In silico mechanistic study of the potential use of NSAIDs to accelerate the healing of patients with pulmonary tuberculosis

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Bachelor's thesis

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ABSTRACT

Background: Pulmonary tuberculosis (PTB) is one of the 10 leading causes of death worldwide. One of the biggest concerns is resistance to antibiotics, the anti-microbial therapy targeting *Mycobacterium tuberculosis* which is used in the current therapeutic regimen against PTB, reason why the number of new multidrug-resistant PTB is increasing worldwide. Due to the long duration and low effectiveness of the treatment in these patients, inflammation remains throughout the months, increasing severity of the disease and leading to an irreversible lung tissue damage and impaired lung function derived from the infection. Thus, non-steroidal anti-inflammatory drugs (NSAIDs), which are host directed therapies, are suggested as a potential therapeutic option to improve the prognosis of PTB patients.

Materials and methods: *In silico* study using the Therapeutic Performance Mapping System technology. First, the molecular characterization of PTB and the NSAIDs acetylsalicylic acid and ibuprofen through manual review of the current scientific literature, specialized databases and official documentation was realised. Then, systems biology based mathematical models were generated, simulating the mechanism of action of NSAIDs in PTB. A mechanistic study at pathway level was carried out using artificial neural networks. Finally, the mechanism of action explaining the impact of NSAIDs in PTB was assessed.

Results and discussion: The two main targets of NSAIDs, COX1 and COX2, had a high predicted relationship with PTB, highlighting their high relationship with inflammation and lung tissue damage. The mechanism of action of NSAIDs showed that by inhibiting COX1 and COX2, the pathological status of some PTB effector proteins, such as NFκB1, ERK1/2, STAT3, MMP2/9 and Bcl2, was reversed.

Conclusion: According to the results obtained in this study, the use of NSAIDs as co-treatment of PTB can accelerate the healing of PTB patients by increasing nitric oxide production, improving macrophage apoptosis, inhibiting hyperinflammation and avoiding pulmonary tissue damage.

Key words: pulmonary tuberculosis (PTB), non-steroidal anti-inflammatory drugs (NSAIDs), ibuprofen, acetylsalicylic acid, *in silico*.

ABBREVIATION LIST

AM	Alveolar macrophage
ANNs	Artificial neural networks
ASA	Acetylsalicylic acid
Bcl2	Apoptosis regulator Bcl-2
COX1	Cyclooxygenase-1
COX2	Cyclooxygenase-2
COXs	Cyclooxygenases
DAPK	Death-associated protein kinase
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing non-integrin 1
EMA	European Medicines Agency
EPAR	European Public Assessment Report
ER	Endoplasmic reticulum
ERK1	Extracellular signal-regulated kinase 1
ERK2	Extracellular signal-regulated kinase 2
FDA	Food and Drug Administration
IBU	Ibuprofen
IFNγ	Interferon gamma
iNOS	Inducible nitric oxide synthase
MAPK	Mitogen-activated protein kinase
Mcl-1	Bcl-2-like protein 3
MDR-TB	Multidrug-resistant Tuberculosis
MEK	Mitogen-activated protein kinase kinase
MMPs	Matrix metalloproteinases
MMP2	Matrix metalloproteinase-2
MMP9	Matrix metalloproteinase-9
MR	Mannose receptor
Mtb	*Mycobacterium tuberculosis*
NFκB1	Nuclear factor NF-kappa-B p105 subunit
NFκB	Nuclear factor NF-kappa-B
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug

PAMP	Pathogen associated molecular pattern
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PI3K	Phosphatidylinositol 3-kinase
PLCβ	Phospholipase Cβ
PRR	Pattern recognition receptor
PTB	Pulmonary tuberculosis
PTGER1	PGE_2 receptor EP1 subtype
RAC1	Ras-related C3 botulinum toxin substrate 1
STAT3	Signal transducer and activator of transcription 3
TB	Tuberculosis
TLR	Toll-like receptor
TNFα	Tumor necrosis factor
TPMS	Therapeutic Performance Mapping System
WHO	World Health Organization

INTRODUCTION

Tuberculosis (TB) is a chronic, life-threatening infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*) infection [1]. According to data provided by World Health Organization (WHO) in 2020, TB is one of the 10 leading causes of death worldwide. In 2019, approximately 10 million people developed TB and it has been responsible for at least 1.4 million deaths [2], since only 10% of infected persons are capable to defeat *Mtb* [3]. Despite *Mtb* can infect anyone in any part of the world, it mainly affects adult men with limited financial resources [2]. The most common TB is pulmonary tuberculosis (PTB), which affects lungs and accounts for about 85% of TB cases [4].

PTB development begins when a healthy person inhales airborne particles containing *Mtb* from an infected person [1]. Once *Mtb* is inside the host, triggers a complex immune response which can be subverted by the pathogen allowing its survival in the host [3]. First, *Mtb* is recognised by diverse immune cells through pattern recognition receptors (PRRs) [5]. This cells phagocyte *Mtb* and trigger an early immune response, where alveolar macrophages (AMs), neutrophils, monocytes and natural killer cells are the major players [6]. *Mtb* is able to survive inside the macrophage by preventing the maturation of phagolysosomes, inhibiting acidification of lysosomes, scavenging oxidative stress, and avoiding apoptosis [3]. Then, the recruitment of B cells and T cells to the site of infection is triggered [6]. This recruitment is used by *Mtb* to complete the formation of the granuloma, which is an immune host-defence mechanism made of innate and adaptive immune cells, but mycobacteria use it to their advantage allowing its replication [7]. When *Mtb* leads the macrophages forming the core of granuloma to necrosis, it causes destruction of the extracellular matrix, triggering extensive tissue damage and excessive inflammation and allowing the spread of *Mtb*, which leads to the magnification of the infection [8] (**Figure 1**).

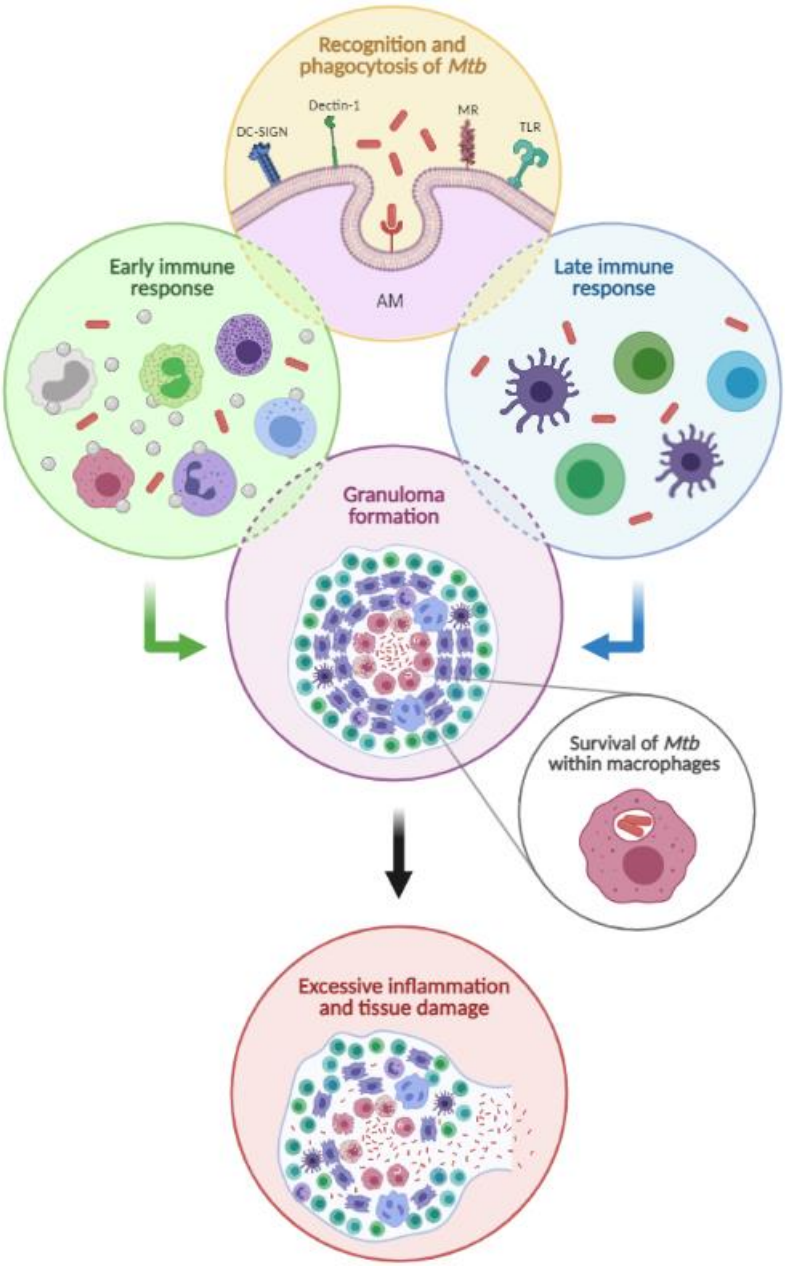

Figure 1. Summary of the most relevant events during PTB development. *Infection by Mtb begins when it is inhaled, reaches the lungs, and is recognized by AMs. Mtb recognition triggers the innate immune response, beginning the granuloma formation. Later, the recruitment of T cells and B cells to the lung is triggered, completing the granuloma formation. In the granuloma, Mtb grows and replicates until Mtb spreads to other organs, causing excessive inflammation and lung tissue damage. Abbreviations: AM, alveolar macrophage; DC, dendritic cell; DC-SIGN, dendritic cell-specific ICAM-3-grabbing non-integrin 1; MR, mannose receptor; Mtb, Mycobacterium tuberculosis; TLR, Toll-like receptor.*

One of the biggest concerns is resistance to antibiotics, anti-microbial therapy targeting the pathogen which is used in the current therapeutic regimen against PTB. The number of new multidrug-resistant TB (MDR-TB) cases in Europe is the highest in the world, representing more than half a million people [9]. Depending on pathogen factors (virulence factors, drug resistance) and host factors (genetic factors, social and economic factors, coinfections,

comorbidities), the response to treatment and the outcome of PTB varies widely [2]. While non-MDR-TB patients are in treatment for at least 6 months, for MDR-TB patients' treatment is much longer and unlikely to be successful. Meanwhile, the inflammation remains throughout the months [2]. This prolonged inflammation is associated with an increased severity of PTB [10], leading to an irreversible lung tissue damage and, consequently, impaired lung function. Even half of the treated and cured patients have some type of pulmonary dysfunction derived from the infection by *Mtb* [11], being impaired lung function, cavities formation, bronchiectasis, and fibrosis [8] some of the most damaging consequences of the hyperinflammatory and sustained response against *Mtb* [12].

Because of the mentioned before, the inhibition of inflammation is suggested as a potential therapeutic option to improve the prognosis of PTB patients [10]. Thus, reducing the duration of treatment and minimizing lung tissue damage and, consequently, the risk of permanent sequelae [13]. Non-steroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (ASA) and ibuprofen (IBU), have powerful anti-inflammatory activity, common side effects, and are one of the most used drugs all over the world [14], since they are safe, simple, and inexpensive. In addition, NSAIDs are host directed therapies, so they do not increase the risk of generating drug-resistance [13]. This is the reason why several studies have suggested the huge potential of NSAIDs as a treatment for PTB [15-18].

