,197	26.9	

Table 1. Characteristics of multidrug-resistant tuberculosis (MDR-TB) and non-MDR-TB patients, from cases in Continental Portugal, for the years of 2000–2016. *TB* tuberculosis, *MDR-TB* multidrug-resistant tuberculosis, *IQR* interquartile range, *HIV* human immunodeficiency virus. [a]Not applicable for age. [b]Mann–Whitney U-test. [c]Prison confinement. [d]Social housing for people with socio-economic vulnerabilities. [e]Data missing for: country of origin (n = 79; 0.1%), alcohol abuse (n = 4642; 8.7%), injectable drug use (n = 4080; 7.6%), reclusion (n = 3935; 7.4%), community residence (n = 4146; 7.8%), homelessness (n = 3981; 7.5%), site of disease (n = 154; 0.3%). [f]Self-reported.

We demonstrated significant heterogeneity in the spatial distribution of the age-standardized notification rates of MDR- and non-MDR-TB at the municipality level. We found a moderate correlation between MDR- and non-MDR-TB standardized notification ratios. We identified 36 high-risk areas for non-MDR-TB and 8 high-risk areas for MDR-TB.

In our study period (2000–2016), the spatial distribution of the age-standardised notification rates of non-MDR-TB ranged from 7.73 to 83.03 notifications per 100,000 population. A high degree of heterogeneity in spatial TB distribution was expected as previously reported in national[8–10] and international[12] spatial studies. The spatial distribution of the age-standardised notification rates of MDR-TB was also heterogeneous (up to fifteen times difference), ranging from 0.08 to 1.20 notifications per 100,000 population across municipalities.

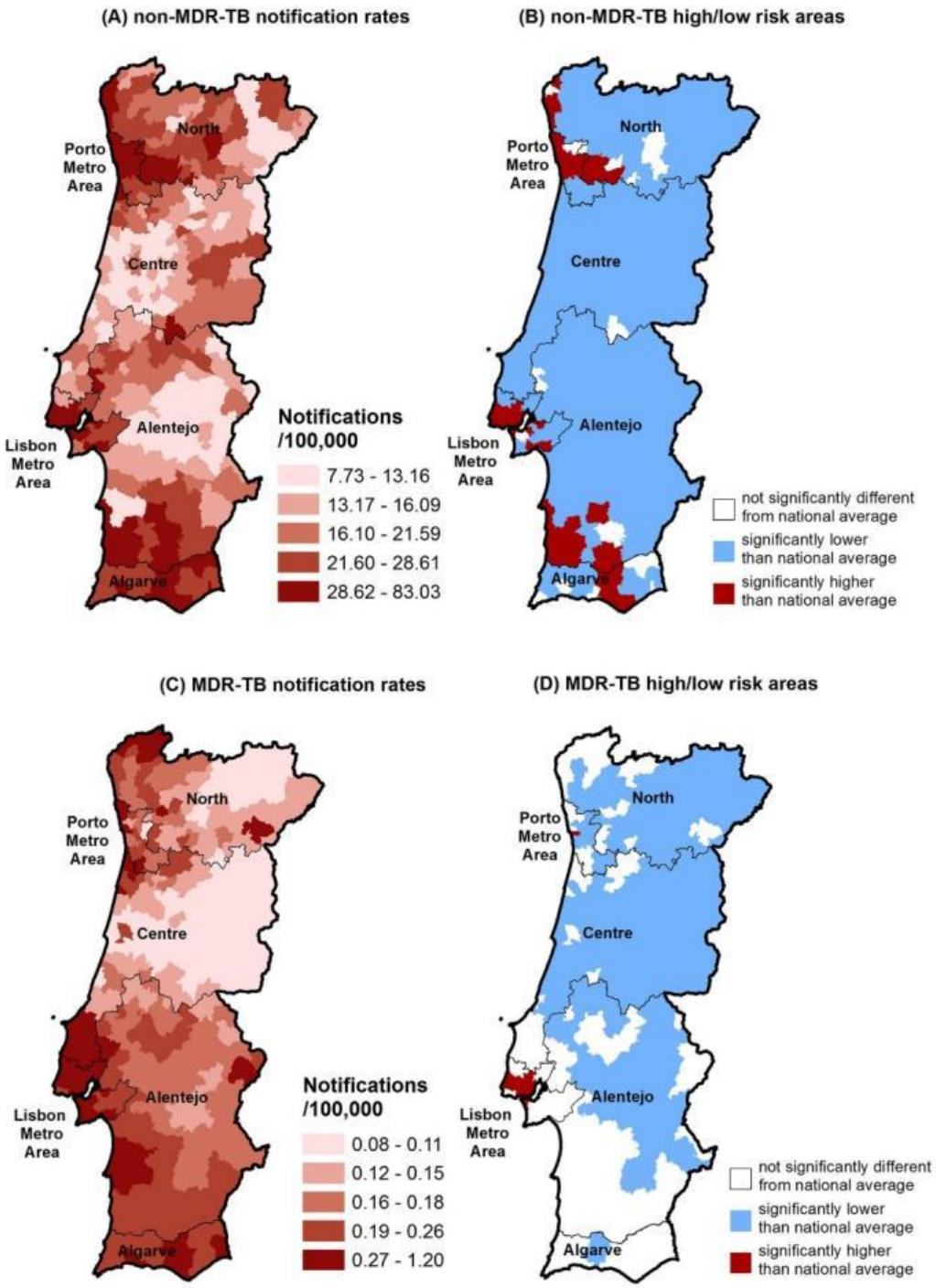

Figure 1. Spatial distribution of the age-standardized notification rates of non-MDR-TB (**A**) and the corresponding delimitation of the high- and low-risk areas (**B**). Spatial distribution of the age-standardized notification rates of MDR-TB (**C**) and the corresponding delimitation of the high- and low-risk areas (**D**). *MDR-TB* multidrug-resistant tuberculosis; high-risk areas are those whose standardized notification ratio is significantly above 1 (i.e., above the country's average); low risk areas are those whose standardized notification ratio is significantly below 1 (i.e., below the country's average).

Patient's characteristics	Total n	High-risk areas for non-MDR-TB but not for MDR-TB		High-risk areas for both MDR- and non-MDR-TB		p-value
		n[a]	IQR or %	n[a]	IQR or %	
Age (years)						
Median (IQR)	32,114	42.0	25	40.0	24	<0.001[b]
Gender						
Female	32,114	4816	31.3	5793	34.6	<0.001
Male		10,562	68.7	10,943	65.4	
Country of origin						
Foreign-born	32,077	1107	7.2	4266	25.5	<0.001
Native		14,255	92.8	12,449	74.5	
HIV status						
Negative	32,114	13,508	87.8	13,240	79.1	<0.001
Positive		1870	12.2	3496	20.9	
Alcohol abuse[f]						
No	28970[e]	11,867	86.1	12,544	82.6	<0.001
Yes		1911	13.9	2648	17.4	
Injectable drug use[f]						
No	29480[e]	12,655	89.8	12,891	83.7	<0.001
Yes		1431	10.2	2503	16.3	
Reclusion[c]						
No	29152[e]	13,895	98.5	14,440	96.0	<0.001
Yes		217	1.5	600	4.0	
Community residence[d]						
No	29005[e]	13,687	97.6	14,259	95.1	<0.001
Yes		331	2.4	728	4.9	
Homelessness						
No	29136[e]	13,940	98.9	14,549	96.7	<0.001
Yes		148	1.1	499	3.3	
Diabetes						
No	32,114	14,654	95.3	15,892	95.0	0.172
Yes		724	4.7	844	5.0	
Silicosis						
No	32,114	15,161	98.6	16,720	99.9	<0.001
Yes		217	1.4	16	0.1	
Previous TB treatment						
No	32,114	13,844	90.0	14,631	87.4	<0.001
Yes		1534	10.0	2105	12.6	
Site of disease						
Pulmonary	32011[e]	11,574	75.5	11,961	71.7	<0.001
Extra-pulmonary		3752	24.5	4724	28.3	

Table 2. Comparison of the characteristics of TB patients between those in high-risk areas only for non-MDR-TB and those in high–risk areas for both MDR- and non-MDR-TB, Continental Portugal, 2000–2016. *TB* tuberculosis, *MDR-TB* multidrug-resistant tuberculosis, *IQR* interquartile range, *HIV* human immunodeficiency virus. [a]Not applicable for age. [b]Mann–Whitney U-test. [c]Prison confinement. [d]Social housing for people with socio-economic vulnerabilities. [e]Data missing for: country of origin (n = 37; 0.1%), alcohol abuse (n = 3144; 9.8%), injectable drug use (n = 2634; 8.2%), reclusion (n = 2962; 9.2%), community residence (n = 3109; 9.7%), homelessness (n = 2978; 9.3%), site of disease (n = 103; 0.3%). [f]Self-reported.

The pronounced spatial heterogeneity of MDR-TB burden has been observed in Moldova (the notified incidence of MDR-TB ranged from 0.5 to 27.2 cases per 100,000 population)[20], China (where the proportion of incident MDR-TB cases varied between 3 and 30%)[21] and Ethiopia (where the standardized morbidity ratio ranged from 0 to 7.0)[22].

We found a moderate correlation between MDR- and non-MDR-TB. We identified 36 high-risk areas for non-MDR-TB and 8 high-risk areas for MDR-TB, which were simultaneously high-risk areas for non-MDR-TB. It was expected that MDR-TB risk areas were comparable with non-MDR-TB risk areas, due to the high probability of acquisition of drug resistance during treatment for TB and the transmission of existing MDR-TB strains in areas with higher rate of transmission of non-MDR-TB. However, only 22% (8/36) of the non-MDR-TB

high-risk areas were also MDR-TB high-risk areas. We compared non-MDR-TB patients from high-risk areas for non-MDR-TB with patients from high-risk areas for both MDR- and non-MDR-TB. Among the characteristics which were most common among the patients from high-risk areas for both MDR- and non-MDR-TB, being HIV infected[23–26], being foreign-born[6,23], homelessness[27] and having history of imprisonment[6], consumption of alcohol[6,25] and injectable drug use[6] have been previously reported as factors associated with MDR-TB development. Previous TB treatment is particularly important risk factor for MDR-TB[23–27].

The role of HIV infection as risk factor for MDR-TB has been inconsistent. In several studies, an association between HIV and MDR-TB disease was not significant or was negative[27,28]. This association was stronger for transmitted than acquired MDR-TB[29,30].

Regarding previous TB treatment, in our study, 40% of MDR-TB patients were previously exposed to anti-tuberculosis drugs. These cannot be assumed to have acquired resistance during treatment. As previously described, 61% of the incidence of MDR-TB among previously treated patients resulted from MDR-TB transmissio[5]. In fact, genetic studies[31–33] suggested that a high percentage of these cases in Portugal were related with the transmission of two stable MDR-TB clusters.

Regarding hotspots of MDR-TB, 7 out of the 8 high-risk areas are located in the Lisbon metropolitan area. Previously identified MDR-TB genetic clusters revealed evidence of transmission of multidrug-resistant strains in this region[31–33].

The strengths of this study are the robust statistical methods used to characterise geographic patterns, taking advantage of the epidemiological characterization of the population over a significant amount of time. This allowed the identification of risk areas for MDR-TB, which are areas for priority action and intervention for the existing national TB control program. We complemented the spatial analysis with quality-assured laboratory data and a detailed epidemiological characterization to evaluate potential risk factors for MDR-TB in the TB risk areas. One possible study limitation is its retrospective design using the national notification system, which limited us in the analysis of the study variables.

In conclusion, we found heterogeneity in the spatial distribution of MDR-TB across municipalities in Portugal. We identified priority areas for intervention against MDR-TB. Our findings suggest that in addition to the development of MDR-TB, transmission of MDR-TB strains occurs in these areas. We propose the inclusion of geographical criteria in the application of molecular drug resistance testing, paying particular attention to screening and early MDR-TB diagnosis in these areas and the performance of routine genotyping of all TB isolates to understand the dynamics of MDR-TB emergence and transmission.

Data availability

The epidemiological and geographical datasets generated during the current study are available from the corresponding author on reasonable request.

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Author contributions
O.O. and A.I.R. conceived and designed the study; O.O., A.I.R. and E.T.K. performed statistical analysis; O.O., T.R., M.C.-N. and R.D. evaluated and interpreted the data; O.O., A.I.R., E.T.K. and T.R. drafted the manuscript and all authors critically reviewed it.

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Competing interests
The authors declare no competing interests.

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Using Bayesian spatial models to map and to identify geographical hotspots of multidrug-resistant tuberculosis in Portugal between 2000 and 2016

Olena Oliveira, Ana Isabel Ribeiro, Elias Teixeira Krainski, Teresa Rito, Raquel Duarte, Margarida Correia-Neves

Supplementary Table S1. The absolute annual number of multidrug-resistant tuberculosis (MDR-TB) and non-MDR-TB cases, in Continental Portugal, from 2000 to 2016.

Year	MDR-TB	Non-MDR-TB	Total
2000	50	4417	4467
2001	43	4315	4358
2002	47	4528	4575
2003	43	4159	4202
2004	56	3804	3860
2005	45	3536	3581
2006	31	3390	3421
2007	47	3078	3125
2008	30	2851	2881
2009	26	2743	2769
2010	28	2622	2650
2011	33	2499	2532
2012	23	2510	2533
2013	16	2312	2328
2014	25	2160	2185
2015	22	2076	2098
2016	18	1834	1852
Total	**583**	**52834**	**53417**

Supplementary Table S2. Comparison of the patient's characteristics between groups: MDR-TB (583 cases) with non-MDR-TB random sample I (583 cases) and MDR-TB (583 cases) with non-MDR-TB random sample II (583 cases).

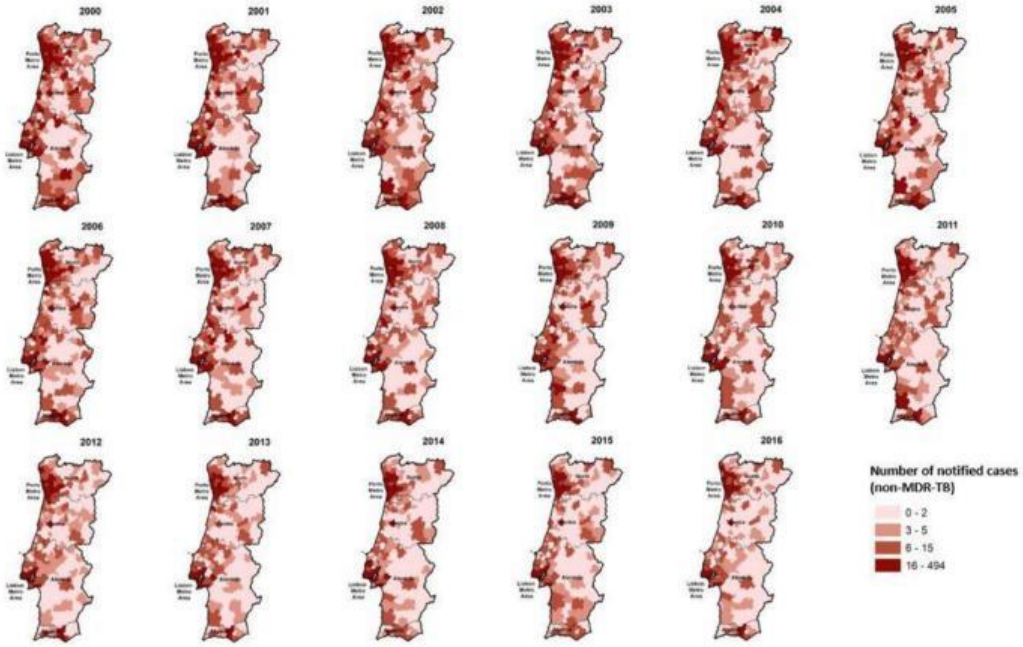
Patient's characteristics		MDR-TB n (IQR or %)	Non-MDR-TB random sample I n (IQR or %)	*p-value*	Non-MDR-TB random sample II n (IQR or %)	*p-value*
Age (years)	Median (IQR)	40.0 (19)	43.0 (26)	0.015	43.0 (26)	0.004
Gender	Female	174 (29.8)	199 (34.1)	0.132	198 (34.0)	0.148
	Male	409 (70.2)	384 (65.9)		385 (66.0)	
Country of origin	Foreign-born	159 (27.3)	85 (14.6)	<0.001	79 (13.6)	<0.001
	Native	424 (72.7)	498 (85.4)		504 (86.4)	
HIV status	Negative	421 (72.2)	522 (89.5)	<0.001	514 (88.2)	<0.001
	Positive	162 (27.8)	61 (10.5)		69 (11.8)	
Alcohol abuse	No	386 (75.5)	436 (82.1)	0. 012	457 (86.1)	<0.001
	Yes	125 (24.5)	95 (17.9)		74 (13.9)	
Injectable drug use	No	415 (79.7)	481 (89.6)	<0.001	492 (91.1)	<0.001
	Yes	106 (20.3)	56 (10.4)		48 (8.9)	
Reclusion	No	481 (93.8)	523 (98.1)	0.001	529 (98.1)	0.001
	Yes	32 (6.2)	10 (1.9)		10 (1.9)	
Community residence	No	480 (94.6)	514 (96.6)	0.212	523 (96.7)	0.192
	Yes	26 (5.1)	18 (3.4)		18 (3.3)	
Homelessness	No	490 (96.3)	524 (98.3)	0.065	532 (98.0)	0.140
	Yes	19 (3.7)	9 (1.7)		11 (2.0)	
Diabetes	No	557 (95.5)	556 (95.4)	1.000	551 (94.5)	0.501
	Yes	26 (4.5)	27 (4.6)		32 (5.5)	
Silicose	No	578 (99.1)	578 (99.1)	1.000	578 (99.1)	1.000
	Yes	5 (0.9)	5 (0.9)		5 (0.9)	
Previous TB treatment	No	348 (59.7)	515 (88.3)	<0.001	529 (90.7)	<0.001
	Yes	235 (40.3)	68 (11.7)		54 (9.3)	
Site of disease	Pulmonary	530 (91.1)	422 (72.5)	<0.001	415 (71.3)	<0.001
	Extra-pulmonary	52 (8.9)	160 (27.5)		167 (28.7)	

TB=tuberculosis; MDR-TB= multidrug- resistant tuberculosis; n= number of cases; IQR= interquartile range; HIV =human immunodeficiency virus.

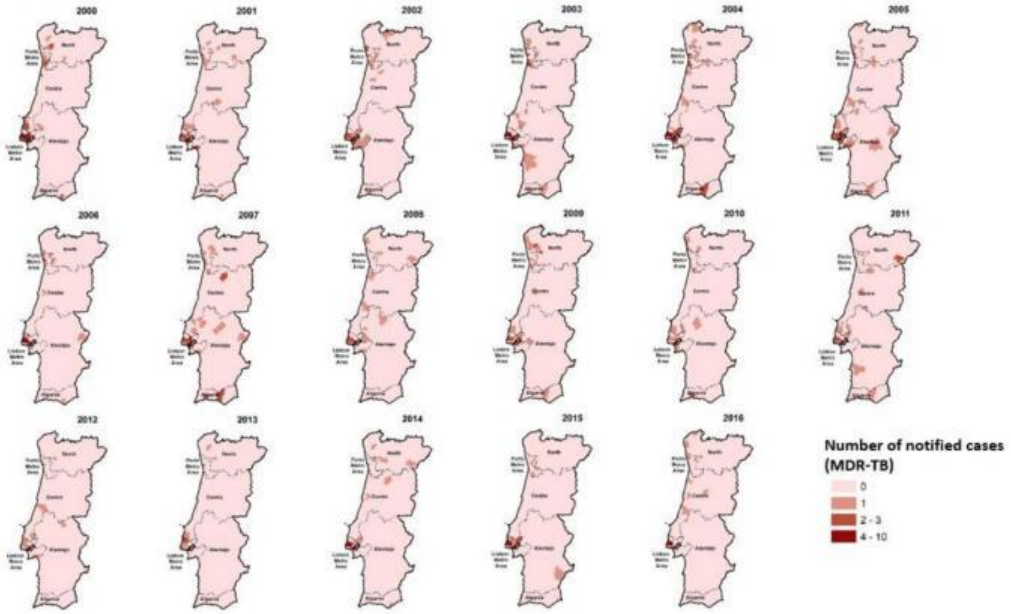
Supplementary Table S3. High-risk areas (standardized notification ratio is significantly above 1, i.e., above the country's average) for non-MDR-TB and MDR-TB, Continental Portugal, 2000-2016.

Region	Municipality	Non-MDR-TB (snr)	MDR-TB (snr)
NORTH	Vila Nova de Cerveira	1.21	nd
	Viana do Castelo	1.10	nd
	Esposende	1.32	nd
	Póvoa de Varzim[a]	1.67	nd
	Vila do Conde[a]	1.84	nd
	Gondomar[a]	1.57	nd
	Vila Nova de Gaia[a]	1.20	nd
	Maia[a]	1.27	nd
	Matosinhos[a]	1.64	nd
	Paredes[a]	1.25	nd
	Penafiel	2.66	nd
	Porto[a,b]	2.24	1.71
	Valongo[a]	1.61	nd
	Marco de Canaveses	1.98	nd
	Espinho[a]	1.24	nd
	Castelo de Paiva	1.14	nd
	Cinfães	1.39	nd
	Resende	1.69	nd
LISBON Metropolitan Area	Cascais	1.20	nd
	Lisboa[b]	1.81	3.49
	Loures[b]	1.25	2.12
	Oeiras[b]	1.32	1.90
	Sintra[b]	1.18	2.50
	Amadora[b]	1.89	2.99
	Odivelas[b]	1.24	2.97
	Almada[b]	1.42	1.79
	Alcochete	1.26	nd
	Moita	1.28	nd
	Setúbal	1.25	nd
ALENTEJO	Sines	1.23	nd
	Aljustrel	1.38	nd
	Almodôvar	1.36	nd
	Odemira	1.08	nd
ALGARVE	Faro	1.18	nd
	Loulé	1.37	nd
	Olhão	1.06	nd

TB=tuberculosis; MDR-TB= multidrug- resistant tuberculosis
snr: standardized notification ratio; nd: not significantly different from country's average.
[a] Porto Metropolitan Area.
[b] High-risk areas for both MDR- and non-MDR-TB.



Supplementary Figure S1. Maps depicting a time series of the number of non-MDR-TB cases by municipality year by year, Continental Portugal, 2000-2016. TB=tuberculosis; MDR-TB= multidrug- resistant tuberculosis; the colour code displays the number of notified cases, from 0 to 494 cases.



Supplementary Figure S2. Maps depicting a time series of the number of MDR-TB cases by municipality year by year, Continental Portugal, 2000-2016. TB=tuberculosis; MDR-TB= multidrug- resistant tuberculosis; the colour code displays the number of notified cases, from 0 to 10 cases.

CHAPTER III

A NATIONWIDE STUDY OF MULTIDRUG-RESISTANT TUBERCULOSIS IN PORTUGAL 2014–2017 USING EPIDEMIOLOGICAL AND MOLECULAR CLUSTERING ANALYSES

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RESEARCH ARTICLE Open Access

A nationwide study of multidrug-resistant tuberculosis in Portugal 2014–2017 using epidemiological and molecular clustering analyses

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Abstract

Background: Increasing multidrug-resistant tuberculosis (MDR-TB) incidence is a major threat against TB eradication worldwide. We aim to conduct a detailed MDR-TB study in Portugal, an European country with endemic TB, combining genetic analysis and epidemiological data, in order to assess the efficiency of public health containment of MRD-TB in the country.

Methods: We used published MIRU-VNTR data, that we reanalysed using a phylogenetic analysis to better describe MDR-TB cases transmission occurring in Portugal from 2014 to 2017, further enriched with epidemiological data of these cases.

Results: We show an MDR-TB transmission scenario, where MDR strains likely arose and are transmitted within local chains. 63% of strains were clustered, suggesting high primary transmission (estimated as 50% using MIRU-VNTR data and 15% considering epidemiological links). These values are higher than those observed across Europe and even for sensitive strains in Portugal using similar methodologies. MDR-TB cases are associated with individuals born in Portugal and evolutionary analysis suggests a local evolution of strains. Consistently the sublineage LAM, the most common in sensitive strains in Europe, is the more frequent in Portugal in contrast with the remaining European MDR-TB picture where immigrant-associated Beijing strains are more common.

Conclusions: Despite efforts to track and contain MDR-TB strains in Portugal, their transmission patterns are still as uncontrolled as that of sensitive strains, stressing the need to reinforce surveillance and containment strategies.

Keywords: Multidrug-resistant tuberculosis, Epidemiology, Transmission, Risk factor

Background

Tuberculosis (TB) remains a high burden disease worldwide with persistent areas where elimination is still a distant goal. Despite declining incidence of TB in the last decades, multidrug-resistant TB (MDR-TB) poses a major threat for WHO's 2035 goal of TB elimination [1]. MDR-TB, defined by resistance of *Mycobacterium tuberculosis* (Mtb) to isoniazid and rifampicin (RR), emerges as consequence of ineffective treatment or incompletion or inappropriate following up of the cases, translating into the evolution of resistant strains with consequent increments in patient morbidity and mortality, and further transmission [2].

WHO estimated a worldwide incidence of around 560,000 cases of MDR/RR-TB per year and a rate of 7.4 cases per 100,000 individuals [3]. In Europe, MDR/RR-TB incidence rate was 12.0 per 100,000 individuals, the highest among the regions considered by WHO [3], but results

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could be biased towards regions with more detailed monitoring programmes on drug-susceptibility testing (DST). MDR-TB notification rate in Portugal has been increasing since 2012 at a rate of 0.8 and 3.7% among new cases and previously treated patients, respectively [4].

TB incidence has been decreasing in Portugal. In 2017, TB notification-rate was 17.8 per 100,000 individuals [5], however 24.8 and 26.0 in the large urban centres of Lisbon and Porto respectively. The proportion of MDR-TB remains at 1% of all TB cases. 80% of MDR-TB patients had no previous TB treatment [5], suggesting mostly primary transmission of MDR strains but without supporting epidemiological studies confirming this scenario.

Previous genetic studies, evaluating clinical isolates collected in Lisbon Health Region, reported high prevalence of MDR-TB with most cases concentrating in two monophyletic clades (Lisbon 3 and Q1) [6, 7]. A database developed by Perdigão and colleagues [8] constitutes the largest collaborative effort to catalogue Mtb diversity in Portuguese-speaking countries. It includes 423 MDR-TB isolates (129 from Portugal) within a larger dataset of 1447 clinical samples, validating Latin-American-Mediterranean (LAM: lineage 4) as the most common sub-lineage in Portugal. These studies are mostly from Lisbon hospitals [6–8], but information on epidemiological characteristics of the cases is lacking, undermining the design of control strategies for preventing and reducing MDR-TB incidence [9].

Although MIRU-VNTR is becoming outdated in the study of TB transmission, mostly due to the overestimation of recent transmission [10–15], it is nevertheless the most used genotyping method implemented across Europe by Public Health authorities in a recent survey [16]. Although conclusions on transmission should be performed with extreme caution, the large body of work accumulated for MIRU-VNTR across Europe and worldwide allows statistics to be methodically compared for regions and scenarios still lacking whole genome data.

In this study we combined an in depth genetic analysis of strain genotyping already published from Portugal [10], with epidemiological data from MDR-TB cases diagnosed between 2014 and 2017, collected as part of the routine functions of Public Health services. We aim to assess the dynamics of MDR-TB emergence and transmission, including the identification of associated risk factors, and last to establish the rate of probable recent transmissions against newly developed resistant strains. These statistics will be compared with results for sensitive strains in Portugal and other MDR-TB transmission scenarios in other developed countries using similar approaches in order to assess the relative efficiency of public health measures for containing MDR-TB in Portugal.

Methods

Data collection

We identified and extracted data from all culture-confirmed MDR-TB cases, diagnosed between 2014 and 2017, from the national TB Surveillance System (SVIG-TB), containing epidemiological, clinical and laboratory data. Epidemiological information collected includes gender, age at time of TB diagnosis, country of origin, place of residence (parish), presence or absence of alcohol or drug misuse, HIV status, description of previous TB treatment and clinical characteristics of TB presentation (site of disease). Information about previous contact of patients with other TB cases was provided by Public Health services, using as linking variables date of birth, sex and place of residence, in order to identify possible epidemiological links between MDR-TB cases.

Mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) genotypes for sensitive and MDR strains were collected from genotypic works that characterised strains isolated from Portugal, namely [7, 17, 18]. Linked epidemiological and MIRU-VNTR data was only possible to MDR-TB cases described between 2014 and 2017.