In a study carried out by Vilaplana *et al.*, an increase in the survival of mice with TB was observed when they were treated with IBU. In addition, a lower number of lung lesions was observed, in concordance with a decrease in the amount of *Mtb* [16]. Byrne *et al.* also realized an *in vivo* study in mice with TB. The study compared the effect of isoniazid and pyrazinamide, two first-line anti-TB drugs, with the effect of ASA and IBU, alone or together with isoniazid on TB. The results showed a greater decrease in the amount of *Mtb* in the lungs when diseased mice were treated with IBU and isoniazid compared to mice that were only treated with pyrazinamide. In contrast, neither ASA with isoniazid nor ASA and IBU individually showed a significant effect in the amount of *Mtb* [17]. Nevertheless, Byrne *et al.* through another *in vivo* study observed that both ASA and IBU administered with pyrazinamide enhanced its effect, increasing its potential to reduce the amount of *Mtb* [18]. These studies report a potential benefit of the incorporation of NSAIDs in the treatment of TB, although more clinical research is needed.

In order to evaluate the efficacy and safety of IBU as a co-treatment of extremely resistant TB, a clinical trial was conducted in Georgia [19]. Currently, one clinical trial is ongoing to evaluate

the effect of ASA and IBU as adjunct therapy added to and compared with the standard of care TB therapy in MDR-TB patients [20].

The beneficial effect of ASA and IBU is possibly mediated by their anti-inflammatory properties. ASA and IBU exerts their anti-inflammatory effects by inhibiting COX1 and COX2 [21]. While ASA acts as an irreversible inhibitor by acetylating the active site of COX1 and COX2 [22], IBU acts as a competitive reversible inhibitor of both COXs [23]. COX1 is constitutively and ubiquitously expressed, while COX2 is induced when there is inflammation [24]. Both cyclooxygenases are involved in the metabolization of arachidonic acid, synthesizing prostaglandin (PG) H_2, which subsequently will be metabolized to prostaglandin E_2 (PGE_2). PGE_2 is involved in inflammatory processes, fever and in the control of infection [13]. High amounts of PGE_2 have been related to better PTB disease progression, due to a down-regulation of the cell-mediated immunity [25]. In addition, high concentrations of PGs increase the expression of metalloproteinases (MMPs), which are highly involved in the deterioration of lung function in PTB [8].

For the reasons stated above, this project purpose that the inclusion of ASA and IBU in the current therapeutic regimen of PTB could accelerate the healing of patients, thus reducing permanent lung damage. The therapeutic mechanism of ASA and IBU in PTB treatment has not yet been determined, therefore in this project, an exhaustive study has been carried out to identify it.

An *in silico* study has been carried out using the Therapeutic Performance Mapping System (TPMS) technology, a systems biology and artificial intelligence-based approach [26]. The TPMS technology creates mathematical models that integrate all available biological, pharmacological and medical knowledge to simulate human physiology *in silico*, allowing to test and evaluate the physiological effects of pharmacological compounds or biological processes at the molecular level. Through the use of artificial intelligence and pattern recognition techniques, TPMS generates mathematical models that are able to suggest mechanistic hypotheses that are in accordance with nature. Various examples of repurposed drugs identified using TPMS approach have been validated *in vitro* and *in vivo*, and one of them is advancing to clinical trials [26-34].

In silico models have many advantages, among them the simulation of real situations, with the possibility of modelling and evaluating each variable. The ability to predict or observe behaviours quickly and cheaply compared to *in vitro* or *in vivo* models, in addition to reducing the use of experimental animals.

HYPOTHESIS AND AIMS

Due to the existence of the great need to reduce the sequelae of the PTB, the main aim of this project was to evaluate if and by which molecular mechanism the inclusion of NSAIDs (considering their recognized therapeutic targets for their indication: COX1 and COX2) in the current therapeutic regimen of PTB could accelerate the healing of patients by avoiding excessive inflammation. Thereby, reducing the development of permanent lung damage and decreasing pulmonary dysfunction.

Furthermore, a secondary aim was to specifically evaluate the potential of ASA and IBU (considering their whole target profile) as treatment for PTB.

To prove the mentioned hypothesis, *in silico* models of patients with PTB have been generated and the effect of ASA and IBU on the pathology has been evaluated at the molecular level.

METHODS

The starting point for this project was the molecular characterization of the pathology (PTB) and the drugs under study (ASA and IBU). PTB was molecularly characterized through manual curation of the current scientific literature regarding this subject. The information found was used to identify its main biological processes (motives), proteins involved in the disease pathophysiology (protein effectors) and to build the protein network around the disease.

High-throughput data was also retrieved from publicly available databases and analysed in order to try to find information to complete the bibliography-based disease characterization and/or to obtain a signature (tissue signature) to include in the models and/or evaluate it afterwards once the mechanisms were predicted.

The drugs were exhaustively reviewed considering available scientific literature, as well as dedicated databases and official documentation to define their targets.

By using the characterization of the disease and the drugs of interest plus the manually curated topology, systems biology based mathematical models were generated, simulating the mechanism of action of the evaluated treatments in PTB. A mechanistic study at pathway level was carried out to identify the motives most affected by the evaluated treatments using artificial neural networks (ANNs), which harbour predictive capacity. Finally, the mechanism of action explaining the impact of NSAIDs in PTB was assessed.

A detailed description of the applied methods is included in the *Annex I - Supplementary methodology*.

RESULTS AND DISCUSSION

PULMONARY TUBERCULOSIS CHARACTERIZATION

PTB literature-based characterization

First, PTB was characterized by searching in the scientific literature. The main pathophysiological processes known to be involved in PTB were described. To make it more understandable, the results were represented in **Figure 2**. Each motive was defined at molecular level and candidate proteins and genes whose activity (or lack thereof) is functionally associated with the development of the condition were considered as condition effectors. The results obtained are shown in **Table 1**.

Table 1. Summary of the motives identified as involved in PTB. *The amount of protein effectors identified in each motive are noted.*

	Motive name	Proteins
1	*Mtb* infection: recognition and phagocytosis	38
2	Early immune response	30
3	Late immune response	48
4	Formation of granulomas	7
5	Survival of *Mtb* within macrophages	66
6	Excessive inflammation and lung tissue damage	42

A total of 205 non-duplicated protein effectors (proteins/genes involved in the development of PTB) were obtained (**Table 1**).

The first motive, *Mtb infection: recognition and phagocytosis,* begins when alveolar epithelial cells, AMs, DCs and neutrophils establish the recognition of the bacteria through a diversity of PRRs and phagocytic receptors [6, 35]. PRRs recognise pathogen associated molecular patterns (PAMPs) that *Mtb* presents. PAMPs recognition by PRRs expressed on immune innate cells is key to the innate immunity providing defence against *Mtb*, by recognizing the pathogen in lung's alveoli and mediating the *Mtb* catchment [5, 36]. Once *Mtb* is inside the lung and detected by immune cells, the pathogen may be phagocyted by AMs, DCs and neutrophils [1].

The second motive, *early immune response*, occurs when innate immune cells go into action. Innate immune cells are the first defence response against the pathogen, which not only determines whether the *Mtb* infection will be established, but also influences its progression by training the adaptive immune response and by regulating inflammation and limiting tissue damage. Despite this, innate immune cells, such as macrophages, where *Mtb* is located, are often niches for bacterial replication, using the innate immune response to their own benefit

and establishing a chronic latent infection inside the phagosome [5, 6, 35, 37, 38]. The main effector innate immune cells are macrophages, neutrophils, DCs, natural killer cells and mast cells [6]. These cells contribute to the formation of the granuloma [39], a principal host-defence system for containing *Mtb* infection [3].

Then, the third motive, *late immune response*, is triggered. The next step in immunity is to activate and recruit lymphocytes to develop adaptive immunity. DCs play a very important role, acting as a much better antigen presenters than macrophages, activating T cells with specific *Mtb* antigens [39] via MHC, costimulatory molecules, and cytokines [5].

Formation of granuloma in the lung is the fourth motive and is a principal host-defence system for containing *Mtb* infection, but it also can act as a reservoir for bacteria [3] as *Mtb* uses granuloma formation to its advantage, allowing intracellular dissemination and greater survival of bacteria [40]. Granuloma is composed of the aggregation of several immune cells, such as macrophages, monocytes, DCs, neutrophils, fibroblasts, epithelioid cells, foamy macrophages, and Langerhans giant cells. This compact and organized core is surrounded by a layer of lymphocytes [7].

In the core of the granuloma occurs the fifth motive, *survival of Mtb within macrophages.* Inside the macrophage, bacteria are isolated in a vacuole named phagosome, which usually will fuse with lysosome leading to the phagolysosome formation [39]. The function of phagosome is to keep bacteria steady to be killed by acid pH, reactive oxygen intermediates, lysosomal enzymes, and toxic peptides from lysosome [41]. However, *Mtb* can inhibit the phagosome maturation and prevent the phagolysosome generation, serving as a niche for its replication [5].

Finally, as the *Mtb* infection progresses and PTB disease advances, the granuloma evolves to form a caseum. Caseous granuloma is composed by epithelioid macrophages covering a necrotic zone build of dead macrophages and other cells with a lymphocytic cuff that comprise both B and T cells [42]. Increased necrosis and decomposition of granuloma cells drive to the accumulation of caseum, which can lead to cavitation of granulomas [7] by destroying the extracellular matrix of the lung. Those cavities are used by *Mtb* to replicate themselves and spread to the airways [35]. Finally leading to the sixth motive, *excessive inflammation and lung tissue damage* (**Figure 2**).

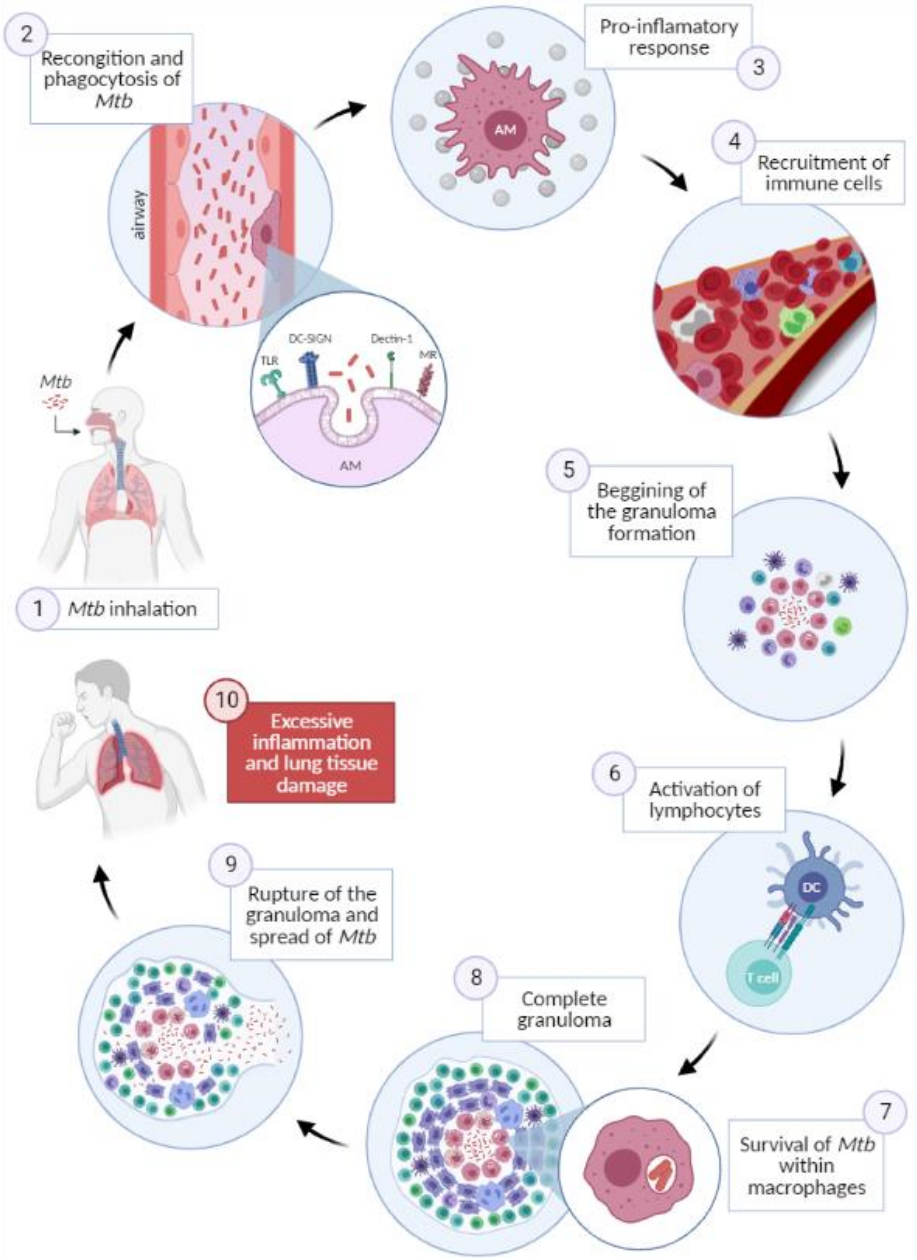

Figure 2. Overview of development of Tuberculosis. *Infection by Mtb begins when it is inhaled, reaches the lungs, and is recognized by AMs. AMs are activated, initiating a cytokine response to recruit immune cells to the site of infection. There begins the granuloma formation. Later, Mtb is transported to the lymph nodes by DCs and monocytes, leading to the recruitment of T cells and B cells to the lung. This completes the granuloma. In the granuloma, Mtb grows and replicates until Mtb spreads to other organs, causing excessive inflammation and lung tissue damage. Abbreviations: AM, alveolar macrophage; DC, dendritic cell; DC-SIGN, dendritic cell-specific ICAM-3-grabbing non-integrin 1; MR, mannose receptor; Mtb, Mycobacterium tuberculosis; TLR, Toll-like receptor.*

PTB high-throughput data evaluation

Having completed the bibliography-based condition characterization, patients' data publicly available has been analysed in order to obtain a molecular fingerprint or signature that can help adjust models and be used to evaluate the modelled drug effects.

Since no relevant high-throughput data were available for PTB that met the stipulated criteria as it could introduce biases, data found were not included in the analysis (detailed information in *PTB high-throughput data evaluation*).

PTB network

Prior to the execution of the analysis, the network around PTB protein effectors was analysed. The network involving all PTB effector proteins and their direct interactors in Anaxomics' human protein network is shown in **Figure 3**.

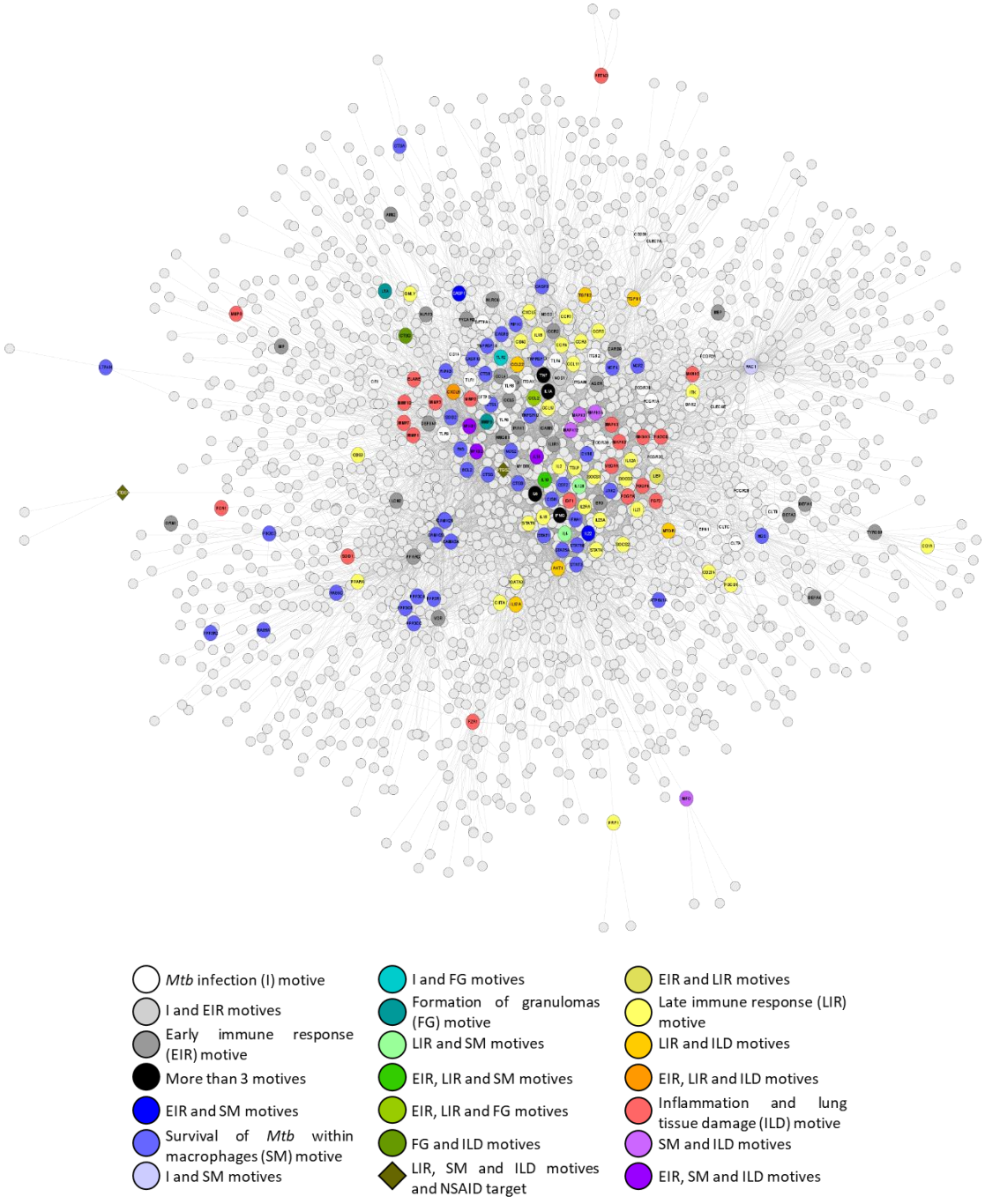

Figure 3. PTB effectors and their direct interactors *according to Anaxomics' human protein network. PTB effectors are shown in the central part and their direct interactors in the distal area.*

A network only showing the PTB effectors directly linked among them was also generated (**Figure 4**). The targets of the drugs under evaluation are also included.

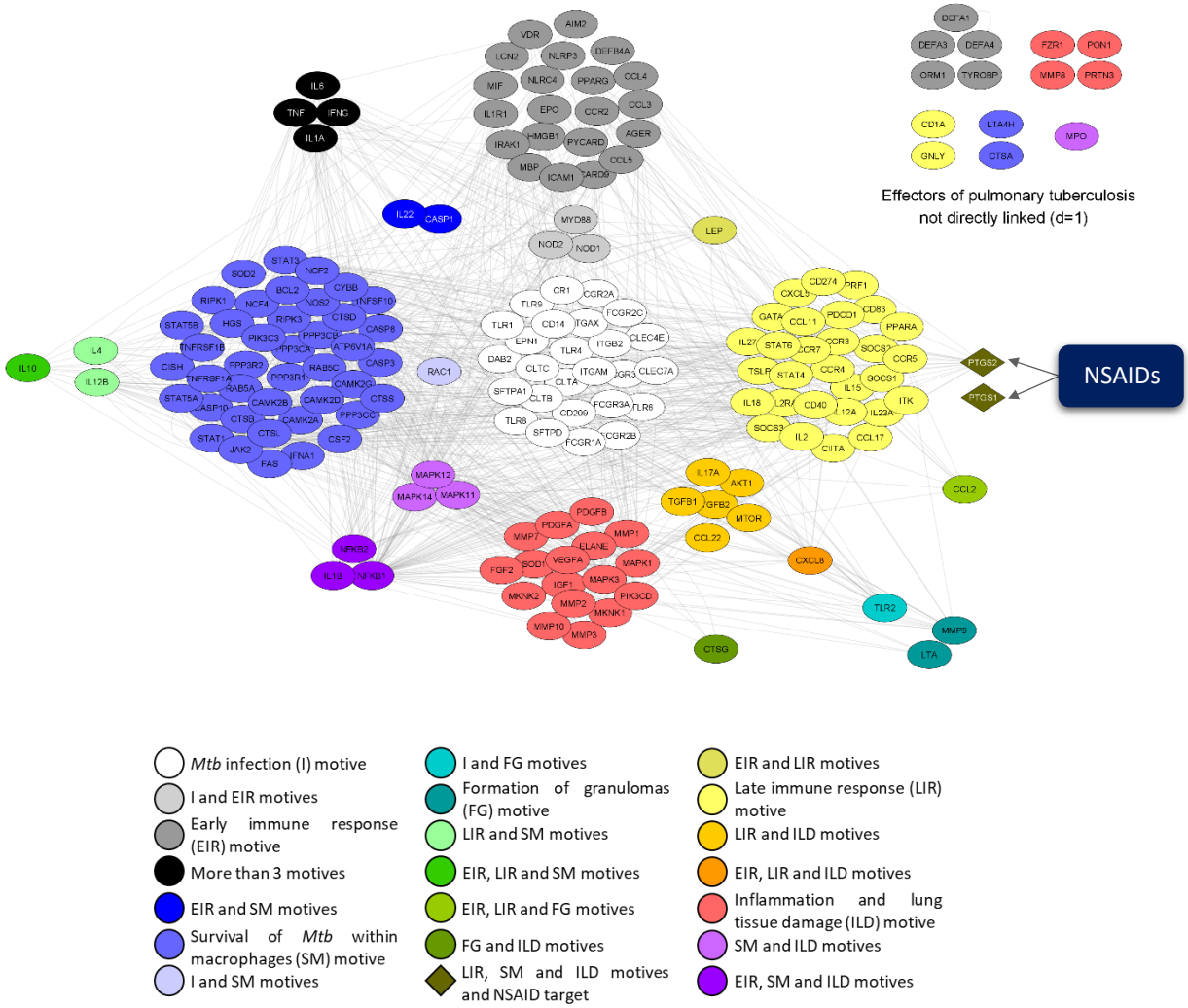

Figure 4. Network of PTB effectors directly linked among them.