Ethical approval

This work was carried out in accordance with the recommendations by the Ethics Sub-commission of Life and Health Sciences from the University of Minho (SECVS 135/2015), by the Ethics Committee for Health of Lisbon and Tagus Valley Region Health Administration (9854/CES/2018) and the Ethics Committee for Health of the Northern Region Health Administration (ARSN 127/2018). All procedures were in accordance with the ethical standards of the responsible committees and with the Helsinki Declaration, as revised in 2008.

Cluster analysis

Clustering analysis was performed as described before in [19]: a cluster was defined as two or more cases with the same MIRU-VNTR profiles. The proportion of recent transmission was calculated by the "n minus one" method [20], using number of cases clustered – number of clusters/number of cases with a strain type.

An epidemiological link was defined between two cases when cases had identified others as contacts, or when cases shared a family or household connection. "Possible" epidemiological links were defined as cases from the same geographical area, with common social or behavioural traits (e.g. workplace, drug use). The number of epidemiological links was used to recalculate an adjusted proportion of recent transmission taking into consideration only those clustered cases further supported by an epidemiological link.

Evolutionary analysis of MDR-TB strains

The phylogenetic reconstruction of MIRU-VNTR profiles was done using median networks [21]. We used two algorithms consecutively implemented in the network software (fluxus engineering), the reduced median followed by the median joining algorithm, as previously described in [18]. We used this hybrid approach weighting the 24 loci according to their allelic diversity [22].

Isolates that present similar MIRU genotypes were referred as genotypically clustered, representing potential episodes of direct TB transmission. Networks were used to construct the most parsimonious trees. Cladograms were visualized in Figtree v.1.3.3 (http://tree.bio.ed.ac.uk/software/figtree/). The classification of the strains in lineages was done using miruvntrplus (http://www.miruvntrplus.org).

Statistical analysis

Data were summarized by descriptive statistics (absolute and relative frequencies or median and range) according to the nature of the variables. Chi-square or Fisher's exact tests were used to evaluate the independence between two categorical variables, while Mann–Whitney U-test compared the distributions of two independent continuous variables. We identified risk factors associated with MDR-TB transmission, comparing patient's socio-demographic and clinical characteristics associated with clustering, that were investigated using logistic regression (comparing cases with unique genotypes against those within clusters). Statistical analyses were performed with SPSS version 18.0 (PASW Statistics 18), and p-values below 0.05 were considered statistically significant.

Results

MDR-TB epidemiology

In Portugal, from January 2014 until December 2017, 8133 TB cases were notified, of which 4175 (51.3%) cases were culture-confirmed and had results of first-line DST (Fig. 1), identifying 77 MDR-TB cases (~ 1% of total cases).

We compared patients with drug-sensitive TB and MDR-TB in the same period (Fig. 2, Additional file 2: Table S1). Patients with MDR-TB were younger: the median age was 43 years (range 20–75) and 55.8% were below 45 years. MDR-TB patients presented higher frequencies of foreign-born individuals (32.9% vs. 15.3%), HIV infected (24.7% vs. 8.0%), alcohol abusers (23.9% vs. 13.2%), injectable drugs users (14.7% vs. 4.6%) and with previous TB treatment (32.9% vs. 7.5%). Among the foreign-born patients, 11 (44%) cases entered the country in the two previous years.

Cluster analysis and potential transmission links

Among 67 MDR-TB cases, 46 cases (69%) were LAM, eight (12%) cases were sublineage Haarlem, two (3%) URAL strains and one (1%) were sublineage X-type. Ten (15%) isolates belonged to Lineage 2, sublineage Beijing. 42 cases were identified in seven MIRU-VNTR clusters ranging from 2 to 14 cases (Table 1; Fig. 3). These MIRU-VNTR clusters showed good correspondence to previously defined clusters on the whole genome level [23] and each MIRU-defined cluster has a set of defined specific mutations in drug-resistance associated genes attesting for their significance as clades. The three largest clusters [5–7], and contained 10, 6 and 14 cases, respectively, with most of these corresponding to Portugal-born individuals, with pulmonary disease, diagnosed in the LTV region. Twenty-five cases presented

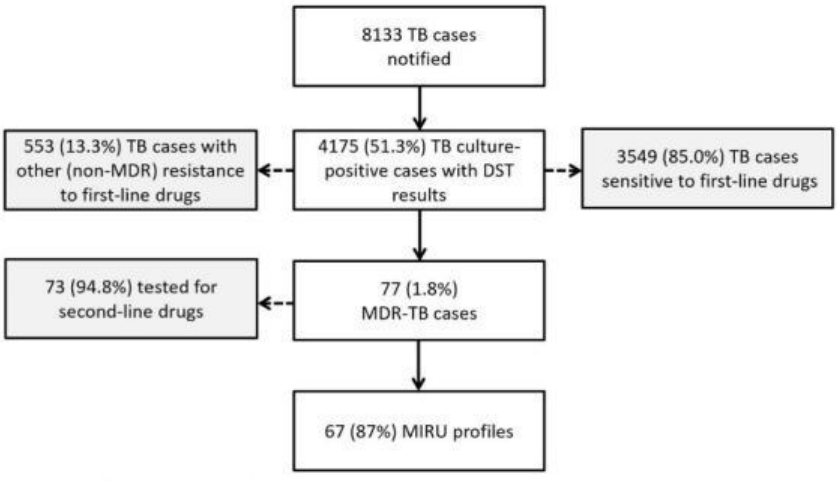

Fig. 1 Tuberculosis cases notified and proportion of multidrug-resistant tuberculosis cases identified in Portugal between 2014 and 2017

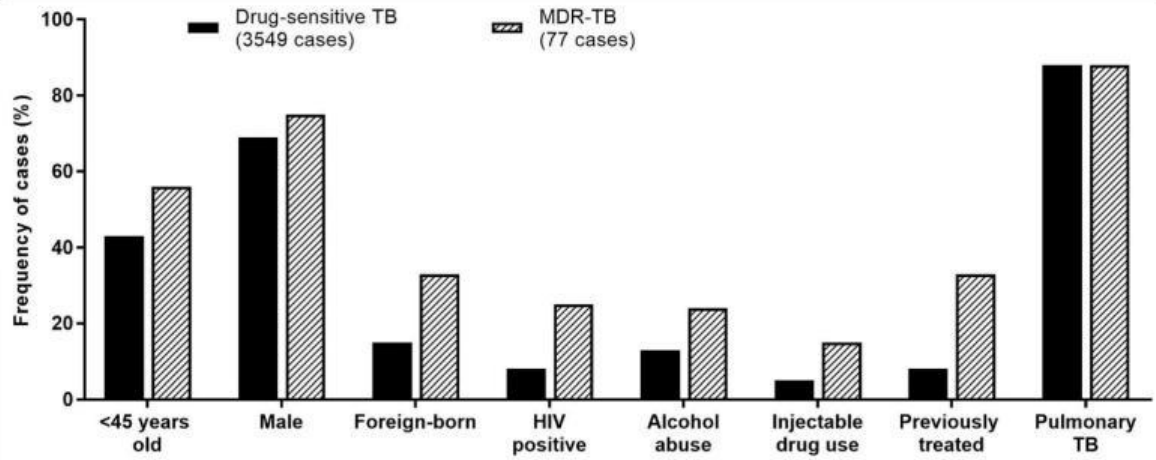

Fig. 2 Differences in prevalence of socio-demographic and clinical characteristics between patients with drug-sensitive tuberculosis ($n = 3549$) and with multidrug-resistant tuberculosis ($n = 77$). All the cases where diagnosed between 2014 and 2017

unique strains, possible outside introductions or newly developed MDR-TB strains (Additional file 3: Table S2). Genetically, the proportion of cases attributable to recent transmission was 52.2%. While this value is strongly overestimated, as MIRU clusters can correspond to clades dating to several years, it allows the identification of circulating clades of MDR strains in the Portuguese scenario.

To corroborate the established genetic connections, we analysed information provided by the Public Health services, identifying 17 links (5 known, 12 possible) between cases within five clusters (Fig. 3):

- In cluster 1, containing four cases from the Northern region, two patients lived in the same parish;

Table 1 Characteristics of MIRU-VNTR clusters, included MIRU profile, identified *Mycobacterium tuberculosis* sub-lineage, patients' country of origin, residence area in Portugal (including North, Central region, Lisbon and Tagus Valley, South and autonomous islands Madeira and Azores), patients' risk factors (identified at diagnosis, namely alcohol abuse, drug misuse, residence in shelters or community residence and history of previous tuberculosis treatment)

Cluster	MIRU-24 loci profile	*M.tuberculosis* sublineage	*n*	Country of origin	Residence area in Portugal	Risk factors	Disease site	Previous TB treatment
1	243,244,331,234 424,153,334,332	Haarlem	4	Portugal	North	1 alcohol abuse	3 pulmonary, 1 extra-pulmonary	No
2	244,233,352,644 425,153,353,823	Beijing	3	Guinea-Bissau	2 LVT, 1 Central	No	1 pulmonary, 1 extra-pulmonary, 1 unknown	1 no, 1 yes, 1 unknown
3	244,233,352,644 425,173,343,723	Beijing	3	Portugal	LVT	Injectable drugs use	Pulmonary	No
4	244,233,352,644 425,183,353,823	Beijing	2	Portugal	1 Madeira, 1 Central	No	Pulmonary	No
5	244,213,132,324 114,142,532,822	LAM	10	7 Portugal, 2 Cape Verde, 1 Mozambique	LVT	3 alcohol abuse, 2 injectable drug use, 1 shelter	7 pulmonary, 2 extra-pulmonary, 1 unknown	5 no, 4 yes, 1 unknown
6	244,213,232,324 116,143,532,822	LAM	6	5 Portugal, 1 Cape Verde	5 LVT, 1 Central	3 alcohol abuse, 2 injectable drug use	Pulmonary	4 no, 2 yes
7	244,213,232,424 116,143,532,822	LAM	14	12 Portugal, 2 Angola	9 LVT, 2 Central, 3 North	4 alcohol abuse, 4 injectable drug use	10 pulmonary, 3 extra-pulmonary, 1 unknown	6 no, 7 yes, 1 unknown

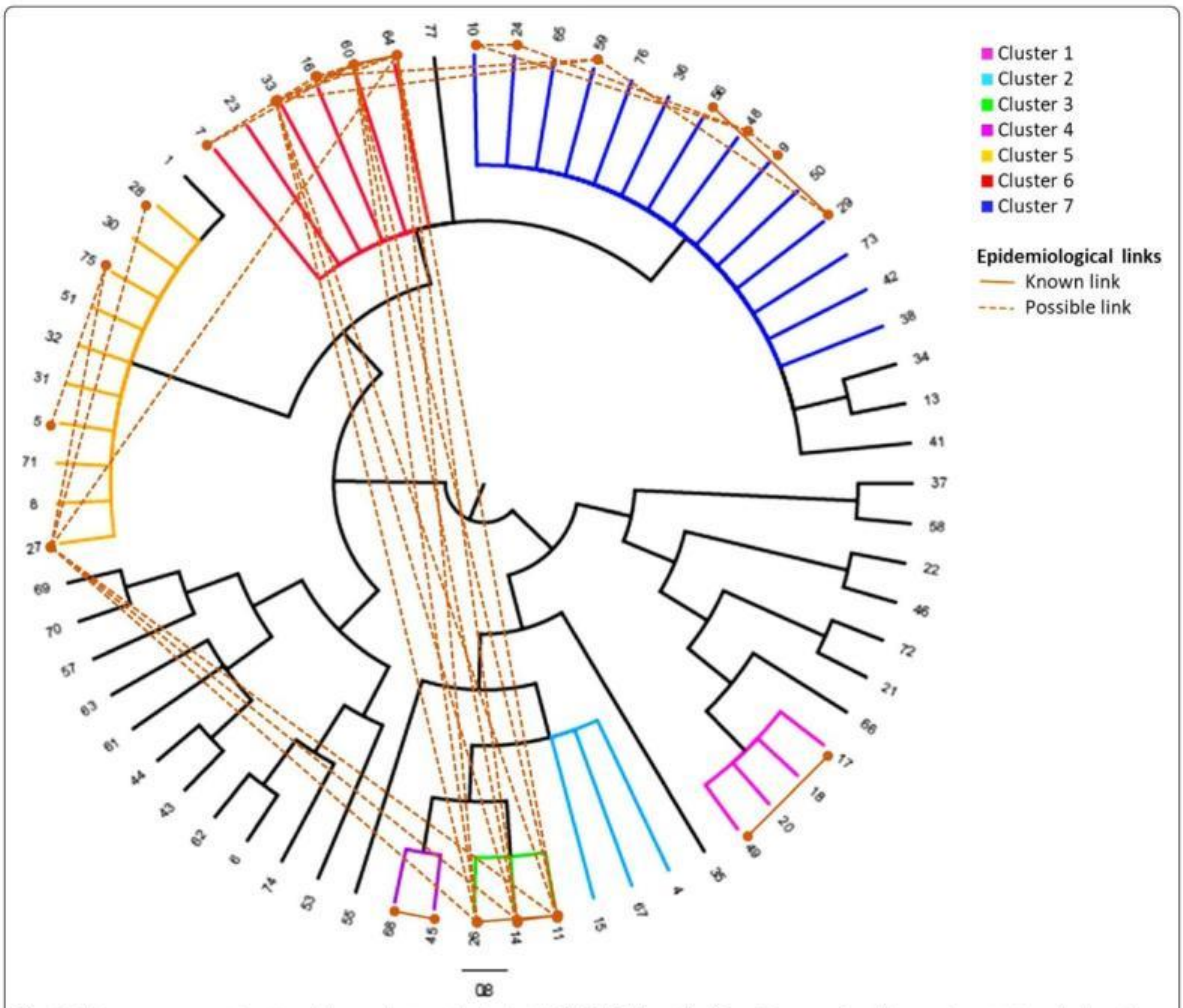

Fig. 3 Cladogram representing the phylogenetic tree obtained with MIRU-VNTR profiles from 67 cases of multidrug-resistant tuberculosis and epidemiological links identified between cases. Clusters 1 to 7 are coloured as follow: pink (cluster 1), light blue (cluster 2), green (cluster 3), purple (cluster 4), orange (cluster 5), red (cluster 6) and dark blue (cluster 7)

- The three patients within cluster 3 were drug-users, attended the same community care facilities and two were close relatives;
- Patients of cluster 4 were friends;
- Two cases of the cluster 6 were relatives; also users of the community care facilities mentioned above for cluster 3;
- Two cases of the cluster 7 were close relatives. One was a civil construction worker with possible contact with two patients of the cluster, also construction workers in the same area.