The **Figure 4** shows a high level of interconnection between PTB effectors and a high number of direct relationships between PTB effectors and NSAIDs targets, which reinforces the intricate molecular mechanisms involved in the disease and the need to explore in detail the potential mechanisms by which NSAIDs could be used as a therapeutic strategy in PTB patients.

DRUGS CHARACTERIZATION

To determine the mechanism of action of the drugs, first, each drug was characterized at the molecular level.

Acetylsalicylic acid characterization

The results of the characterization of the targets and off-targets of ASA are shown in **Table 2**.

As shown in **Table 2**, two ASA targets and eleven off-targets were determined. On the one hand, nine proteins are directly inhibited by ASA: COX1, COX2, AKR1C1, EDNRA, GRP78, RSK2, IKKβ, PKC2 and CFTR. On the other hand, ASA directly activates four proteins: AMPK2, AMPK, p53 and NOS3.

Table 2. Acetylsalicylic acid proposed drug targets and off-targets. *It is also indicated the effect of the drug over the proposed target (red arrow (↓) indicates an inhibition and green arrow (↑) an activation), whether the candidate has been proposed as drug target according to the explored databases, as well as the bibliographic reference/s justifying the proposed drug target. The drug targets with pharmacological action against the approved indication are shaded in grey.*

UniProt ID	Protein Name	Short Name	Effect	DrugBank	References
P23219	Prostaglandin G/H synthase 1	**COX1**	↓	Yes	[43, 44]
P35354	Prostaglandin G/H synthase 2	**COX2**	↓	Yes	[43, 44]
Q04828	Aldo-keto reductase family 1 member C1	**AKR1C1**	↓	Yes	[45]
P54646	5'-AMP-activated protein kinase catalytic subunit alpha-2	**AMPK2**	↑	Yes	[46, 47]
Q9Y478	5'-AMP-activated protein kinase subunit beta-1	**AMPK**	↑	Yes	[46, 47]
P25101	Endothelin-1 receptor	**EDNRA**	↓	Yes	[48]
P04637	Cellular tumor antigen p53	**p53**	↑	Yes	[49]
P11021	Endoplasmic reticulum chaperone BiP	**GRP78**	↓	Yes	[50, 51]
P51812	Ribosomal protein S6 kinase alpha-3	**RSK2**	↓	Yes	[52]
O14920	Inhibitor of nuclear factor kappa-B kinase subunit beta	**IKKβ**	↓	Yes	[50, 53, 54]
P29474	Nitric oxide synthase, endothelial	**NOS3**	↑	No	[55-57]
Q05513	Protein kinase C zeta type	**PKC2**	↓	No	[58]
P13569	Cystic fibrosis transmembrane conductance regulator	**CFTR**	↓	No	[59, 60]

Ibuprofen characterization

The results of the characterization of the targets and off-targets of IBU are shown in **Table 3**.

Two targets and five off-targets were found for IBU, as shown in **Table 3**. Four of them, COX1, COX2, CFTR and ASIC1, are directly inhibited by IBU, while the remaining three, PPARγ, PPARα and AMPK2, are directly activated by the drug.

Table 3. Ibuprofen proposed drug targets and off-targets. *It is also indicated the effect of the drug over the proposed target (red arrow (↓) indicates an inhibition and green arrow (↑) an activation), whether the candidate has been proposed as drug target according to the explored databases, as well as the bibliographic reference/s justifying the proposed drug target. The drug targets with pharmacological action against the approved indication are shaded in grey.*

UniProt ID	Protein Name	Short Name	Effect	DrugBank	References
P23219	Prostaglandin G/H synthase 1	**COX1**	↓	Yes	[61]
P35354	Prostaglandin G/H synthase 2	**COX2**	↓	Yes	[61-63]
P13569	Cystic fibrosis transmembrane conductance regulator	**CFTR**	↓	Yes	[59, 60]
P37231	Peroxisome proliferator-activated receptor gamma	**PPARγ**	↑	Yes	[64]
Q07869	Peroxisome proliferator-activated receptor alpha	**PPARα**	↑	Yes	[65]
P78348	Acid-sensing ion channel 1	**ASIC1**	↓	No	[66-68]
P54646	5'-AMP-activated protein kinase catalytic subunit alpha-2	**AMPK2**	↑	No	[69-71]

As can be seen in both **Table 2** and **Table 3**, both ASA and IBU have COX1 and COX2 as main targets, which are the most recognized, and also a set of off-targets that could also participate in the therapeutic efficacy of the treatments.

MECHANISTIC EVALUATION OF THE POTENTIAL RELATIONSHIP BETWEEN NSAIDS AND PTB

The possible mechanistic relationship between the drugs under study and the proteins included in the molecular characterization of PTB has been evaluated by means of ANNs analysis [72].

ANNs evaluate the relationship among protein sets or regions inside Anaxomics' network, providing a predictive score that quantifies the probability of the existence of a functional relationship between the evaluated regions. Each score (from 0 to 100%) is associated with a p-value that describes the probability of the result being a true positive (**Table 4**).

Table 4. ANNs values and their associated p-values. *Category division of ANNs score according to probability of being a true positive result.*

ANNs score	Likelihood of the predicted relationship	Associated p-value
≥92	VERY HIGH	<0.01
78-91	HIGH	0.01-0.05
71-77	MEDIUM-HIGH	0.10-0.05
38-70	MEDIUM	0.20-0.10
<38	LOW	>0.20

Relationship between COX1 and COX2 and PTB

Because of NSAIDs, including both ASA and IBU, exert inhibition on both COX1 and COX2 [73], the relationship between the major targets of ASA and IBU and PTB was evaluated using ANNs. In order to identify the specific processes of PTB in which the drugs under study may be involved, the relationship between COX1, COX2 and PTB motives was also evaluated. To know which of the COXs is more related to PTB and its motives, COX1 and COX2 were also evaluated individually. The results are shown in **Table 5**.

Table 5. Summary of the ANNs results for COX1 and COX2 (as a set and individually) on PTB and on each of its motives*, according to the probability of a relationship by means of ANNs analysis.*

Target Name	PTB	*Mtb* infection: recognition and phagocytosis	Early immune response	Late immune response	Formation of granulomas	*Mtb* survival within macrophages	Excessive inflammation and tissue damage
COXs	86%	8%	13%	91%	8%	88%	92%
COX1	66%	3%	6%	79%	4%	74%	76%
COX2	72%	6%	36%	84%	14%	80%	83%

It should be noted that the results show a high predicted relationship between COX1 and COX2 as a set (COXs) and PTB. When the major targets of ASA and IBU were individually evaluated, greater involvement of COX2 with PTB was observed, showing a medium-high relationship with PTB. On the other hand, COX1 presented a medium predicted relationship. As already mentioned (*see section Introduction*), COX1 is constitutively and ubiquitously expressed, while COX2 is induced in stress situations, such as infection, predominantly in macrophages, monocytes, and fibroblasts [24]. Therefore, it is not surprising that there is a greater relationship between COX2 and PTB than between COX1 and the pathology, since COX2 is expressed precisely in situations of inflammation.

COXs also showed a high predicted relationship with the *Late immune response* and *Mtb survival within macrophages* motives. While a very high relationship between COXs and the *Excessive inflammation and tissue damage* motive was observed. Regarding each COX individually, a high predicted relationship was observed between both COX1 and COX2 and the *Late immune response* motive. In the same way, a high relationship between COX2 and *Mtb survival within macrophages* and *Excessive inflammation and tissue damage* motives was observed, while, in contrast, COX1 presented a medium-high predicted relationship with both motives.

About the results obtained for the *Late immune response* motive, a study realized in mice found that during the late phase of *Mtb* infection, the concentration of PGE_2 was four times higher than in the initial phase. This PGE_2 production, which is catalysed by COX1 and COX2, contributes to a defective immune response, allowing the progression of PTB [25]. In the same way, another study carried out with mice concluded that PGE_2 is one of the main mediators that leads to a down-regulation of the cellular immune response [74]. Thus, confirming the high predicted relationship between both COXs (as a set and individually) and the *Late immune response* motive.

Regarding the relationship observed with the *Mtb survival within macrophages* motive, in physiological conditions, when macrophages are activated by IFNγ to fusion phagosomes with lysosomes [75], they generate an oxidative stress environment by the action of NADPH oxidase 2 and inducible nitric oxide synthase (iNOS) [76]. But in *Mtb* infection, several *Mtb* proteins inhibit iNOS expression, reducing oxidative stress and allowing *Mtb* to survive [77]. This could be because, as already mentioned, during the late phase of infection the concentration of PGE_2 increases, contributing to the survival of the mycobacterium. Exercising the opposite mechanism that was observed during the initial phase of infection, where a stable concentration of PGE_2 contributes to the control of PTB through the adequate expression of iNOS [25]. Furthermore, PGE_2 has also been seen to inhibit the killing of *Mtb* [13]. Therefore, the scientific evidence supports the results obtained in relation to the *Mtb survival within macrophages* motive.

In addition, a high contribution of polymorphonuclear leukocytes in excessive inflammation and tissue damage caused in the late stage of infection was described [21, 78]. This is partly due to the synthesis of PGs by COX1 and COX2, with a greater contribution from the latter isoform, which predominates in these cells [21]. A review about MMPs in TB shows that PGE_2 increase the expression of MMPs, which are closely related to lung damage [79]. Thus,

reinforcing the existence of a very high relationship between COX1, COX2 and the *Excessive inflammation and tissue damage* motive.

On the contrary, COX1 and COX2 showed a low predicted relationship with the rest of the motives: *Mtb infection: recognition and phagocytosis*, *Early immune response* and *Formation of granulomas*.

Considering the results obtained from ANNs, the use of NSAIDs as co-therapy for PTB could be effective by reducing the sustained inflammation and avoiding the subsequent lung damage. It has been observed that the main motives on which it would act would be *Late immune response*, *Mtb survival within macrophages* and *Excessive inflammation and tissue damage*, which are most interesting ones to prevent the sequelae of PTB.

Otherwise, the first motive, *Mtb infection: recognition and phagocytosis* is a process that cannot be avoided, unless the drug is a vaccine, thus it is logical that there is not a high relationship between the targets of the NSAIDs and the mentioned motive. Regarding the *Early immune response* motive, TB is usually detected in late stages, when it passes from the latent phase to the active phase, and the patient begins to show symptoms [9]. Therefore, it is difficult to act at this early stage, so it was also expected that COXs did not act on this motive, *Early immune response*. About the predicted relationship with the *Formation of granulomas* motive obtained with ANNs, is not conclusive, because it is a phenomenon that occurs during the *Early immune response* and culminates in the *Late immune response*, occurs throughout the disease development process, so there is very little scientific evidence that speaks about specific effectors of that specific motive. Only seven effector proteins were included in that motive in the characterization, therefore the results should be interpreted with caution.

Another important point to make is that the results of the ANNs does not specify whether the proteins have a positive or negative relationship with the disease, although thanks to the evidence provided it has been observed that in late stages of the infection, where we want to intervene, the majority contribution of both COX1 and COX2 is negative for the host, thus contributing to disease progression.

The results referring to COX1 and COX2 are the same for **Table 5**, **Table 6** and **Table 7**.

Relationship between ASA and PTB

Once the major targets of NSAIDs (COX1 and COX2) were evaluated, the relationship between the drugs considering their whole target profile and PTB were also assessed. The relationship between the drug targets and PTB motives was also evaluated in order to identify the specific processes of PTB in which the drugs under study, considering their whole target profile, may be involved. The results obtained from the ASA analysis are shown in **Table 6**.

Table 6. Summary of the ANNs results for ASA and its targets on PTB and on each of its motives, *according to the probability of a relationship by means of ANNs analysis. The drug targets with pharmacological action against the approved indication are shaded in grey.*

Drug and Target Name	PTB	*Mtb* infection: recognition and phagocytosis	Early immune response	Late immune response	Formation of granulomas	*Mtb* survival within macrophages	Excessive inflammation and tissue damage
ASA	77%	27%	50%	74%	35%	82%	87%
COX2	72%	6%	36%	84%	14%	80%	83%
COX1	66%	3%	6%	79%	4%	74%	76%
RSK2	64%	13%	16%	59%	86%	36%	54%
p53	63%	19%	49%	65%	69%	35%	63%
NOS3	58%	4%	9%	14%	61%	43%	63%
PKC2	39%	4%	3%	25%	56%	58%	64%
EDNRA	36%	3%	11%	6%	4%	11%	7%
IKKβ	35%	20%	67%	66%	70%	68%	66%
AMPK	20%	7%	4%	36%	53%	11%	35%
AMPK2	20%	7%	4%	36%	53%	11%	35%
AKR1C1	6%	3%	4%	3%	3%	34%	3%
CFTR	0%	0%	0%	0%	0%	0%	0%
GRP78	0%	0%	0%	0%	0%	0%	0%

The results show a medium-high predicted relationship between ASA (considering its whole target profile) and PTB, showing a high relationship with the *Mtb survival within macrophages* and *Excessive inflammation and tissue damage* motives.

The relationship between COX1, COX2 and PTB has been reviewed in the previous section (*see section Relationship between COX1 and COX2 and PTB*).

Regarding the off-targets of ASA, the one that showed a greater relationship with some of the motives of PTB was ribosomal protein S6 kinase alpha-3 (RSK2), which presents a high relationship with the *Formation of granulomas* motive, although it has a medium predicted relationship with PTB, and with the *Late immune response* and *Excessive inflammation and tissue damage* motives. RSK2 acts downstream of ERK1 and ERK2 as a repressor of the pro-

apoptotic function of DAPK, which is an important apoptosis regulator [80]. Despite the result obtained in **Table 6**, which shows a low relationship with the *Mtb survival within macrophages* motive, RSK2 function could be related to this motive, since inhibition of apoptosis could allow the survival of *Mtb* [81]. Thus, ASA could inhibit RSK2 apoptosis repressor function by downregulating its expression [52]. In this way, leading to enhanced clearance of *Mtb* by restoring the pro-apoptotic function of DAPK. Beyond this, RSK2 has not been linked to PTB. Therefore, more investigations will be necessary to determine the possible role of RSK2 in the pathology.

Similarly, the off-targets p53, NOS3, and PKC2 also show a medium predicted relationship with PTB, while EDNRA, IKKβ, AMPK, AMPK2, AKR1C1, CFTR, and GRP78 have a low predicted relationship.

Cellular tumor antigen p53 (p53) presents a medium predicted relationship with the *Excessive inflammation and tissue damage* motive. p53 regulates processes such as DNA repair, cell cycle arrest and cell death. Activation of p53 is triggered by oxidative stress, upregulating inflammation and apoptosis [82]. Considering that apoptosis is the main function that p53 could exert in relation to PTB, it would have been expected to obtain a greater relationship between p53 and the *Mtb survival within macrophages* motive. However, p53 also contributes to inflammation [82], so its involvement in *Excessive inflammation and tissue damage* motive was predictable. Otherwise, *Mtb* is able to avoid the generation of oxidative stress [77] inhibiting this way apoptosis, which is essential to kill mycobacteria [81]. However, Lim *et al*. showed how greater activation of p53 improved the elimination of *Mtb* in mice lungs by triggering macrophage apoptosis [82]. For this reason, the use of ASA as a co-treatment of PTB could contribute to an improvement of macrophage apoptosis by upregulating p53 [49], despite the fact that the ANNs has shown a low predicted relationship between p53 and the *Mtb survival within macrophages* motive because it has not found a direct mechanistic relationship.

Regarding the results obtained from endothelial nitric oxide synthase (NOS3), this protein shows a remarkable medium relationship with the *Formation of granulomas*, *Mtb survival within macrophages* and *Excessive inflammation and tissue damage* motives. In the PTB context, the expression of NOS3 increases in the inflammatory zone of the granuloma, contributing to nitric oxide (NO) production [83]. In a murine study, the dual role of NO in PTB was detailed. On the one hand, NO contributes to the activation of macrophages, while on the other hand, it inhibits the activity of NFκB, thus preventing hyperinflammation [84]. Thus, the

relationship between NOS3 and the motives obtained is confirmed. Therefore, ASA could help combat *Mtb* by upregulating NOS3 [55-57], since while the host response against *Mtb* is improved, it is controlled that this response is not excessive.

In the same way, protein kinase C zeta type (PKC2) also shows a medium relationship with the *Formation of granulomas*, *Mtb survival within macrophages* and *Excessive inflammation and tissue damage* motives. PKC2 participates in the PI3K and MAPK signaling pathways and is also involved in the activation of NFκB, cell proliferation and the inflammatory response, among others [85]. In *Mtb* infected macrophages, PKC2 activity is crucial in the upstream regulation of ERK1 and ERK2 activation and TNFα expression. An essential role of PKC2 as intermediary in NFκB pathway activation has also been determined, being essential for its proper activation [86]. When ASA is administered acts inhibiting PKC2 [58], thus preventing the activation of ERK1, ERK2, and NFκB. On the one hand, as already mentioned, adequate regulation of NFκB will prevent the hyperinflammation [84], reducing lung tissue damage. On the other hand, a proper regulation of ERK1 and ERK2 will contribute to stop or slow down the development of sequelae derived of PTB, according to the results obtained in the **Figure 5** (s*ee section Mechanism of action of NSAIDs in PTB*). This is because ERK1 and ERK2 are positive regulators of the expression of MMPs, one of the main proteins responsible for the degradation of the extracellular matrix, and consequently for lung tissue damage [8, 87]. Demonstrating a substantial connection with the *Excessive inflammation and tissue damage* motive.

Endothelin-1 receptor (EDNRA), which shows a low predicted relationship with PTB and all its motives, acts as a receptor for endothelin-1, an endothelium-derived vasoconstrictor peptide. In a study carried out in a murine model by Correa *et al.*, it was observed that *Mtb* can cleavage endothelin-1 through the secretion of enzymes. The results suggest a possible role for the endothelin pathway in the pathogenesis of *Mtb*, being able to modulate the host's immune response against *Mtb* through EDNRA signalling. EDNRA blocking possibly boosted vasodilation, allowing greater infiltration of immune cells at the site of infection, and increasing lung tissue damage without appreciating a decrease in bacterial load [88]. Thus, an imbalance in EDNRA signalling could result in increased survival of *Mtb* in the lung. As shown in **Table 2**, ASA directly inhibits EDNRA [48], therefore it could contribute to PTB progression or at least, it could not reverse the pathological action of EDNRA protein. Although the literature suggests a possible connection between EDNRA and PTB, ANNs has shown a low predicted relationship because it has not found a direct mechanistic relationship.

Even though inhibitor of NFκB kinase subunit beta (IKKβ) shows a low relationship with PTB, IKKβ shows between 66-70% of predicted relationship with the *Early immune response*, *Late immune response*, *Formation of granulomas*, *Mtb survival within macrophages* and *Excessive inflammation and tissue damage* motives. It is a fairly high relationship, so this off-target could considerably contribute to the pharmacological action of ASA against PTB. IKKβ is part of the NFκB signalling pathway, acting as an activator of this transcription factor. Zhou *et al.* described the role of IKKβ in the control of *Mtb* infection in a recent publication. IKKβ is part of the NFκB signalling pathway, which is activated by inflammatory signals, such as TNFα. Active IKKβ could contribute to a better response against *Mtb* [89]. Despite this, in late stages of the disease, one of the most damaging processes for the host is hyperinflammation [10]. This hyperinflammation leads to tissue damage and helps *Mtb* spreading, contributing to its increased survival inside the host [8]. Therefore, it is to be expected that IKKβ is related to both *Mtb survival within macrophages* and *Excessive inflammation and tissue damage* motives. In addition to inflammation, NFκB signalling is responsible for regulating other processes such as innate and adaptive immune responses [90]. Furthermore, granuloma formation is completed when the core is surrounded by a layer of lymphocytes, which are adaptive immune cells [7]. An inhibition of IKKβ by ASA [50, 53, 54] would reduce NFκB signalling, and consequently, the inflammation. More in-depth studies would be needed on the possible pharmacological role that target IKKβ would have in PTB.

AMP-activated protein kinase (AMPK), which involves both α and β subunits and has shown a low predicted relationship with PTB, is an essential metabolic regulator that activates catabolic pathways to produce energy in response to stress, including metabolic stress, infection, and inflammation. AMPK is also involved in the activation of autophagy, an important process in host immunity and in anti-microbial responses. In addition, the autophagy process improves the maturation of the phagosome, necessary for the formation of the phagolysosome. The phagolysosome, as mentioned before (s*ee section PTB literature-based characterization),* is necessary for the proper killing of *Mtb*. Thus, studies have determined the role of AMPK in the regulation of host defence against *Mtb*. During infection, *Mtb* can inhibit AMPK, whose activity is required to autophagy activation, and consequently for phagolysosome formation [91]. Taking this into account, increased activity of AMPK and AMPK2 by the action of ASA [46, 47] could counteract the inhibition exerted by *Mtb*, increasing this way autophagic function and improving the elimination of mycobacteria. Again, the scientific evidence shows a relationship between AMPK, AMPK2 and PTB that the ANNs could not show, since it has not found a direct mechanistic relationship.

Aldo-keto reductase family 1 member C1 (AKR1C1) is a cytosolic aldo-keto reductase that modulates the metabolism of hormones such as androgens, estrogens and progestins [92]. No scientific evidence has been observed linking the activity of AKR1C1 with PTB and AKR1C1 has shown a low predicted relationship with PTB, therefore, the inhibition of the protein by ASA [45] seems not to be involved in PTB modulation.