The proportion of cases attributable to recent transmission after adjustment for detected epidemiological links was 14.9%.

Risk factors associated with clustering

To identify particular risk factors underlying active transmission of MDR-TB, we compared clustered cases (likely recent transmission cases) against unique cases. In the univariate analysis, being born in Portugal (OR 3.67; 95% CI 1.24–10.88; $p = 0.019$) and alcohol abuse (OR 10.15; 95% CI 1.22–84.39; $p = 0.032$) were associated with clustering (Table 2). Nevertheless, the table shows that alcohol abuse is a good identifier for clustered cases and its low frequency within the unique cases turns the logistic regression model impractical. After adjustment for gender and age, being Portugal-born was the only independently clustering-associated variable (adjusted OR 3.64; 95% CI 1.16–11.47; $p = 0.027$).

Table 2 Assessment of patient's characteristics and risk factors that could be associated with multidrug-resistant *Mycobacterium tuberculosis* clustering of cases, considering the cases reported between 2014 and 2017

MDR-TB patient Characteristic		Unknown	Unique ($n = 25$)	Clustered ($n = 42$)	Univariate analysis	
			n (%)	n (%)	OR (95% CI)	*p*-value
Age group	< 45 years old	0	16 (64.0)	21 (50.0)	Ref	0.267
	≥45 years old		9 (36.0)	21 (50.0)	1.78 (0.64–6.91)	
Gender	Female	0	7 (28.0)	9 (21.4)	Ref	0.543
	Male		18 (72.0)	33 (78.6)	1.43 (0.46–4.47)	
Country of origin	Foreign-born	1	12 (50.0)	9 (21.4)	Ref	**0.019**
	Native		12 (50.0)	33 (78.6)	**3.67 (1.24–10.88)**	
HIV status	Negative	4	21 (87.5)	27 (69.2)	Ref	0.109
	Positive		3 (12.5)	12 (30.8)	3.11 (0.78–12.46)	
Alcohol abuse	No	6	22 (95.7)	26 (68.4)	Ref	**0.032**
	Yes		1 (4.3)	12 (31.6)	**10.15 (1.22–84.39)**	
Injectable drug use	No	8	20 (87.0)	29 (80.6)	Ref	0.525
	Yes		3 (13.0)	7 (19.4)	1.61 (0.37–6.98)	
TB treatment history	Never treated	4	15 (62.5)	25 (64.1)	Ref	0.898
	Previously treated		9 (37.5)	14 (35.9)	0.93 (0.33–2.68)	
Site of disease	Pulmonary	4	24 (42.9)	32 (57.1)	n/a	n/a
	Extra-pulmonary		0 (0.0)	7 (100.0)		

CI confidence interval, *OR* odds ratio, *Ref* reference
Statistically significant values are indicated in bold

Genetic contextualization of Portuguese MDR-TB strains

Aiming at a clearer picture of emergence and spread of Mtb strains, we collected and combined published Portuguese MIRU-VNTR data [7, 10, 18].

The evolutionary networks are shown in Fig. 4 and Additional file 1: Figure S1 Networks represent genotypes (circles), where branch length is proportional to genetic distance. Size of the circles is proportional to frequency of cases with the same genotype, being hypothetical clusters of transmission. MDR clusters (Table 1) are highlighted in the figures. Genotypes from strains resistant to at least one second-line anti-TB drug often appear within clusters (Fig. 4) but with individual strains scattered across the network, similarly to sensitive and first-line resistant strains. One aspect to explore with caution, given the low resolution of MIRU-VNTR markers, is that deeper clusters often include sensitive, first-line resistant and MDR strains within the Portuguese population (being cluster 1 the exception). A common ancestry of MDR and sensitive strains in the same area suggests that MDR strains are likely the result of evolution of strains occurring within local chains of transmissions in Portugal and not brought from abroad, often without time for differentiation between both in MIRU markers.

From the network analysis, MDR-TB cluster distribution is similar to the pattern observed with drug-sensitive strains, as described, for example, for Porto Urban area [18], with the existence of several prominent clusters in both suggesting active transmission, or at least the maintenance of several circulating MDR-TB clades within the population. Moreover, general clustering statistics are very similar to that dataset, corresponding to 63% of the MDR-TB cases being clustered, against 59.7% in the drug-sensitive TB cases within Porto ($p = 0.883$, not significant). Considering both datasets, the percentage of cases attributed to recent transmission based on MIRU-VNTR data is 52.2% against 43.8%, higher in MDR cases. While the statistics are overestimated using MIRU-VNTR they are directly comparable and both suggest the circulation of specific Mtb clades within the population in terms of sensitive and MDR strains.

For refining the geography of MDR-TB, we inspected the origin of the drug-resistance cases. The main clusters described in Lisbon [7], are also present in the Centre and Northern regions of the country. Also, while many of the single genotypes are present in immigrants (Additional file 1: Figure S1) possible recent introductions, clustered cases, often clustering with sensitive (Fig. 4), are mostly present in autochthonous individuals, reinforcing a possible evolution and transmission of MDR strains within indigenous transmission chains [18].

History of previous TB treatment

Twenty-three (34%) of the 67 MDR-TB cases with MIRU-VNTR profiles reported previous TB treatment, lacking DST and genotypic data on these past infections.

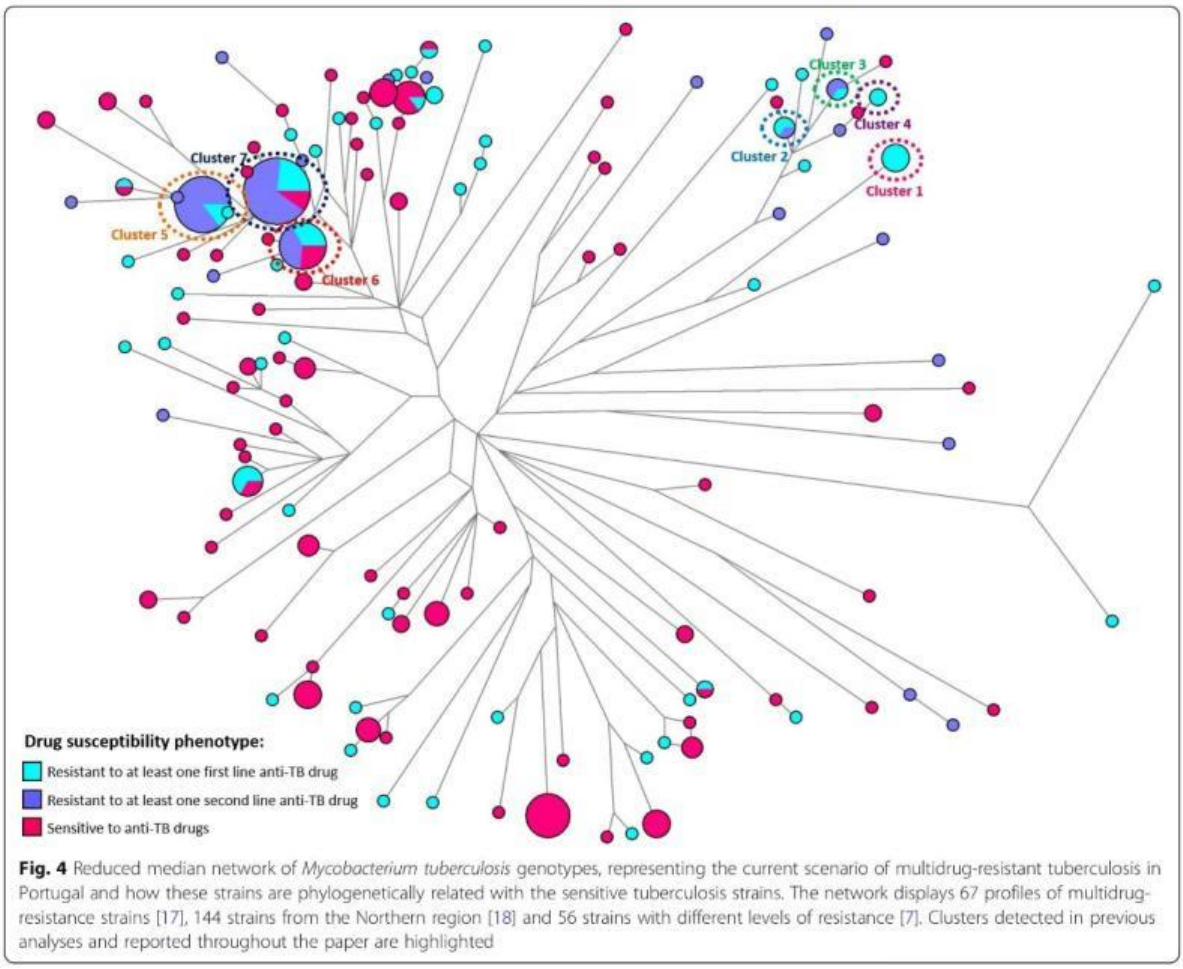

Fig. 4 Reduced median network of *Mycobacterium tuberculosis* genotypes, representing the current scenario of multidrug-resistant tuberculosis in Portugal and how these strains are phylogenetically related with the sensitive tuberculosis strains. The network displays 67 profiles of multidrug-resistance strains [17], 144 strains from the Northern region [18] and 56 strains with different levels of resistance [7]. Clusters detected in previous analyses and reported throughout the paper are highlighted

One individual reported three previous TB treatments. This individual was Portuguese, inmate and injectable drug user. He perished during the fourth treatment with a strain resistant to all first-line drugs and 6 s-line drugs tested, appearing isolated in the genetic networks, pinpointing this case expectedly as one of emergence of resistance. Six other cases reported two previous treatment courses but display no specific evolutionary pattern, being either clustered or isolated.

Within the 23 MDR-TB cases that reported a previous TB treatment, nine had an unique genotype, appearing isolated in the network, possibly representing independent evolution of MDR strains. In opposite, 14 genotypes were in clusters, likely transmission events. The frequency of individuals with previous TB treatment in clustered genotypes is basically the same as the frequency in non-clustered individuals, 36 and 38%, respectively (Table 2). This might reflect the fact that risk of reinfection and inadequate treatment are likely associated with the same risk groups, making the individuals likely vessels for either acquiring new strains or development of MDR.

Discussion

There are various initiatives worldwide aiming to contain and minimise the impact of MDR-TB. Nevertheless, an integrative scenario of MDR-TB transmission dynamics in Portugal is virtually inexistent. During a 4-year period (2014–2017) MDR-TB patients were younger, with higher prevalence of foreign-born individuals, HIV infected, alcohol abusers, injectable drug users and with previous TB treatment, when compared with drug-sensitive patients. Previous TB treatment is known as a strong risk factor for MDR-TB [24–27], and young age, HIV infection, being foreign-born and frequent consumption of alcohol have also been reported in several countries associated with development of MDR-TB [24, 25, 27, 28]. Several of these risk factors are underlying ineffective completion of previous treatments.

We identified seven MIRU-VNTR clusters, where the three largest clusters belonged to sublineage LAM, mostly corresponding to Portugal-born individuals, with pulmonary disease, diagnosed in the LTV region. Being Portugal-born was the independent risk factor statistically associated with MDR-TB recent transmission, suggesting that contrarily to the scenario in other developed countries MDR-TB is not strongly associated with foreign individuals. This trend is also supported by the frequency of the main genotyped lineages.

According to the European Centre for Disease Control (ECDC) surveillance report, Beijing was the most common genotypic lineage among MDR-TB strains isolated in 2015 in Europe (60.0%), followed by LAM (17.6%) [29], despite LAM being more prevalent in sensitive strains (as in Ireland [30] and Belgium [31], for example) . By contrast, here, the most common genotypic lineage in MDR-TB was LAM (69%), followed by Beijing (15%) and Haarlem (12%), similarly to a study on sensitive strain in Northern Portugal (LAM represented 61.1%) [18]. The similar prevalence of LAM in sensitive and MDR-strains strengthens the hypothesis that MDR-TB in Portugal is evolving within autochthonous chains of transmission and being transmitted similarly to drug-sensitive TB.

Clusters 5, 6 and 7 correspond to previously identified MDR-TB clusters Q1, Lisboa3-A and Lisboa3-B in the LTV region [6, 7], reflecting continuous circulation of these strains in the region extending to other regions of Portugal and abroad (Q1 and Lisboa3-B were included in cross-border clusters reported by ECDC, being present in the UK and France [29]). While we suggested an origin of these clusters within the autochthonous transmission chains, determining exact source and direction of transmission between countries would require higher discriminatory power and deeper epidemiological investigations [32].

The proportion of clustered cases in our study (63%) was generally higher than most studies using similar methodologies, for example in the UK, Switzerland and USA [19, 33, 34] but similar to a Portuguese sensitive sample (59.7%) following the same criteria [18]. We estimated recent transmission to 52.2% using MIRU-VNTR data and 14.9 after adjustment for epidemiological data. While MIRU-VNTR data largely overestimates recent transmission estimates [16, 35], the epidemiological data likely underestimates direct transmission given the difficulty in assessing all relevant epidemiological information using only conventional contact tracing data in complex transmission chains [18]. Nevertheless, these 14.9% were still twice as high as what was reported in UK [19] and Switzerland [34] using similar approaches. The higher prevalence of MDR-TB (above 50%) displaying no previous treatment provides evidence for high rate of primary transmission of MDR-TB in Portugal, also supported by a higher estimated rate of transmission in MDR than sensitive strains in Portugal, against the expected European trend. Independently of the clustering being overestimated, in the sense that MIRU clusters could date up to decades in some instances, it reflects the existence of circulating genetic clades being maintained in the population.

Conclusion

This study has limitations in terms of analysed period and genotyping, but it offers nevertheless a strong effort to assess MDR-TB scenario in Portugal using a more detailed combined MIRU-VNTR and epidemiological analysis. While the recent transmission rates are widely overestimated by the application of MIRU-VNTR data, there is nevertheless a striking scenario that relates to a high percentage of circulating strains from the same clades (when compared with sensitive strains and MDR-TB in other countries) than what could be expected given the public health efforts to contain MDR-TB. Taking into account the possible scenarios of emergence of MDR-TB, it is necessary to readjust measures to decrease transmission, including for example, improvement of earlier diagnosis and better adherence to treatment in specific regions of the country, including the support of community institutions focusing on specific population groups, and a faster and integrative genotyping protocol for early identification of clustered cases.