It should be noted that CFTR and GRP78 have a 0% of relationship with PTB or any of its motives. CFTR and GRP78 have been able to be evaluated and, according to the results obtained from the ANNs, their mechanistic relationship with PTB and each of their motives would be non-existent. One the one hand, one study carried out by Liang *et al.* shows that endoplasmic reticulum chaperone BiP (GRP78), which is essential in protein folding and quality control in the endoplasmic reticulum (ER), is increased during *Mtb* infection, acting as a sign of ER stress [93]. Therefore, the administration of ASA, which down-regulates GRP78 activity [50, 51], could reduce ER stress, and oppose the action of *Mtb*. On the other hand, cystic fibrosis transmembrane conductance regulator (CFTR) mediates the transport of chloride ions across the cell membrane [94]. No scientific evidence has been observed directly linking CFTR and PTB and has shown a 0% of relationship with PTB or any of its motives, thus CFTR inhibition by ASA [59, 60] would not seem to directly modulate PTB.

Considering these results, ASA could be a good option as co-treatment for PTB, since the modulation it exerts on all its targets and off-targets, to a greater or lesser extent, is positive for the patient. Except for EDNRA, whose inhibition could be beneficial for PTB development. In any case, the relationship predicted by ANNs between EDNRA and the pathology is low, so it would have little influence on its therapeutic potential. Anyway, the activation/inhibition constant of each target and off-target should be determined in future studies.

Relationship between IBU and PTB

The relationship between IBU considering its whole target profile and PTB was also evaluated. The relationship between the drug targets and PTB motives was also assessed in order to identify the specific processes of PTB in which the drugs under study may be involved. The results obtained from the IBU analysis are shown in **Table 7**.

Table 7. Summary of the ANNs results for IBU and its targets on PTB and on each of its motives, *according to the probability of a relationship by means of ANNs analysis. The drug targets with pharmacological action against the approved indication are shaded in grey.*

Drug and Target Name	PTB	*Mtb* infection: recognition and phagocytosis	Early immune response	Late immune response	Formation of granulomas	*Mtb* survival within macrophages	Excessive inflammation and tissue damage
IBU	45%	4%	55%	61%	42%	70%	66%
PPARγ	73%	4%	87%	7%	86%	33%	54%
COX2	72%	6%	36%	84%	14%	80%	83%
PPARα	72%	10%	60%	88%	71%	70%	64%
COX1	66%	3%	6%	79%	4%	74%	76%
AMPK2	20%	7%	4%	36%	53%	11%	35%
ASIC1	4%	3%	3%	3%	53%	8%	5%
CFTR	0%	0%	0%	0%	0%	0%	0%

The results show a medium predicted relationship between IBU (considering its whole target profile) and PTB.

Both ASA and IBU share the targets COX1 and COX2, and the off-targets CFTR and AMPK2. All four proteins are directly inhibited by the action of these drugs. Therefore, the rationale made for ASA on the previous section (s*ee section Relationship between ASA and PTB*) can also be applied to IBU. In addition, CFTR has been able to be evaluated and, according to the results obtained from the ANNs, their mechanistic relationship with PTB and each of their motives would be non-existent.

Concerning the remaining off-targets of IBU, those that showed a greater relationship with some of the motives of PTB were PPARγ and PPARα. PPARα presented high predicted relationship with the *Late immune response* motive and a medium-high relationship with the *Formation of granulomas* motive. Although it presents a very similar relationship in *Mtb survival within macrophages* and *Excessive inflammation and tissue damage* motives. Further studies will be needed to discover its therapeutic potential. Furthermore, a high relationship

was observed between PPARγ and the *Early immune response* and *Formation of granulomas* motives.

Regarding peroxisome proliferator-activated receptor gamma (PPARγ), is a transcription factor that regulates inflammation and fatty acid metabolism. PPARγ is expressed in immune cells [95] and AMs, which play a crucial role in PTB development acting as niches for *Mtb* replication. The study carried out *in vitro* by Liu *et al.* concluded that *Mtb*, through proteins expressed in its cell wall, induces the expression of PPARγ. This increased PPARγ expression leads to inflammation and contributes to the pathogenesis of *Mtb* [96]. Thus, PPARγ expression is induced by *Mtb* through a mechanism that leads to the downregulation of macrophage activity, which are crucial in the *Early immune response* [3]. Several studies carried out in human and murine macrophages have observed that a blockage of PPARγ reduces the growth of *Mtb*, while its activation leads to increased replication of mycobacteria. Furthermore, PPARγ is associated with the production of PGE_2 and indirectly inhibits apoptosis through the activation of anti-apoptotic Mcl-1 [95], therefore, a greater relationship should be observed with the *Mtb survival within macrophages* motive. Its function has also been related to the *Formation of granulomas* motive, in which are also involved the macrophages. Thus, taking all together, the activation of PPARγ by IBU [64] in advanced stages of PTB could increase the detriment of patients' condition.

In contrast, peroxisome proliferator-activated receptor alpha (PPARα), which is downregulated in peripheral blood mononuclear cells from PTB patients [97], is constitutively expressed and antagonizes the functions of NFκB, acting as a negative regulator of inflammation. A study realized in mouse by Kim *et al.* established a crucial role for PPARα in anti-mycobacterial responses. Noting that an increased PPARα activity improved autophagy, phagosome maturation and the immune response against mycobacteria in bone marrow derived macrophages from mice [95]. Therefore, it would be expected to obtain a relevant relationship with the *Mtb survival within macrophages*, *Late immune response* and *Excessive inflammation and tissue damage* motives. Thus IBU, by activating PPARα [65] could help improve anti-mycobacterial responses.

Instead, the remaining off-targets, AMPK2, ASIC1 and CFTR, showed a low predicted relationship with PTB and with all its motives, except for the *Formation of granulomas* motive, where AMPK2 and ASIC1 presented a medium-low relationship.

Regarding acid-sensing ion channel 1 (ASIC1) functions as proton-gated sodium channel in the central nervous system and is involved in pain perception and in neuroinflammation [66]. No

evidence has been found to link ASIC1 with PTB and has shown a low predicted relationship with PTB and its motives, therefore, the inhibition of ASIC1 by IBU [66-68] would not be related to PTB, although it could intervene in inflammation and pain.

Taking these results into account, IBU could be a good option as a co-treatment for PTB, since the aim of the NSAIDs administration is to combat the *Mtb survival within macrophages* and *Excessive inflammation and tissue damage* motives, in which IBU presents a relationship of 70% and 66%, respectively. One drawback is the activation that IBU exerts on PPARγ, since this protein acts in favour of mycobacteria, helping to its survival. In any case, to know the contribution of each target and off-target in the pharmacological effect of IBU, further studies will be needed to determine its activation/inhibition constant.

MECHANISM OF ACTION OF NSAIDS IN PTB

Mathematical models simulating PTB have been built considering the target profile of NSAIDs with pharmacological action for the approved indication (COX1 and COX2) and PTB molecular definition. As a result, the molecular mechanisms by which NSAIDs, through the modulation of COX1 and COX2, can be effective in PTB treatment, were obtained. The results are shown in **Figure 5**.

COX1 and COX2 have been considered as the only targets of ASA and IBU because they are the most recognized targets of the NSAIDs [73] and have the best-defined therapeutic effect.

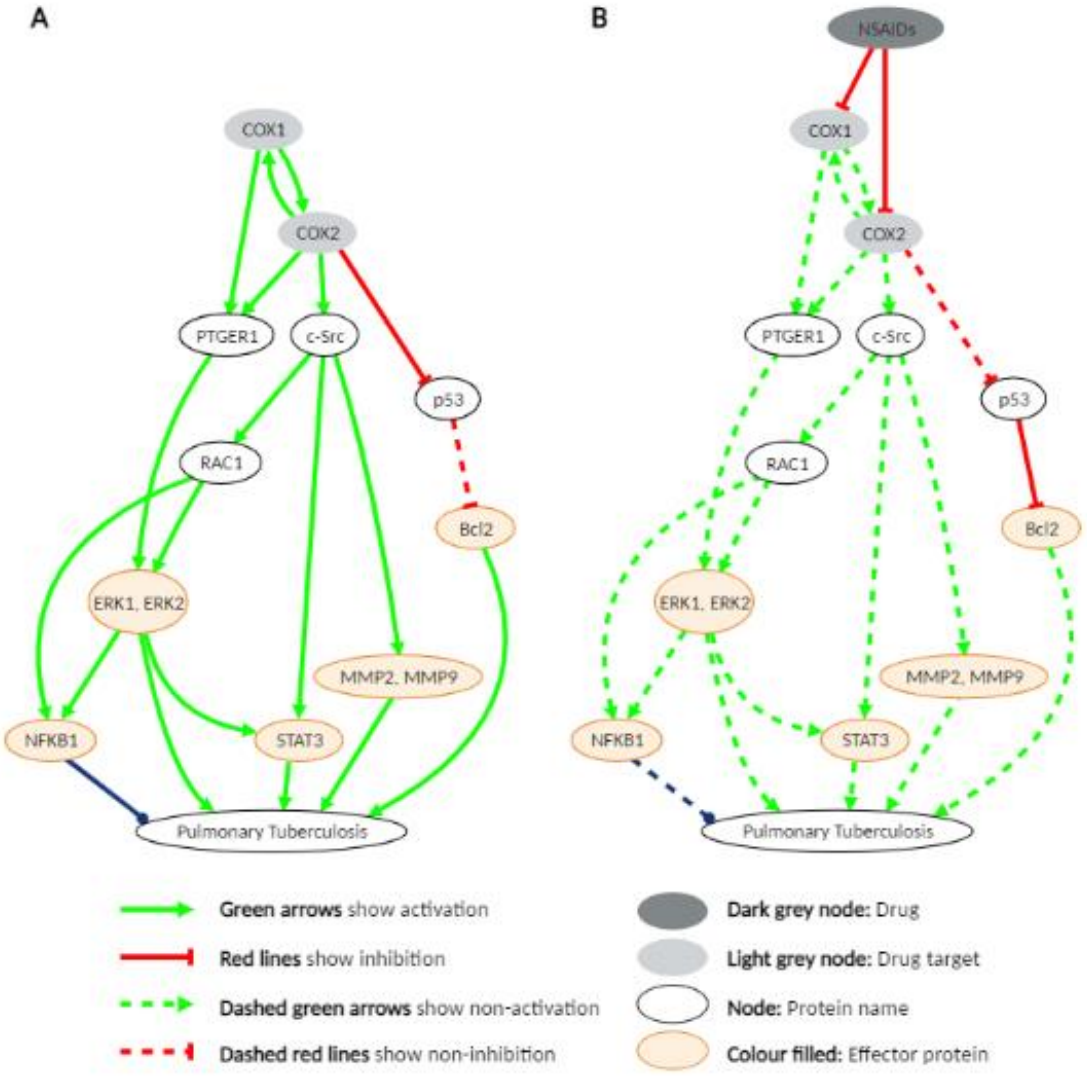

***Figure 5. Potential mechanism of action of NSAIDs in PTB**. A. The COX1 and COX2 signalling pathway is represented in the pathological state of PTB. B. Potential mechanism of action of NSAIDs by which they could reverse the pathological state of PTB effectors. Blue line shows contribution to and fight against the development of PTB.*

As previously mentioned, COX1 and COX2 are direct targets of NSAIDs, and are inhibited by these drugs [73]. COX1 and COX2 are two enzymes that catalyse the formation of PGE_2 [21]

and can act by forming a dimer [98]. The PGE_2 signalling pathway is triggered through the EP1 receptor (PTGER1) [22]. Therefore, ASA and IBU could prevent the development of PTB through the following predicted mechanisms:

Inactivation of PTGER1:

PTGER1, through the activation of the PLCβ pathway which leads to the activation of protein kinase C, finally activates ERK1 and ERK2 [99], two kinases that contribute to the development of PTB by acting as positive regulators of the expression of MMPs [87]. In addition, ERK1 and ERK2 induce the transcription factors NFκB1 [100] and STAT3 [101]. On the one hand, NFκB1 in the early stages of infection mediates key responses to limit the growth of *Mtb* in the granuloma [102], and if NFκB1 is not correctly activated, it will not exert proper control over bacterial growth. On the other hand, in the late stages of infection, NFκB1 triggers inflammation contributing to hyperinflammation harmful for the patient and beneficial for *Mtb* [84], reason for which NFκB1 activity has been represented with a blue line. Therefore, a non-activation of the transcription factor NFκB1 in advanced stages of the infection could be beneficial for the patient. Similarly, STAT3 plays key roles in the control of inflammation and immunity [103] and reduces NO production, allowing the survival of *Mtb*. Therefore, the inhibition of STAT3 will allow the adequate production of NO, a key anti-mycobacterial molecule in the defence of the host against *Mtb* [77]. Consequent to inactivated PTGER1, the signalling pathway to activate ERK1 and ERK2 will not be triggered [99], thus reversing the pathologic state of the effector proteins of PTB.