Additional files

Additional file 1: Figure S1. Reduced median network, representing the current scenario of multidrug-resistant TB in Portugal and how these strains are grouped together with the sensitive TB strains. The network displays 67 profiles of *Mycobacterium tuberculosis* strains [17], 144 strains from the Northern region [18] and 56 strains with different levels of resistance [7]. The migratory status of the cases is highlighted: green cases are natives from Portugal, yellow are foreigners and grey are individuals that on diagnosis (or publication) did not disclosure migratory status. Clusters detected in previous analyses and reported throughout the paper are highlighted. (DOCX 572 kb)

Additional file 2: Table S1. Assessment of patient's characteristics of drug-sensitive tuberculosis and multidrug-resistant tuberculosis, considering the cases reported between 2014 and 2017. (DOCX 20 kb)

Additional file 3: Table S2. Characteristics of non-clustered cases, including the ID displayed in Fig. 3, identified *Mycobacterium tuberculosis* lineage and sub-lineage, patients' country of origin, residence area in Portugal (including North, Central region, Lisbon and Tagus Valley, South and autonomous islands Madeira and Azores), patients' risk factors (identified at diagnosis, namely alcohol abuse, drug misuse, residence in shelters or community residence and history of previous TB episodes and consequent treatment). (DOCX 20 kb)

Abbreviations

SVIG: Surveillance System; TB: Tuberculosis; CI: Confidence interval; DST: Drug susceptibility testing; ECDC: European Centre for Disease Control; HIV: Human immunodeficiency virus; LAM: Latin-American-Mediterranean; LTV: Lisbon and Tagus Valley; MDR-TB: Multidrug-resistant tuberculosis; MIRU-VNTR: Mycobacterial interspersed repetitive units–variable number tandem repeat; Mtb: *Mycobacterium tuberculosis*; OR: Odds ratio; PASW: Predictive

Analytics Software; RR: Rifampicin-resistant; SPSS: Statistical Package for the Social Sciences; TB: Tuberculosis; WHO: World Health Organization

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Authors' contributions
RD and MCN conceptualized and designed the study; CC contextualised the findings within a public health perspective and handled the database; OO did data collection; OO and RG did statistical analysis; TR did genetic analysis; OO and TR drafted the manuscript and all authors critically reviewed it. All authors have read and approved the final manuscript.

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Availability of data and materials
The genetic datasets analysed during the current study are available within the manuscripts referenced by us and also in http://cplp-tb.ff.ulisboa.pt/. The raw epidemiological dataset generated and analysed during the current study is not publicly available and it is property of the TB National programme. All the relevant information analysed is contained within this manuscript.

Ethics approval and consent to participate
This work was carried out in accordance with the recommendations by the Ethics Sub-commission of Life and Health Sciences from the University of Minho (SECVS 135/2015), by the Ethics Committee for Health of Lisbon and Tagus Valley Region Health Administration (9854/CES/2018) and the Ethics Committee for Health of the Northern Region Health Administration (ARSN 127/2018). All procedures were in accordance with the ethical standards of the responsible committees and with the Helsinki Declaration, as revised in 2008. Administrative authorization was obtained from the TB National programme to access SVIG-TB data. Informed consent was waived because only routinely collected data and anonymised at their source were used.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Additional File 1

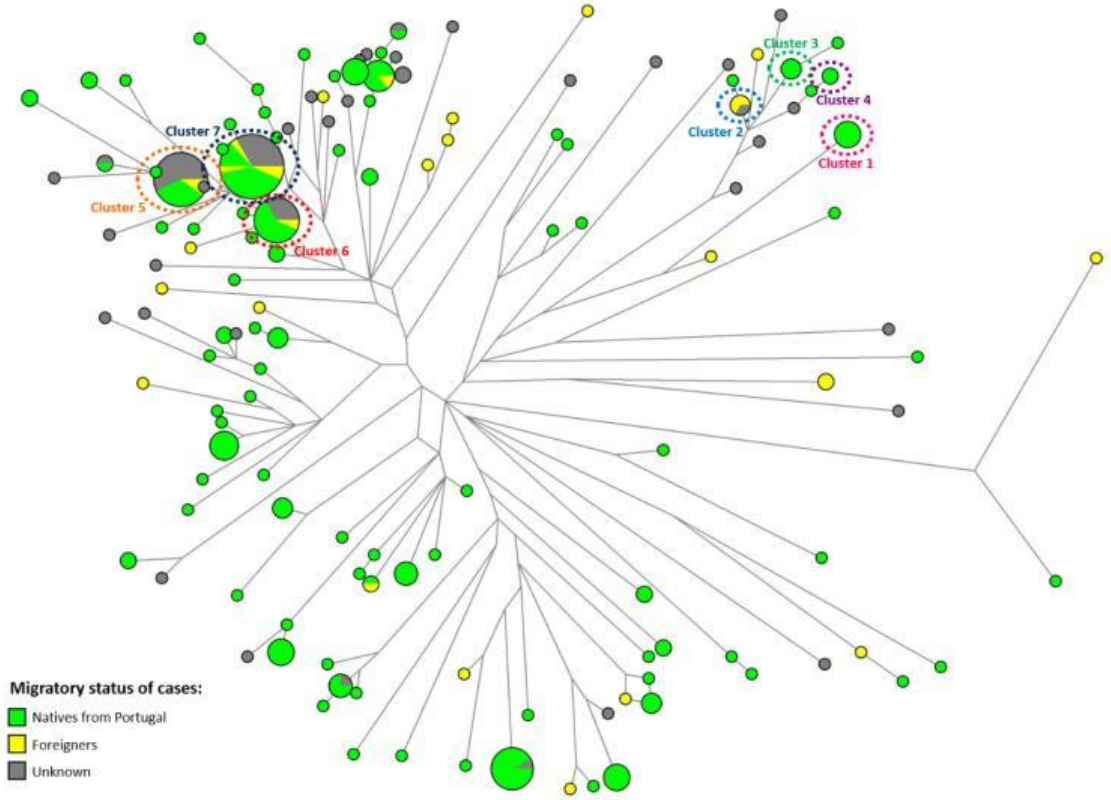

Figure-S1. Reduced median network, representing the current scenario of multidrug-resistant TB in Portugal and how these strains are grouped together with the sensitive TB strains. The network displays 67 profiles of *Mycobacterium tuberculosis* strains (10), 144 strains from the Northern region (12) and 56 strains with different levels of resistance (7). The migratory status of the cases is highlighted: green cases are natives from Portugal, yellow are foreigners and grey are individuals that on diagnosis (or publication) did not disclosure migratory status. Clusters detected in previous analyses and reported throughout the paper are highlighted.

Additional File 2

Table-S1. Assessment of patient's characteristics of drug-sensitive tuberculosis and multidrug-resistant tuberculosis, considering the cases reported between 2014 and 2017.

Patients characteristics		Unknown	Total	Drug-sensitive TB nº (%)	MDR-TB nº (%)	p-value
Age, years	Median (min, max)	0	3626	48 (0-96)	43 (20-75)	0.111
Age group	<45 years old ≥45 years old	0	3626	1538 (43.3) 2011(56.7)	43(55.8) 34(44.2)	**0.038**
Gender	Female Male	0	3626	1098(30.9) 2451(69.1)	19(24.7) 58(75.3)	0.292
Country of origin	Foreign-born Native	1	3625	543(15.3) 3006(84.7)	25(32.9) 51(67.1)	**<0.001**
HIV status	Negative Positive	4	3622	3265(92.0) 284(8.0)	55(75.3) 18(24.7)	**<0.001**
Alcohol abuse	No Yes	218	3408	2898(86.8) 439(13.2)	54(76.1) 17(23.9)	**0.014**
Injectable drug use	No Yes	223	3403	3183(95.4) 152(4.6)	58(85.3) 10(14.7)	**<0.001**
TB treatment history	Never treated Previously treated	4	3622	3283(92.5) 266(7.5)	49(67.1) 24(32.9)	**<0.001**
Site of disease	Pulmonary Extra-pulmonary	4	3622	3113(87.7) 436(12.3)	64(87.7) 9(12.3)	1.000

Additional File 3

Table-S2. Characteristics of non-clustered cases, including the ID displayed in Figure-3, identified *Mycobacterium tuberculosis* lineage and sub-lineage, patients' country of origin, residence area in Portugal (including North, Central region, Lisbon and Tagus Valley, South and autonomous islands Madeira and Azores), patients' risk factors (identified at diagnosis, namely alcohol abuse, drug misuse, residence in shelters or community residence and history of previous TB episodes and consequent treatment).

ID Figure-3	Lineage	Sub-lineage	Country of origin	Residence area in Portugal	Risk factos	Site of disease	TB treatment history
1	4	LAM	Portugal	LTV	Drug use, Prison	Pulmonary	Yes
6	4	LAM	Belarus	LTV	No	Pulmonary	No
13	4	LAM	Portugal	LTV	Alcohol abuse	Pulmonary	No
21	4	Haarlem	Portugal	North	Drug use, Homeless	Pulmonary	No
22	4	X	Andorra	North	No	Pulmonary	Yes
41	4	LAM	Guinea	LTV	Community residence	Pulmonary	Yes
34	4	LAM	Portugal	LTV	No	Pulmonary	No
37	4	URAL	Ukraine	LTV	No	Pulmonary	No
35	2	Beijing	Portugal	LTV	Drug use, prison	Pulmonary	Yes
43	4	LAM	Portugal	North	No	Pulmonary	Yes
44	4	LAM	Portugal	North	No	Pulmonary	No
46	4	Haarlem	Moldova	Algarve	No	Pulmonary	No
57	4	LAM	Angola	LTV	No	Pulmonary	Yes
61	4	LAM	Angola	LTV	No	Pulmonary	No
55	2	Beijing	Ukraine	LTV	Community residence	Pulmonary	Yes
62	4	LAM	Portugal	Central	No	Pulmonary	Yes
53	4	LAM	Portugal	Central	No	Pulmonary	No
66	4	Haarlem	Angola	Central	No	Pulmonary	No
69	4	LAM	Angola	LTV	No	Pulmonary	No
77	4	LAM	Portugal	LTV	No	Pulmonary	No
72	4	Haarlem	Portugal	North	No	Pulmonary	No
70	4	LAM	Angola	North	No	Pulmonary	No
58	4	URAL	Portugal	Central	No	Pulmonary	No
74	4	LAM	Angola	Central	No	Pulmonary	Yes
63	4	LAM	Unknown	LTV	Unknown	Unknown	Unknown

CHAPTER IV

EVALUATION OF DRUG-RESISTANT TUBERCULOSIS TREATMENT OUTCOME IN PORTUGAL, 2000-2016

PloS one. 2021; 16(4):e0250028.

PLOS ONE

RESEARCH ARTICLE

Evaluation of drug-resistant tuberculosis treatment outcome in Portugal, 2000–2016

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Data Availability Statement: The data cannot be shared publicly because include sensitive patient information and because they are owned by a third-party organization, the Directorate-General of Health of Portugal. Data are available from the National Program for the area of Tuberculosis of the Directorate-General of Health of Portugal (contact via geral@dgs.min-saude.pt) for researchers who meet the criteria for access to confidential data. The authors did not have any special privileges in accessing the data that other researchers would not have.

Abstract

Treatment of drug-resistant tuberculosis (TB), which is usually less successful than that of drug-susceptible TB, represents a challenge for TB control and elimination. We aimed to evaluate treatment outcomes and to identify the factors associated with death among patients with MDR and XDR-TB in Portugal. We assessed MDR-TB cases reported for the period 2000–2016, using the national TB Surveillance System. Treatment outcomes were defined according to WHO recommendations. We identified the factors associated with death using logistic regression. We evaluated treatment outcomes of 294 MDR- and 142 XDR-TB patients. The treatment success rate was 73.8% among MDR- and 62.7% among XDR-TB patients ($p = 0.023$). The case-fatality rate was 18.4% among MDR- and 23.9% among XDR-TB patients. HIV infection (OR 4.55; 95% CI 2.31–8.99; $p < 0.001$) and resistance to one or more second-line injectable drugs (OR 2.73; 95% CI 1.26–5.92; $p = 0.011$) were independently associated with death among MDR-TB patients. HIV infection, injectable drug use, past imprisonment, comorbidities, and alcohol abuse are conditions that were associated with death early on and during treatment. Early diagnosis of MDR-TB and further monitoring of these patients are necessary to improve treatment outcome.

Introduction

Tuberculosis (TB) treatment success (the percentage of cured patients and those with treatment completed) is one indicator for monitoring implementation of the End TB Strategy. Globally, the recommended target level for 2025 is above 90% [1]. In the European Union, the treatment success rate for the 45,499 TB cases treated in 2017 was 67.6%, still standing far from the established goal. Moreover, the latest surveillance data (2018) for patients with drug-resistant TB shows lower treatment success rates: 48.1% for multidrug-resistant TB (MDR-TB), defined by resistance of *Mycobacterium tuberculosis* (Mtb) to isoniazid and

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Competing interests: The authors have declared that no competing interests exist.

Abbreviations: CI, confidence interval; DOT, Directly Observed Therapy; DST, drug susceptibility testing; ECDC, European Centre for Disease Prevention and Control; HIV, human immunodeficiency virus; INSA, Instituto Nacional de Saúde Ricardo Jorge; IQR, interquartile range; MDR-TB, multidrug-resistant tuberculosis; Mtb, *Mycobacterium tuberculosis*; OR, odds ratio; pre-XDR_{FQ}-TB, pre-extensively drug-resistant tuberculosis resistant to either a fluoroquinolone; pre-XDR_{SLID}-TB, pre-extensively drug-resistant tuberculosis resistant to second-line injectable drugs; TB, tuberculosis; WGS, whole-genome sequencing; WHO, World Health Organization; XDR-TB, extensively drug-resistant tuberculosis.

rifampicin, and 37.4% for extensively drug-resistant TB (XDR-TB), defined as MDR-TB plus resistance to at least one of the fluoroquinolones and one of the injectable drugs [2]. Death is a more frequent unfavorable outcome among those with MDR- or XDR-TB than with susceptible TB (17.1%, 21.8%, and 6.9%, respectively) [2].