Inactivation of c-Src:

COX2 is also able to enhance the c-Src activity. The PGE_2 synthesized by COX1 and COX 2 is received by the EP2 and EP4 receptors. These receptors, by activating β-arrestin, manage to activate c-Src. [99]. c-Src plays a key role in cell motility, proliferation, and survival [104] and enhance the activity of RAC1 [105]. RAC1 is a GTPase whose function is essential in immunity and has anti-microbial function [106]. RAC1 expression is downregulated in PTB disease [107]. This GTPase finally triggers the MEK/ERK signalling pathway, enhancing the activity of ERK1 and ERK2 [108]. Both ERK1 and ERK2 kinases can induce the expression of NFκB1 [109] and STAT3 [110], as explained before. In addition, c-Src can induce the activity of both MMP2 and MMP9 [111]. MMP2 breaks down components of the extracellular matrix and deposits them around inflamed or damaged tissue. In PTB, this results in fibrosis and cavity formation, leading to pulmonary dysfunction [8]. MMP9 is secreted by neutrophils and macrophages [112] and it also degrades the components of the extracellular matrix, one of the characteristic actions of

neutrophils [113]. Consequent to inactivated c-Src, RAC1 and, consequently, both MMP2 and MMP9 will not be activated, in this way, reducing the consequences of the disease, such as the formation of cavities and pathologies derived from the tissue damage generated during the infection.

Activation of p53:

COX2 can inhibit the expression of p53 [114]. p53 regulates processes such as programmed cell death and, when active, triggers apoptosis [115] by inhibiting Bcl2 activity [116]. In PTB, there is an increase in Bcl2 activity that results in an inhibition of apoptosis of macrophages infected with *Mtb*, increasing the survival of mycobacterium [3]. Therefore, the inhibition of the anti-apoptotic protein Bcl2 by the activity of p53, will improve the elimination of *Mtb* [82]. This is because, as already explained, macrophage apoptosis is one of the essential mechanisms that the host has to fight against *Mtb*, so that an optimal apoptotic activity will improve the response against *Mtb* [81].

Finally, once the drug acts and spreads it signaling to the effector proteins, the pathological state of these effectors is reversed.

Further studies are needed to evaluate the mechanism of action of ASA and IBU considering their whole target profile.

CONCLUSIONS

Based on the results obtained by using ANNs, NSAIDs could exert beneficial effects in PTB patients through the inhibition of COX1 and COX2, acting on the *Late immune response*, the *Mtb survival within macrophages*, and the *Excessive inflammation and tissue damage* motives. Considering the whole target profile of ASA and IBU, ASA could exert a greater benefit in patients with PTB, and in addition to COX1 and COX2, additional ASA and IBU targets, such as IKKβ and PPARα respectively, could also exert potential benefits in PTB treatment, although further studies are needed to evaluate the inhibition/activation constants of each off-target and its contribution to the pharmacological action of each drug.

In addition, according to the results obtained from the sampling methods, the use of NSAIDs as co-treatment of PTB can accelerate the healing of PTB patients through the blockade of COX1 and COX2 by increasing NO production, improving macrophage apoptosis, inhibiting hyperinflammation and avoiding pulmonary tissue damage. A mechanistic evaluation of each NSAID considering the off-targets that would contribute the most (for example, PPARα in IBU) would be interesting to increase the understanding or behaviour of each particular NSAID in PTB and thus, finish evaluating which of all of them would be the more optimal.

Finally, one of the main limitations in this study is the impossibility of evaluating anti-TB treatment together with NSAIDs at the same time. This is because TPMS technology can only evaluate human-directed therapies, and antibiotic is a pathogen-directed therapy, therefore, it should be evaluated in future studies.

ANNEX I - SUPPLEMENTARY METHODOLOGY

A proprietary Anaxomics technology has been used: Therapeutic Performance Mapping System (TPMS). TPMS technology exploits the latest advances in systems biology to accelerate biomedical research, drug discovery and development [26].

The following sections describe in detail the steps performed in this project.

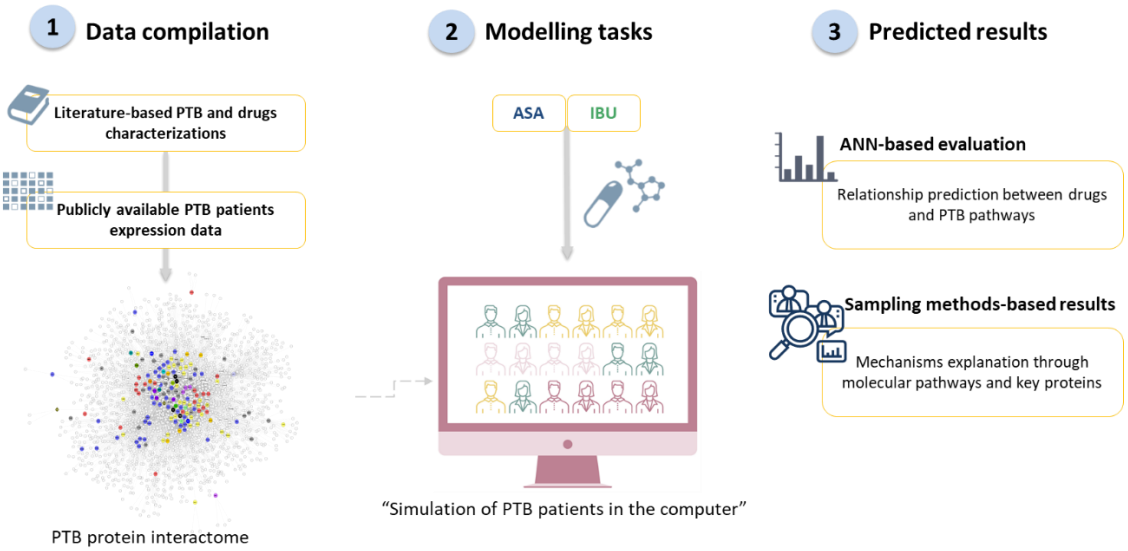

Figure 6. Schematic representation of the project execution.

PULMONARY TUBERCULOSIS CHARACTERIZATION

PTB bibliography-based characterization

PTB was molecularly characterized through manual curation of the current scientific literature regarding this subject. Reviews of the past 5 years on the molecular pathogenesis and pathophysiology of the condition available on PubMed database (*https://www.ncbi.nlm.nih.gov/pubmed*) by date February 2nd, 2021 were retrieved and assessed seeking to identify the main pathophysiological processes known to be involved in this condition (**Table 1**). These processes are referred to as 'motives', classified into causative motives, which are directly related to the onset or pathophysiology of the condition characterized; and manifestative motives, that are a consequence of the pathology. To do the search, all the normalized nomenclatures of PTB were considered. The specific search performed was:

(pulmonary[Title]) AND (tuberculosis[Title] OR tuberculoses[Title] OR phtisis[Title] OR consumption[Title] OR consumptions[Title]) AND (molecular[Title/Abstract] OR pathophysiology[Title/Abstract] OR pathogenesis[Title/Abstract])

By using this approach, 6 different motives were described for PTB pathophysiology (**Table 1**).

Subsequently, each pathophysiological process has been further characterized at protein level. The results of this search were evaluated at the abstract level, and if molecular information describing the condition pathophysiology was found, were thoroughly reviewed to identify protein/gene candidates to be condition effectors, e.g., proteins whose activity (or lack thereof) is functionally associated with the development of the condition. The effectors were documented in an Excel where was recorded the following: effector protein name, gene name, UniProtID, motive, reference (that links the protein to the motive and condition), evidence (a brief description), and causative effect. The causative effect can be 1 when the activation or overactivation causes PTB, -1 when the inhibition or fewer activation causes the condition and 9 when there is not information about the action of the protein.

If the evidence of the involvement of a candidate in the condition was judged not consistent enough for it to be considered an effector, an additional PubMed search was performed specifically for the candidate, including all the protein names according to UniProtKB (e.g.: *(pulmonary[Title]) AND (tuberculosis[Title] OR tuberculoses[Title] OR phtisis[Title] OR consumption[Title] OR consumptions[Title]) AND ("Mitogen-activated protein kinase 1"[Title/Abstract]) OR "MAP kinase 1"[Title/Abstract] OR "MAPK 1"[Title/Abstract] OR ERT1[Title/Abstract] OR "Extracellular signal-regulated kinase 2"[Title/Abstract] OR ERK-2[Title/Abstract] OR "MAP kinase isoform p42"[Title/Abstract] OR p42-MAPK[Title/Abstract] OR "Mitogen-activated protein kinase 2"[Title/Abstract] OR "MAP kinase 2"[Title/Abstract] OR "MAPK 2"[Title/Abstract] OR PRKM1[Title/Abstract] OR PRKM2[Title/Abstract]).* If novel candidates were identified in this phase, they were included as effectors following the same criteria and protocol. When information specifically addressing PTB could not be found, general information concerning PTB was also included in the characterization, in order to ensure a complete molecular characterization of the pathophysiology of the disease.

PTB high-throughput data evaluation

Having completed the bibliography-based condition characterization, patients' data publicly available has been analysed in order to obtain a molecular fingerprint or signature that can help adjust models and be used to evaluate the modelled drug effects.

Gene expression data regarding the condition of interest was identified in Gene Expression Omnibus (GEO) public repository [117]. Specifically, the following query was performed on March 22nd, 2021:

- GEO: *(pulmonary tuberculosis[All Fields])*

The data was filtered by organism (*Homo Sapiens*), entry type (*Series*), experiment type (*Expression profiling by array*, *Protein profiling by protein array, Expression profiling by high throughput sequencing*) and sample type (*tissue*). GEO search retrieved 31 results. The following selection criteria were followed: having a significant number of samples of patients with PTB without comorbidities (n > 50), age range between 18-65 years and samples obtained from a population of various ethnicities or Westerners.