The treatment of MDR/XDR-TB requires the use of bactericidal and bacteriostatic drugs for long periods. Treatment success depends not only on the choice of an effective treatment regimen but also on the patient monitorisation and the management of therapeutics adverse events and comorbidities, potential drug-drug interactions, and even the patient's tolerability to the drug regimen implemented [3].

In Portugal, TB cases are managed mainly in TB Outpatient Centres. As a specific strategy to MDR-TB control, in 2007, the National Reference Centre was created to monitor and support the treatment of MDR/XDR-TB cases, producing the national guidelines and recommendations. Later, to support the implementation of these standard procedures, to decentralize this approach and to facilitate accessibility to all, the Regional Reference Centres were created. Although these Centres currently operate in each of the seven health regions of the country, they started working at different times. While the first Centre opened in the Northern Region in 2009 [4,5], the Centre in Lisbon and Tagus Valley Region, with the highest TB burden in the country, started to work only in 2013 [6]. In Portugal, all TB patients receive free treatment from the National Health System under the National Tuberculosis Program. Hospitalization is the first choice at the start of MDR-TB treatment, and the patient remains hospitalized until smear sputum conversion. The Regional Reference Centres are responsible for the clinical management of patients during the entire treatment course, including the choice of the treatment regimen. Adequate regimens are based on current MDR-TB treatment guidelines [7,8] and adjusted according to the clinical and microbiological response along with drug susceptibility testing (DST) results. Directly Observed Therapy (DOT) is provided throughout the treatment at the primary care level [4,5].

Following our earlier analysis evaluating treatment outcomes of MDR-TB patients in Portugal [9], here we updated information to give a complete assessment of the disease along 17 years of cases reported in Portugal. Furthermore, we assessed case-fatality rate and identified the factors associated with death in MDR- and XDR-TB patient groups.

Methods

Data collection

We selected MDR-TB cases diagnosed in Portugal from January 2000 until December 2016 from the national TB Surveillance System (SVIG-TB). We evaluated patients with known treatment outcomes and second-line drug resistance profiles, dividing those into groups: MDR- and XDR-TB cases.

Information collected included demographic and clinical characteristics of each case: age, sex, country of origin, addictions (e.g., drug or alcohol abuse), HIV status, living conditions (e.g., being a prisoner, living in a community residence, homelessness), comorbidities (diabetes, silicosis, chronic obstructive pulmonary disease, liver disease or neoplasia), previous TB treatment and site of infection. We also collected dates of the onset of symptoms, diagnosis of TB, and treatment initiation.

Diagnosis and treatment

MDR-TB diagnosis requires a positive culture or detection of both acid-fast bacilli by microscopy and an Mtb-specific nucleic acid amplification testing, followed by detection of resistance to isoniazid and rifampicin by genotypic and phenotypic methods [2]. We included in our

study culture-positive MDR-TB cases tested for resistance to first- and second-line anti-TB drugs by phenotypic methods, the conventional gold standard. All tests were performed in laboratories integrated into the national network, periodically certified and checked. Mtb strains that revealed resistance to isoniazid and rifampicin were tested for second-line anti-TB drugs in the TB National Reference Laboratory (Instituto Nacional de Saúde Ricardo Jorge: INSA) in Porto, which is also a World Health Organization (WHO) Supranational Reference Laboratory.

HIV testing is done routinely, using an opt-out strategy (patients were informed that an HIV test will be conducted and that they may decline or defer) [10]. The treatment regimen is designed according to WHO guidelines. Treatment begins with a standard or empirical regimen until DST for second-line drugs results are available. Afterwards, individually tailored regimens that take into account the drug resistance patterns are used. Treatment continues for at least 18 months after culture conversion [11–13]. The patients are hospitalized until smear conversion and then followed in one of the Regional Reference Centre. Directly observed treatment is performed during the entire treatment.

Treatment outcomes

The treatment outcomes were defined according to WHO recommendations as *cured*, *treatment completed*, *treatment failed*, *death*, and *lost to follow-up* [7,14], and reported to the National TB Surveillance System. The sum of *cured* (treatment completed and three or more consecutive cultures taken at least 30 days apart are negative after the intensive phase) and *treatment completed* (treatment completed but no record that three or more consecutive cultures taken at least 30 days apart are negative after the intensive phase) is considered as treatment success. That is, treatment success includes *treatment completed* with or without three or more consecutive cultures taken at least 30 days apart, which are negative after the intensive phase. Although in our study no cured was registered during the studied period, it will not affect estimated success rate. *Treatment failure*, *death* and *lost to follow-up* were considered as unfavorable treatment outcomes.

The percentage of cases for each outcome was determined considering the total number of patients who started treatment over studied period.

Statistical analysis

We describe patient's characteristics through absolute and relative frequencies for categorical variables as gender, country of origin, HIV status, alcohol abuse, injectable drug use, prisoner, community residence, homelessness, comorbidity, chest radiography, previous TB treatment, site of disease. Median with interquartile range (IQR) was used for continuous variables as age (years), delay in diagnosis and treatment (days) and duration of treatment (months). Delay in diagnosis and treatment was defined as the period from the date of the onset of symptoms until the diagnosis of TB and treatment initiation. We compared the prevalence of these characteristics between patients grouped according to second-line drug resistance profile and between MDR-TB patients that died within and after the first six months of treatment, using the Chi-squared test (or Fisher's test, if appropriate) for categorical variables. The Mann–Whitney U-test (or median test) was used to compare continuous variables. We estimated treatment success rate by year and we compared their medians before and after 2008, year that corresponds to half of the study period and when some measures of MDR-TB control were taken in Portugal. We also estimated treatment success rate over the study period and we compared it among patient groups. We used the Chi-squared test (or Fisher's test, if appropriate) to compare patient groups' treatment outcomes. Univariate and multivariate logistic

regression was conducted to identify death factors during treatment among patients in each drug resistance categories. Crude and adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were determined. Statistical analyses were performed with SPSS version 18.0 (PASW Statistics 18), and p-values below 0.05 were considered statistically significant.

Ethical considerations

Ethical approval and informed consent were not required, as the patient data, collected by an official national surveillance system, were anonymized following the ethical research guidelines in Portugal.

Results

Dataset characterization

In Portugal, from January 2000 until December 2016, 576 MDR-TB cases were diagnosed. We evaluated 436 cases, excluding patients with unknown treatment outcomes and second-line drug resistance profiles. Of them, 294 (67.4%) cases were MDR-TB, and 142 (32.6%) cases were XDR-TB (Fig 1).

Demographic and clinical characteristics of the patients are shown in Table 1. Compared to MDR-TB, XDR-TB patients presented higher prevalence of alcohol abuse (34.1%), injectable drug use (30.8%), past or present imprisonment (12.7%) and previous TB treatment (54.9%) (Table 1).

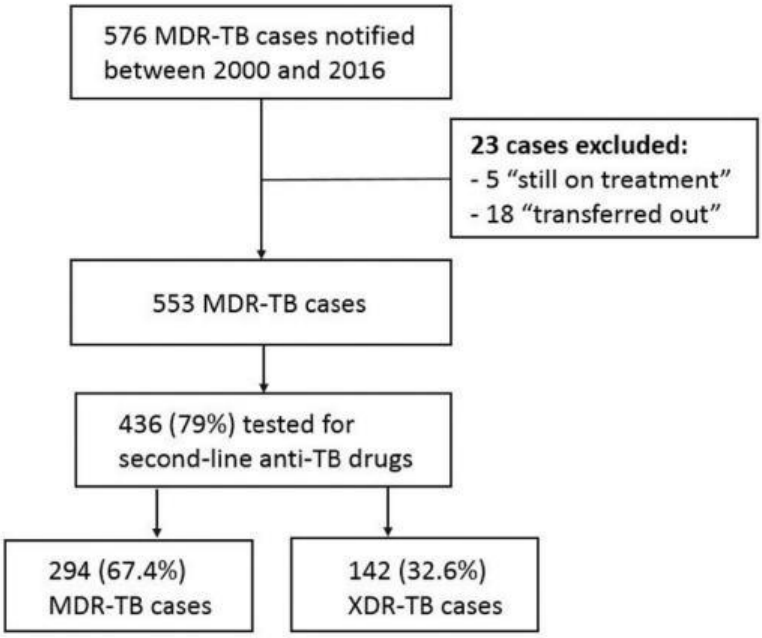

Fig 1. Flowchart of the cases included in the analysis, considering MDR-TB cases reported between 2000 and 2016 in Portugal. MDR-TB = multidrug-resistant tuberculosis; XDR-TB = extensively drug-resistant tuberculosis.

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Table 1. Characteristics of multidrug-resistant and extensively drug-resistant tuberculosis patients, considering the cases reported in Portugal between 2000 and 2016 (n = 436).

Patient's characteristics		Total N	MDR-TB		XDR-TB		p-value
			n or M	% or IQR	n or M	% or M	
Categorical variables							
Gender	Female	436	93	31.6	37	26.1	0.280
	Male		201	68.4	105	73.9	
Country of origin	Native	435[a]	213	72.7	114	80.3	0.110
	Foreign-born		80	27.3	28	19.7	
HIV status	Negative	436	224	76.2	98	69.0	0.138
	Positive		70.0	23.8	44	31.0	
Alcohol abuse[b]	No	387[a]	203	77.8	83	65.9	**0.018**
	Yes		58	22.2	43	34.1	
Injectable drug use[b]	No	392[a]	224	85.5	90	69.2	**0.001**
	Yes		38	14.5	40	30.8	
Imprisonment	No	383[a]	247	96.1	110	87.3	**0.003**
	Yes		10	3.9	16	12.7	
Community residence[b]	No	380[a]	244	95.3	118	95.2	1.000
	Yes		12	4.7	6	4.8	
Homelessness	No	380[a]	248	96.9	120	96.8	1.000
	Yes		8	3.1	4	3.2	
Comorbidities	No	436	252	85.7	121	85.2	1.000
	Yes		42	14.3	21	14.8	
Chest radiography	No cavitation	401[a]	123	44.7	58	46.0	0.892
	Cavitation		152	55.3	68	54.0	
Previous TB treatment	No	436	187	63.6	64	45.1	**<0.001**
	Yes		107	36.4	78	54.9	
Site of disease	Pulmonary	435[a]	278	94.6	131	92.9	0.643
	Extra-pulmonary		16	5.4	10	7.1	
Continuous variables							
Age (years)	Median, IQR	436	41.0	20.0	40.0	16.0	0.611
Delay in TB diagnosis and treatment (days)	Median, IQR	333[a]	73.0	84.0	71.0	63.0	0.941
Treatment duration (months)	Median, IQR	436	20.4	13.2	23.4	10.7	**<0.001**

n = number of cases; M = median; IQR = interquartile range; HIV = human immunodeficiency virus; TB = tuberculosis; MDR-TB = multidrug-resistant tuberculosis; XDR-TB = and extensively drug-resistant tuberculosis.

[a] Data missing for: Country of origin (n = 1; 0.2%), alcohol abuse (n = 49; 11.2%), injectable drug use (n = 44; 10.1%), prisoners (n = 53; 12.2%), community residence (n = 56; 12.8%), homelessness (n = 56; 12.8%), chest radiography (n = 35; 8.0%), site of disease (n = 1; 0.2%), delay in TB diagnosis and treatment (n = 103; 23.6%).

[b] Self-reported.

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Treatment and treatment outcomes

In our study, median TB diagnostic delay, which was calculated for patients with available date for the onset of symptoms (n = 333; 76.4%), was 72 days. There were no significant differences in TB diagnostic delay between MDR- and XDR-TB (73 days and 71 days; $p = 0.941$; Table 1).

All 436 patients started treatment on the day of diagnosis of TB. Initial treatment regimen data was available for 414 (95.0%) patients. Of them, 170 (41.1%) received second-line anti-TB drugs in the initial treatment regimen.

Duration of treatment among MDR-TB patients was 20.4 months and 23.4 months among XDR-TB patients ($p < 0.001$) (Table 1).

Table 2. Treatment outcomes among multidrug-resistant and extensively drug-resistant tuberculosis patients who started treatment between 2000 and 2016 (n = 436).

Treatment outcomes		Total		MDR-TB		XDR-TB		*p*-value
		n	%	n	%	n	%	
Treatment success	Treatment completed	306	70.2	217	73.8	89	62.7	**0.023**
Unfavorable outcomes	Treatment failed	16	3.7	8	2.7	8	5.6	0.213
	Lost to follow-up	26	6.0	15	5.1	11	7.7	0.381
	Death	88	20.2	54	18.4	34	23.9	0.218

n = number of cases; MDR-TB = multidrug-resistant tuberculosis; XDR-TB = and extensively drug-resistant tuberculosis.

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The treatment success rate tended to increase since 2000 to 2016 (S1 Fig). We compared the medians of treatment success rate before and after 2008 and we found no statistically significant difference between them (67.5% and 78.6% respectively; $p = 0.153$).

The treatment success rate over the study period was superior among MDR than XDR-TB patients (73.8% and 62.7%; $p = 0.023$; Table 2). Among unfavorable treatment outcomes, *death* was more frequent than *treatment failure* or *loss to follow-up* in both groups. The case-fatality rate among MDR- and XDR-TB patients was 18.4% and 23.9% respectively ($p = 0.218$; Table 2).

Additionally, we evaluated treatment outcomes among MDR-TB patients, dividing cases into groups: MDR-TB without additional second-line drug resistance, MDR-TB with additional resistance to one or more second-line injectable drugs (pre-XDR_{SLID}-TB) and MDR-TB with additional resistance to one or more fluoroquinolones (pre-XDR_{FQ}-TB) (S1 Table).

We found that pre-XDR_{SLID}-TB patients had worse treatment outcome than patients from other drug-resistance groups: the treatment success rate was 55.6% ($p = 0.002$) and the case-fatality rate was 31.0% ($p = 0.065$) among them (S1 Table). However, due to the small numbers of patients in with pre-XDR_{SLID}- and pre-XDR_{FQ}-TB, we decided not to go ahead with looking for factors associated with death in these groups, but to include these resistance profiles as independent variables in the analysis of factors associated with patient death among MDR- TB.

Factors associated with patient death

We assessed separately factors associated with patient death among MDR- and XDR-TB patients (Tables 3 and 4).

Among MDR-TB patients, HIV infection (OR 4.14; 95% CI 2.21–7.74; $p < 0.001$), injectable drug use (OR 6.30; 95% CI 2.98–13.34; $p < 0.001$), presence of comorbidities (OR 2.31; 95% CI 1.11–4.81; $p = 0.026$) and resistance to one or more second-line injectable drugs (OR 2.68; 95% CI 1.29–5.59; $p = 0.008$) were significantly associated with death in univariate analysis. HIV infection (OR 4.55; 95% CI 2.31–8.99; $p < 0.001$) and resistance to one or more second-line injectable drugs (OR 2.73; 95% CI 1.26–5.92; $p = 0.011$) remained independently associated with death in multivariate analysis (Table 3).

Injectable drug use (OR 2.69; 95% CI 1.15–6.33; $p = 0.023$) and history past or present of imprisonment (OR 3.30; 95% CI 1.10–9.87; $p = 0.033$) were significantly associated with death among patients with XDR-TB in univariate analysis. However, these associations were not confirmed in the multivariate analysis (Table 4).

The elapsed time between beginning of treatment and death

In the cases for which death was reported, the treatment duration until death was 9.5 months among MDR-TB patients and 13.1 months among XDR-TB patients (Table 5). In addition, 22

Table 3. Factors associated with death among patients with multidrug-resistant tuberculosis (n = 294).

Factors	Death		Univariate analysis		Multivariate analysis	
	No n (%)	Yes n (%)	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Age (years), median (IQR)	41.0(18)	40.5(29)	1.02(0.99–1.04)	0.111		
Gender						
Female	80(86.0)	13(14.0)	Ref			
Male	160(79.6)	41(20.4)	1.58(0.80–3.11)	0.189		
Country of origin						
Native	174(81.7)	39(18.3)	Ref			
Foreign-born	65(81.2)	15(18.8)	1.03(0.53–1.99)	0.931		
HIV status						
Negative	196(87.5)	28(12.5)	Ref		Ref	
Positive	44(62.9)	26(37.1)	4.14(2.21–7.74)	**<0.001**	4.55(2.31–8.99)	**<0.001**
Alcohol abused						
No	166(81.8)	37(18.2)	Ref			
Yes	48(82.8)	10(17.2)	0.94(0.43–2.02)	0.963		
Injectable drug used						
No	196(87.5)	28(12.5)	Ref			
Yes	20(52.6)	18(47.4)	6.30(2.98–13.36)	**<0.001**		
Imprisonment						
No	208(84.2)	39(15.8)	Ref			
Yes	6(60.0)	4(40.0)	3.56(0.96–13.19)	0.058		
Community residence						
No	204(83.6)	40(16.4)	Ref			
Yes	10(83.3)	2(16.7)	1.02(0.22–4.83)	0.980		
Homelessness						
No	207(83.5)	41(16.5)	Ref			
Yes	7(87.5)	1(12.5)	0.72(0.09–6.02)	0.763		
Comorbidities						
No	211(83.7)	41(16.3)	Ref			
Yes	29(69.0)	13(31.0)	2.31(1.11–4.81)	**0.026**		
Chest radiography						
No cavitation	98(79.7)	25(20.3)	Ref			
Cavitation	131(86.2)	21(13.8)	0.63(0.33–0.19)	0.153		
Previous TB treatment						
No	156(83.4)	31(16.6)	Ref			
Yes	84(78.5)	23(21.5)	1.38(0.76–2.51)	0.296		
Site of disease						
Pulmonary	228(82.0)	50(18.0)	Ref			
Extra-pulmonary	12(75.0)	4(25.0)	1.52(0.47–4.91)	0.484		
Pre-XDR_{SLID}-TB						
No	190(85.6)	32(14.4)	Ref		Ref	
Yes	31(68.9)	14(31.1)	2.68(1.29–5.59)	**0.008**	2.73(1.26–5.92)	**0.011**
Pre-XDR_{FQ}-TB						
No	213(81.3)	49(18.7)	Ref			
Yes	27(84.4	5(15.6)	0.81(0.30–2.20)	0.672		

n = number of cases; OR = odds ratio; CI = confidence interval; IQR = interquartile range; HIV = human immunodeficiency virus; TB = tuberculosis; pre-XDR_{SLID}-TB = resistance to one or more second-line injectable drugs; pre-XDR_{FQ}-TB = resistance to one or more fluoroquinolones.

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Table 4. Factors associated with death among patients with extensively drug-resistant tuberculosis (n = 142).

Factors	Death		Univariate analysis		Multivariate analysis	
	No n (%)	Yes n (%)	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
Age (years), median (IQR)	39.0(17)	43.5(17)	1.02(0.99–1.05)	0.309		
Gender						
Female	32(86.5)	5(13.5)	Ref			
Male	76(72.4)	29(27.6)	2.44(0.87–6.88)	0.091		
Country of origin						
Native	88(78.2)	26(22.8)	Ref			
Foreign-born	20(71.4)	8(28.6)	1.35(0.54–3.43)	0.523		
HIV status						
Negative	76(77.6)	22(22.4)	Ref			
Positive	32(72.7)	12(27.3)	1.30(0.57–2.93)	0. 534		
Alcohol abused						
No	68(81.9)	15(18.1)	Ref			
Yes	31(72.1)	12(27.9)	1.76(0.74–4.19)	0.205		
Injectable drug used						
No	75(83.3)	15(16.7)	Ref		Ref	
Yes	26(65.0)	14(35.0)	2.69(1.15–6.33)	**0.023**	2.19(0.80–5.96)	0.125
Imprisonment						
No	89(80.9)	21(19.1)	Ref		Ref	
Yes	9(56.2)	7(43.8)	3.30(1.10–9.87)	**0.033**	2.24(0.66–7.61)	0.198
Community residence						
No	94(79.7)	24(20.3)	Ref			
Yes	5(83.3)	1(16.7)	0.78(0.09–7.02)	0.827		
Homelessness						
No	97(80.8)	23(19.2)	Ref			
Yes	2(50.0)	2(50.0)	4.22(0.56–31.54)	0.161		
Comorbidities						
No	90(74.4)	31(25.6)	Ref			
Yes	18(85.7)	3(14.3)	0.48(0.13–1.76)	0.270		
Chest radiography						
No cavitation	48(82.8)	10(17.2)	Ref			
Cavitation	49(72.1)	19(27.9)	1.86(0.79–4.41)	0.158		
Previous TB treatment						
No	51(79.7)	13(20.3)	Ref			
Yes	57(73.1)	21(26.9)	1.45(0.66–3.18)	0.360		
Site of disease						
Pulmonary	99(75.6)	32(24.4)	Ref			
Extra-pulmonary	8(80.0)	2(20.0)	0.77(0.16–3.83)	0.753		

n = number of cases; OR = odds ratio; CI = confidence interval; IQR = interquartile range; HIV = human immunodeficiency virus; TB = tuberculosis.

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(40.7%) of the MDR-TB patients versus 8 (23.5%) of the XDR-TB patients died within the first six months (Table 5).

We compared the demographic characteristics of MDR-TB patients who died within the first six months of treatment with patients who died after that period (S2 Table). Alcohol abuse (60.0%), injectable drug use (55.6%), and comorbidities (61.5%) were more frequent among MDR-TB patients that died within the first six months, although these differences were

Table 5. Treatment duration until patient death among multidrug-resistant and extensively drug-resistant tuberculosis patients in cases where death was reported (n = 88).

Categories	Time in months, median (IQR)	Death	
		Within the first six months of treatment, n (%)	After the first six months of treatment, n (%)
MDR-TB	9.5(14.0)	22(40.7)	32(59.3)
XDR-TB	13.1(17.7)	8(23.5)	26(76.5)

IQR = interquartile range; n = number of cases; MDR-TB = multidrug-resistant tuberculosis; XDR-TB = and extensively drug-resistant tuberculosis.

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not statistically significant. The proportion of MDR-TB patients without additional second-line injectable drug resistance was significantly higher among them (56.2%; $p = 0.020$) (S2 Table).

Discussion

This study evaluated treatment outcomes of a cohort of 436 patients with drug-resistant TB diagnosed in Portugal within 17 years, using national TB surveillance data. We paid special attention to death during treatment and identified factors associated with it in MDR- and XDR-TB patient groups.

We found that treatment was more successful for MDR-TB than XDR-TB patients. The case-fatality rate was highest among XDR-TB patients. Death during treatment occurred earlier for patients with MDR-TB than for the ones with XDR-TB; 40.7% of them died within the first six months of treatment. HIV infection and resistance to one or more second-line injectable drugs were independently associated with death among MDR-TB patients.

In our study, the overall treatment success rate among MDR/XDR-TB was 70.2%, below the 2020 target of the action plan for the WHO European Region (75%) [15]. However, treatment success rate among MDR-TB patients (77.9%) was higher than one the reported for the European Union by the European Centre for Disease Prevention and Control (ECDC) (48.1%) [2]. In this meta-analysis, 74 different studies were from several locations (60%) [16] and a study from Brazil (58,1%). Still, it was lower than reported by European countries, like Italy [17] and the Netherlands [18] (81.3% and 88%, respectively).

The treatment success rate among XDR-TB patients (62.8%) was higher than the one reported by the ECDC (37.4%) [2], in the meta-analysis mentioned above [16] and a study from Brazil [19] (26% and 18.6%, respectively).

Death was more frequent among unfavorable treatment outcomes in both patient groups. A higher frequency of *death* among MDR-TB patients was also reported by ECDC [2], while *lost to follow-up* was more frequently reported in the meta-analysis mentioned above [16], in Brazil [20] and China [21]. The case-fatality rate in our study was 18.4% that is equal to the rate reported in a meta-analysis (18%) [16], but is higher than reported by ECDC (17.1%) [2] and shown in Brazil (14.3%) [19] and China (2.8%) [21]. Nevertheless, in Pakistan, the rate was even higher (19.8%) [22].

Among XDR-TB patients, contrary to our results, *treatment failure* was a more frequent unfavorable outcome reported by ECDC [2] and shown in a meta-analysis [16] and Brazil [19]. However, our case-fatality rate (23.4%) is lower than was shown in Brazil (30.0%) [19], but is higher than was reported by ECDC (21.8%) [2] and in a meta-analysis (21%) [16].

We found that HIV infection was independently associated with death among MDR-TB patients. Although HIV infection was less prevalent among MDR-TB patients than among XDR-TB patients (23.8% *vs*. 31.0%), HIV infected patients of this group were 4.6 times more likely to die. This finding is consistent with the results described previously in several studies.

A meta-analysis noted an increase in deaths among HIV-MDRTB co-infected patients in low-income regions compared with high-income regions [23]. In India and Tanzania, HIV infection was also associated with death [24,25]. Toxicity and adverse events from antiretroviral therapy (ART) coupled with MDR-TB, therapy's side effects can be accountable for poor treatment outcome in this patient group. According to WHO, antiretroviral therapy must be started as early as possible (within the first eight weeks) after the beginning of anti-TB treatment, irrespective of CD4 T cell count [13]. However, a study carried out in South Africa found that ART use before MDR-TB treatment was significantly associated with higher case-fatality rate than when ART was initiated after the beginning of MDR-TB treatment [26]. Unfortunately, we do not assess information about antiretroviral therapy or CD4 cell counts because this information is not reported to our TB Surveillance System.

Resistance to one or more second-line injectable drugs was also independently associated with death among MDR-TB patients. We included this variable in the analysis of factors associated with death after observing the low treatment success rate (55.6%) and the high case-fatality rate (31.0%) among these patients that is contradictory to what has been demonstrated in previous studies [27,28].

The second-line injectable drugs (amikacin, kanamycin, and capreomycin) are some of the core second-line drugs used in the intensive phase of treatment of MDR-TB, which varied from 6 (WHO guidelines, 2008) [11] to 7–8.5 months (WHO guidelines, 2011) [12]. However, the role in treatment and the importance of resistance to each of these drugs is not the same. On the one hand, resistance to capreomycin [29] and kanamycin [9] were independently associated with unfavorable outcome in some studies. On the other hand, a recent meta-analysis showed that amikacin provided modest benefits in treatment, while kanamycin and capreomycin were associated with unfavorable outcomes [30]. Thus, according to the new WHO consolidated guidance of 2019, kanamycin and capreomycin are not included in longer treatment regimens [13].

Among XDR-TB patients, injectable drug use and being prisoner were associated with death in univariate analysis. However, all seven prisoners, who died, were also injectable drug users. Thus, a combination of these two factors should be considered as a risk situation for death in this group of patients.

Finally, we found that 40.7% of the MDR-TB patients, who died during treatment, died in the first six months. These patients represent more than half (56.2%) of the patients without additional second-line drug resistance who died during treatment. Alcohol abuse (60.0%), injectable drug use (55.6%), and comorbidities (61.5%) were also most frequent among them. These findings indicate that patients' death is not due to resistance to second-line injectable drugs but due to their addictions and comorbidities.

One of the strengths of this study is that it evaluated treatment outcomes of a cohort of drug-resistant TB patients over a significant amount of time. These patients were also previously characterised for the genetics of the pathogen [31] and for their spatial distribution in Portugal [32]. The knowledge about risk factors for death generated in this study will improve patients' clinical management, enhancing treatment success, which is another strength. One of the study's limitations is a restriction of the studied variables due to retrospective study design and using only the SVIG-TB date. Thus, we could not assess the effect of important variables on lethality, such as antiretroviral therapy and CD4 T cell count.

In conclusion, our findings suggest that factors and conditions as HIV infection, injectable drug use, alcohol abuse and comorbidities are most often associated with death early on and during treatment. This suggests the need for early diagnosis of MDR-TB and further monitoring of patients that present those characteristics from treatment initiation. Furthermore, it is

also required careful assessment of the relationship between early death and delayed MDR-TB diagnosis and clinical status of the patient that should grant further investigation.