GRAPHICAL REPRESENTATION OF PTB PATHOPHYSIOLOGY

PTB representations were performed using BioRender (BioRender.com). Two figures are provided (**Figure 1** and **Figure 2**).

A schematic representation of PTB motives was created for visualisation purposes and to facilitate their understanding.

GRAPHICAL REPRESENTATION OF PTB NETWORK

Network representations were performed using Cytoscape[1] software [118]. Two network representations are provided (**Figure 3** and **Figure 4**).

A protein-protein interaction network was created for visualisation purposes including PTB characterization (s*ee section Pulmonary tuberculosis characterization*) and proteins directly interacting with PTB effectors.

The networks contain links obtained from database-derived interactions between the proteins (including the links used for any of the networks used for model construction in Anaxomics) extracted from: KEGG [119], BioGRID [120], Intact [121], REACTOME [122], TRRUST [123], and HPRD [124].

[1] Open-source network visualisation software downloadable from: http://www.cytoscape.org/download.php

DRUGS CHARACTERIZATION

The drug molecular characterization consists of the identification of drug targets and off-targets. The drugs evaluated in the study are acetylsalicylic acid and ibuprofen.

In order to characterize the drugs and incorporate this information into its mechanism of action, an in-depth review of the following official documents (EMA - European Medicines Agency: European Public Assessment Report (EPAR) and FDA - Food and Drug Administration: Multidisciplinary review and Chemistry review, product monograph) for each drug was carried out. When available, target information was retrieved from specialised databases such as DrugBank [125], Stitch [126] and SuperTarget [127].
Furthermore, available literature regarding known targets of the drugs of interest in PubMed to date February 24th, 2021 was retrieved and assessed. The information was managed as already reported for the condition characterization. The specific searches performed were the following:

- **Ibuprofen:** (ibuprofen[*Title*] OR ibuprophen[*Title*]) AND (target OR "activity assay" OR "binding assay" OR inhibitor) AND (inhibition OR binding OR substrate OR action OR "mechanism of action")
- **Acetylsalicylic acid**: (aspirin[*Title*] OR ASA[*Title*] OR "acetylsalicylic acid"[*Title*] OR "2-acetoxybenzoic acid"[*Title*] OR acetylsalicylate[*Title*] OR "2-acetoxybenzenecarboxylic acid"[Title]) AND ("target" OR "activity assay" OR "binding assay" OR "inhibitor") AND ("inhibition" OR "binding" OR "substrate" OR "action" OR "mechanism of action")

Targets and off-targets were documented in in two different sheets of an Excel, one different Excel for each drug. The first sheet where the targets were documented, the following was recorded: DrugBank ID of the drug, name of the drug, UniProtID of the target/off-target, target/off-target protein name, target/off-target gene name, reference, evidence, effect of the drug over the target/off-target and DrugBank pharmacological action. The effect of the drug over the target/off-target can be 0 when the drug is a modulator, 1 when the drug is an agonist, potentiator or activator, and -1 when the drug is an antagonist or inhibitor.

Once the search was carried out and all the information was compiled, the information obtained was compared with the information from Anaxomics' databases in order to contrast the information.

SYSTEMS BIOLOGY - MATHEMATICAL MODELS AROUND DRUG TARGETS AND PTB

For the generation of mathematical models, Anaxomics builds a biological map including all the proteins included in the human proteome. Data is obtained from public and private external databases (KEGG [119], BioGRID [120], Intact [121], REACTOME [122], TRRUST [123], and HPRD [124]) and the manual curation of scientific literature.

The network is then enriched with functional information of the proteins (whether they participate in pathological processes, are drug targets…). Then, the models are trained with a proprietary Truth Table containing public available data. In brief, to train the mathematical models a collection of known input-output physiological signals is used. The molecular description of these input-output physiological signals mainly derives from literature mining and a compendium of databases that accumulate biological and clinical knowledge (such as microarray databases e.g. GEO, Phosphorylation databases e.g. PHOSIDA, 2D gel databases, Biological Effectors Database) and drug databases (e.g. DrugBank). That is:

- Model inputs - e.g. information about drugs obtained from its characterization (*see section Drugs characterization*), since they inhibit or activate one or more nodes of the model (their targets) triggering a perturbation through the system.
- Model outputs – e.g. molecular characterization of the biological processes of interest (up-regulated or down-regulated proteins, after the treatment).

The collection of known input-output physiological signals generates a list of physiological rules or principles found to apply to particular pathophysiological conditions. These set of rules, the “truths”, are collated to form the Truth Table that every constructed mathematical model must satisfy.

Two different modelling approaches were employed with the aim of generating the different mechanisms of action: ANNs (with predictive capacity) and sampling-based methods (with descriptive capacity).

Artificial neural networks (ANNs)

Once the mathematical models were built, their predictive power was exploited, through an ANNs strategy [72], to rank drugs and drug targets according to their mechanistic relationship with PTB and its associated motives, as detailed in [26, 128].

Specifically, the mechanistic relationship between COX1 and COX2 (as a set and individually) and PTB and each of their motives has been evaluated. In the same way, the mechanistic

relationship of ASA and IBU and each of their off-targets with PTB and their motives has been evaluated.

To generate each ANNs, an input set and an output set were generated.

Relationship between COX1 and COX2 and PTB

In **Table 5**, the predicted relationship between COX1 and COX2 (as a set and individually) and PTB and each of its motives was evaluated. To perform this ANNs-based analysis, an input set with the UniProtID of COX1 and COX2 was generated in a text document. And an output set was also generated in another text document with the UniProtID of each of the proteins identified as effectors of PTB, classified according to the motives in which they were assigned.

Specifically, a set called PTB that included all the PTB effectors was generated, another set was also generated for the first motive (*Mtb infection: recognition and phagocytosis*), called PTB_Motive1, in which all the PTB effectors that are classified in that motive were included. Similarly, equivalent sets (with different name) were generated for each of the 5 remaining motives. An example is shown in **Table 8**.

Table 8. Example of how to do the input set and the output set to obtain the results of Table 5.

INPUT SET	
Set name	**UniProtID**
COXs	COX1
COXs	COX2
COX1	COX1
COX2	COX2

OUTPUT SET	
Set name	**UniProtID**
PTB	Protein 1
PTB	Protein 2
PTB	Protein n
PTB_Motive1	Protein 1
PTB_Motive1	Protein 3
PTB_Motive1	Protein n
PTB_MotiveX	Protein 2
PTB_MotiveX	Protein 4
PTB_MotiveX	Protein n

Considering the input and output sets abovementioned, the ANNs evaluated the potential mechanistic relationship between COX1 and COX2 (as a set and individually) and PTB and its associated motives.

Relationship between ASA, IBU and PTB

The **Table 6** shows the predicted relationship between ASA (considering its whole target profile) and PTB and each of its motives according to ANNs results. To perform this analysis, an input set was generated with the UniProtID of each ASA target (and off-target), and an output

set with the UniProtID of the proteins identified as effectors of PTB, classified according to the motives in which they were identified, as explained in the previous point.

Regarding the input set, a set called ASA that included all the targets and off-targets of ASA was generated. This set is used to evaluate the predicted relationship of ASA (considering its whole target profile) with PTB and each of its motives.

In order to evaluate the predicted relationship of each target/off-target of ASA with PTB and each of its motives, other sets were generated for each of the targets/off-targets, called ASA_Target1. Similarly, equivalent sets (with different names) were generated for each of the remaining targets and off-targets. The same protocol was followed to obtain the results of **Table 7**, but with the IBU targets and off-targets. An example is shown in **Table 9**.

Table 9. Example of how to do the input set and the output set to obtain the results of Table 6 and Table 7.

INPUT SET	
Set name	**UniProtID**
ASA	Target 1
ASA	Target 2
ASA	Target n
ASA_Target 1	Target 1
ASA_Target 2	Target 2
ASA_Target n	Target n

OUTPUT SET	
Set name	**UniProtID**
PTB	Protein 1
PTB	Protein 2
PTB	Protein n
PTB_Motive1	Protein 1
PTB_Motive1	Protein 3
PTB_Motive1	Protein n
PTB_MotiveX	Protein 2
PTB_MotiveX	Protein 4
PTB_MotiveX	Protein n

Sampling methods - Mechanism of action of NSAIDs

TPMS sampling-based methods [26] can be used for describing with high capability all plausible relationships between an input (or stimulus, in this case NSAIDs) and an output (or response, in this case PTB).

Although this type of network would generate a large number of possible mechanistic solutions, it can be limited by constraints and restrictions that must be respected: the topology of the protein network, the functional, medical and biological information stored in Anaxomics' databases, and the available data about the drug (known effects on the target and target biology). Various different approaches and optimisation systems [129] can be used for such a purpose, from those based on randomised systems (such as a Montecarlo-based system [72]) to those based on information derived from the topology of the network, in order to

solve each parameter of the equation, e.g. the weights associated to the links between the nodes in the human protein network.

From this base set of valid mathematical solutions, Anaxomics technology identifies the most probable mechanisms of action that achieve a physiological response when the system is stimulated with a specific stimulus. Not all solutions are used for the analysis. Only mechanisms of action that are plausible from the standpoint of currently accepted scientific knowledge are considered.

These models are then composed of several plausible mechanistic solutions, which allows us to explore the predicted activity of each protein (ranged between [-1,1]) within the drugs' mechanisms of action and the most frequent pathways occurring between NSAIDs and the PTB molecular definition.

The sampling methods analysis allowed the evaluation of the impact of the therapy on the disease processes and each individual protein effector. These models simulate the ability of the treatment to revert the proteins altered in PTB (e.g., the ability of the treatment to return these PTB proteins to a non-pathological state in terms of protein activity).

Once the mechanism of action generated by the mathematical model was obtained, each link was manually validated according to the information contained in the scientific literature. That is, each link between proteins (A → B) was manually curated by searching for scientific evidence contextualized with PTB, or failing that, with one of its motives. Thus, ensuring that the predicted interaction between protein A and protein B were plausible in a patient with PTB.

Then, when the mechanism of action was curated, certain links were validated, forced, or forbidden, and it was reintroduced into the system to generate another mechanism of action with the corrections made. A link was validated if the interaction between the two proteins occurred in the context of the condition. A link was forbidden if the interaction was not condition contextualized, if it happened in the opposite way (B → A) or if it happened with the opposite sign, that is, instead of protein A activating protein B, protein A inhibiting protein B. Finally, a link was forced if the way or sign of a forbidden link was corrected, to force the system to learn the correctly interaction.

ANNEX II - ADDITIONAL INFORMATION

To facilitate the understanding of the text, the common name of some proteins has been used (**Table 10**).

Table 10. Common names of the targets and off-targets of the drugs with their corresponding gene name.

Gene name	Synonym
HSPA5	GRP78
IKBKB	IKKβ
PPARA	PPARα
PPARG	PPARγ
PRKAA2	AMPK2
PRKAB1	AMPK
PRKCZ	PKC2
PTGS1	COX1
PTGS2	COX2
RPS6KA3	RSK2
TP53	p53

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