Supporting information

S1 Fig. Treatment success rate by year, 2000–2016.
(TIF)

S1 Table. Treatment outcomes by drug resistance categories who started treatment between 2000 and 2016 (n = 436). n = number of cases; MDR-TB = multidrug-resistant tuberculosis; pre-XDR_{SLID}-TB = pre-extensively second-line injectable drug-resistant tuberculosis; pre-XDR_{FQ}-TB = pre-extensively fluoroquinolone-resistant tuberculosis; XDR-TB = and extensively drug-resistant tuberculosis. [a] Treatment success included only "Treatment completed" because no cured was registered. [b] Fisher's Exact Test.
(DOCX)

S2 Table. Characteristics of multidrug-resistant tuberculosis patients who died within and after the first six months of treatment (n = 54). [a] Not applicable for age. n = number of cases; IQR = interquartile range; HIV = human immunodeficiency virus; TB = tuberculosis.
(DOCX)

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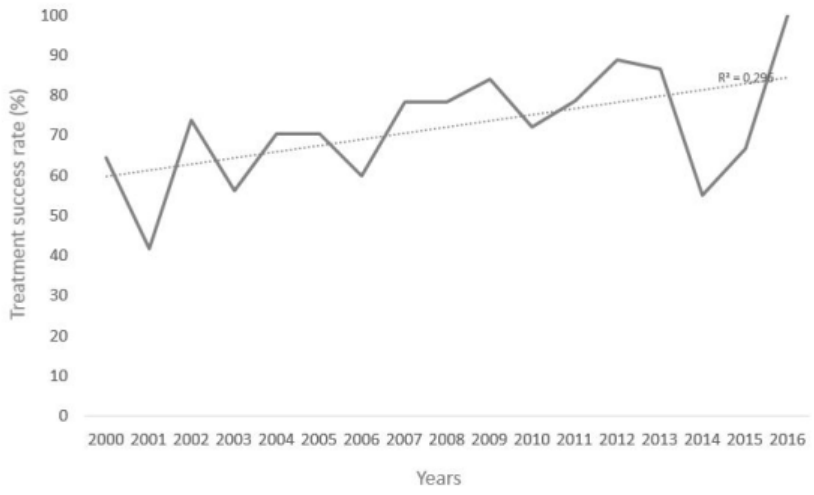
Supporting information



S1 Fig. Treatment success rate by year, 2000-2016.

S1 Table. Treatment outcomes by drug resistance categories who started treatment between 2000 and 2016 (n=436).

Treatment outcomes	Total		MDR-TB		Pre-XDR$_{SLID}$-TB		Pre-XDR$_{FQ}$-TB		XDR-TB		*p*-value[b]
	n	%	n	%	n	%	n	%	n	%	
Treatment success[a]											
Treatment completed	306	70.2	169	77.9	25	55.6	23	71.9	89	62.8	0.002
Unfavourable outcomes											
Treatment failed	16	3.7	5	2.3	3	6.7	0	0	8	5.6	0.147
Lost to follow-up	26	6.0	8	3.7	3	6.7	4	12.5	11	7.7	0.107
Death	88	20.2	35	16.1	14	31.0	5	15.6	34	23.9	0.065

n= number of cases; MDR-TB=multidrug-resistant tuberculosis; pre-XDR$_{SLID}$-TB =pre-extensively second-line injectable drug-resistant tuberculosis; pre-XDR$_{FQ}$-TB= pre-extensively fluoroquinolone-resistant tuberculosis; XDR-TB=and extensively drug-resistant tuberculosis.

[a] Treatment success included only "Treatment completed" because no cured was registered.

[b] Fisher's Exact Test

S2 Table. Characteristics of multidrug-resistant tuberculosis patients who died within and after the first six months of treatment (n=54).

Patient's characteristics		Death				*p*-value
		Within the first six months of treatment		After the first six months of treatment		
		n[a]	IQR or %	n[a]	IQR or %	
Age (years)	Median, IQR	44.0	27.0	39.5	30.0	0.481
Gender	Female	5	38.5	8	61.5	1.000
	Male	17	41.5	24	58.5	
Country of origin	Native	18	46.2	21	53.8	0.319
	Foreign-born	4	26.7	11	73.3	
HIV status	Negative	9	32.1	19	67.9	0.290
	Positive	13	50.0	13	50.0	
Alcohol abuse	No	12	32.4	25	67.6	0.150
	Yes	6	60.0	4	40.0	
Injectable drug use	No	8	28.6	20	71.4	0.128
	Yes	10	55.6	8	44.4	
Imprisonment	No	15	38.5	24	61.5	1.000
	Yes	2	50.0	2	50.0	
Community residence	No	18	45.0	22	55.0	1.498
	Yes	0	0.0	2	100.0	
Homelessness	No	17	41.5	24	58.5	0.429
	Yes	1	100.0	0	0.0	
Comorbidities	No	14	34.1	27	65.9	0.153
	Yes	8	61.5	5	38.5	
Chest radiography	No cavitation	13	52.0	12	48.0	0.099
	Cavitation	5	23.8	16	762	
Previous TB treatment	No	15	48.4	16	51.6	0.295
	Yes	7	30.4	16	69.6	
Site of disease	Pulmonary	20	40.0	30	60.0	1.000
	Extra-pulmonary	2	50.0	2	50.0	
Pre-XDR_{SLID}-TB	No	18	56.2	14	43.8	**0.020**
	Yes	2	14.3	12	85.7	
Pre-XDR_{FQ}-TB	No	21	42.9	28	57.1	0.638
	Yes	1	20.0	4	80.0	

[a] Not applicable for age.

n= number of cases; IQR= interquartile range; HIV =human immunodeficiency virus; TB=tuberculosis.

CHAPTER V

FINAL REMARKS

In Portugal, since 2000, several measures were taken in response to resurgence of TB and the emergence of MDR-TB that resulted in a decrease in their burden. However, this decrease must be accelerated in order to achieve the goal of TB eradication by 2035, for which MDR-TB presents a major obstacle. For this, it becomes necessary the revision of the MDR-TB scenario in Portugal, by assessing different aspects of the disease in terms of transmissibility, incidence, local strategies, and current outcomes. The work presented in this thesis contributes to augment the knowledge in the MDR-TB field and could prove even more important in face of the negative impact of the COVID-19 pandemic on TB control that is foreseen in the coming years.

We found significant heterogeneity in the spatial distribution of MDR- and non-MDR-TB and that the distribution of MDR- and non-MDR-TB were moderately correlated at the municipality level across Portugal (Chapter II) [1]. We identified 36 high-risk areas for non-MDR-TB and 8 high-risk areas for MDR-TB and uncover the fact that all 8 high-risk areas for MDR-TB were simultaneously high-risk areas for non-MDR-TB. However, these 8 areas represent only 22 % of the non-MDR-TB high-risk areas. A very important detail is that 7 out of the 8 high-risk areas are located in the Lisbon metropolitan area, where active transmission of MDR-TB was previously reported based on genetic studies [2, 3] and as highlighted in Chapter III [4].

We show an MDR-TB transmission scenario, where MDR strains likely arose and are transmitted within local chains of transmission (Chapter III) [4]. Among 7 identified genetic clusters, the 3 largest clusters belong to sublineage LAM that is in contrast with the remaining European MDR-TB picture, where immigrant-associated Beijing strains are more common [5]. However, these 3 largest clusters, which included 71% of clustered cases, corresponds to the previously described MDR-TB clusters as Q1, Lisboa3-A and Lisboa3-B in the LTV region [2, 3].

Based on genetic information we estimated that 52.2% of MDR-TB cases were attributable to recent transmission (i.e., within the previous 2 years). Although this percentage dropped to 14.9% after adjustment with epidemiological data, it was twice as high as what was reported in some European countries [6, 7]. However, our estimation of recent transmission is probably underestimated. The number of epidemiological links between clustered cases must be higher than what we were able to identify. According to an investigation carried out in UK, 33% of epidemiological links were identified through routine contact tracing and 67% following MIRU-VNTR cluster investigation [8]. In our study we used epidemiological data only from routine contact tracing collected by Public Health services through epidemiological surveys. Data from cluster investigation, which would allow to identify the epidemiological

link between cases within the same genotypic cluster, was absent because this investigation was not performed at the time of diagnosis.

Currently, in Portugal, WGS technique is being implemented for genomic characterization of multidrug-resistant Mtb isolates [9], which is very advantageous for TB surveillance. However, this powerful tool, which provide a greater genetic discrimination, must be complemented by epidemiological data from contact tracing and real-time cluster investigation [10] to obtain a "real-time surveillance" and fully interpret transmission dynamics in each region.

We suggest the performance of routine genotyping of all TB isolates to understand the dynamics of MDR-TB emergence and transmission. In Portugal, genotyping of Mtb isolates is carried out only if an outbreak is suspected [11, 12]. Genotyping of all Mtb isolates and cluster investigations has been routinely applied a decade ago in low TB incidence countries of Europe such as England, the Netherlands, Denmark, Sweden, Finland and Norway [10, 13-15]. Additionally, for example, in England, a routine WGS service was launched in 2017 and WGS is now being used routinely in the TB diagnostic pathway [16]. The delay of diagnosis of MDR-TB increases the likelihood of its transmission in the community [17]. In our study, median TB diagnostic delay was 72 days that is comparable to 74 days of diagnosis delay of TB in Portugal in 2019, which has been increasing in the last decade [18] and will probably increase even more due to the negative impact of the COVID-19 pandemic. For this reason, we suggest that in municipalities that we have identified as high-risk areas rapid drug resistance testing will be used in case of any suspicion and diagnosis of TB [1] and not just in case of suspicion of MDR-TB, as recommended so far by the Direção-Geral da Saúde (DGS) [19]. Meanwhile, the use of WGS technique, at least in identified high-risk areas, could help solve some important needs. This is because WGS, in addition to its important role in identifying transmission chains [8], allows simultaneous identification of mycobacterial species and first- and second-line drug resistance in a short time [20]. This approach would also be effective in TB control in the Lisbon and Tagus Valley (LVT) region where TB presents one of the highest burden of the country (23.7 and 20 cases per 100 000 population, respectively) [18].

In addition to the importance of early MDR-TB diagnosis, it is also very important to start and complete an appropriate course of anti-TB treatment. Successful treatment, in addition to clinical benefit for patients, also reduces transmission of MDR-TB within the population and mortality among patients. The treatment success rate that we estimated for MDR/XDR-TB (70.2%) (Chapter IV) [21] was higher than previously reported (62.1%) [22], but it is still below the 2020 target of the action plan for the WHO European Region (75%) [23]. The increase in success rate is most probably associated with the

functioning of the Region Reference Centres, as was demonstrated in the Northern region of the country [24] and by the decrease of TB notification rate in Portugal since 2002 [18]. However, the treatment success rate among MDR-TB patients only (77.9%) was higher than the reported for the European Union by the European Centre for Disease Prevention and Control (ECDC) (48.1%) [25], but it was lower than reported by other European countries, like Italy [26] and the Netherlands [27] (81.3% and 88%, respectively).

When we evaluated the treatment outcomes within drug-resistant patient groups, we observed that 18.4% of MDR-TB patients and 23.9% of XDR-TB patients died during treatment. In EU, according to surveillance data regarding 2018, 17.1% of MDR-TB patients and 21.8% of XDR-TB patients died during treatment [25] and that is compatible with our findings. We also observed that 40.7% of the MDR-TB patients, who died during treatment, died in the first 6 months, which probably reflects the patient's clinical condition since the beginning of treatment and non-response to treatment.

Comparing TB patient characteristics, we observed that the patients from high-risk areas for both MDR- and non-MDR-TB had a higher prevalence of several factors directly or indirectly associated with increased risk/susceptibility to TB, namely: HIV infection, foreign-born, history of imprisonment, consumption of alcohol and injectable drugs, previous TB treatment, living in a community residence or be homeless [1]. In fact, these factors, which are known as potential risk factors for MDR-TB [28-32], were most common among patients with MDR-TB in our spatial [1] and clustering studies [4].

Regarding HIV infection, a meta-analysis by Eldholm and colleagues demonstrated that HIV co-infection does not significantly impact the transmissibility of Mtb and does not affect the rate of Mtb drug resistance evolution within patients [33]. Accordingly, HIV infection is often mostly associated with primary rather than with acquired MDR-TB [34-36]. However, HIV co-infection accelerates progression from latent infection to active TB and it is likely to reactivate an infection by MDR strain acquired more recently following community or institutional transmission in settings with high MDR-TB prevalence [34, 35].

In our studies, HIV infection was more prevalent among clustered comparing with unique MDR-TB cases (30.8% and 12.5% respectively), but HIV infection was not associated with MDR-TB recent transmission [4]. Moreover, HIV infection was independently associated with death during treatment (in the period after the first 6 months of treatment) among MDR-TB patients [21]. This finding is consistent with the results described previously: HIV infection was associated with death in India [37] and Tanzania [38]; and an increase in deaths among MDR-TB patients co-infected with HIV in low-income countries like South

Africa, Peru and Belarus, compared with high-income countries like United States and Netherlands was noted in a meta-analysis [39].

The fact of being previously treated with anti-tuberculosis drugs, cannot be assumed as evidence for acquisition of resistance during current treatment. According to a population-based transmission modelling analysis, 61.3% of the incidence of MDR-TB among previously treated patients resulted from new episodes of infection with MDR-TB strains [40].

Although foreign-born individuals were frequent among MDR-TB patients in our studies, recent transmission of MDR-TB was associated with Portugal-born individuals. Alcohol abuse was not independently associated with transmission, but was a good identifier for Portugal-born clustered cases: 92% of clustered cases with alcohol abuse were Portugal-born [4].

Besides that, alcohol abuse, injectable drugs use and others comorbidities (like diabetes, silicosis, chronic obstructive pulmonary disease, liver disease or neoplasia) were most frequent among MDR-TB patients, who died within the first six months of treatment and could probably be associated with early death.

In conclusion our study corroborates the need for individuals belonging to vulnerable groups such as HIV infected, alcohol abusers and injectable drug users, to be granted special attention both in early diagnosis and in monitoring of potential anti-tuberculosis treatment resistance.

This work had its limitations due to its retrospective design that limited our evaluation only to the analysis of the data collected routinely. For example, it would be very important to evaluate the association of TB and MDR-TB with socio-economic deprivation that will be our next challenge. Also it is necessary to develop a prospective study to evaluate the appearance of TB/MDR-TB among contacts over the years that it was not possible in the present work.

In conclusion, we showed that continuous transmission of MDR-TB occurs in municipalities that we have identified as high-risk areas. Taking into account our results we suggest that strategies for TB and subsequently MDR-TB control in Portugal should be reviewed and adjusted to accelerate the decrease of disease incidence. This readjustment will also be an asset to respond to the expected negative impact of COVID-19 pandemic.

The main strategy should be focused on active search for cases of TB disease and latent infection, and early detection of drug-resistance, through more extensive contact investigation and longer follow-up (more than 2 years), cluster investigations and systematic screening, particularly among vulnerable groups. We are aware that this decision to change strategies and implement new measures must be

taken at the political-economic level because it requires investment in human resources, equipment and materials. The measures as we propose require strengthening of Public Health mainly, which has a predominant role in the control and prevention of transmission TB.

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