ADVANCES IN HEALTH AND DISEASE

Advances in Health and Disease

Volume 57

Lowell T. Duncan
Editor

Nova Medicine & Health

NOVA

Advances in Health and Disease

This is a numbered series focused on the latest research in health and disease.

Advances in Health and Disease

Advances in Health and Disease. Volume 54
Lowell T. Duncan (Editor)
2022. ISBN: 978-1-68507-868-3 (Hardcover)
2022. ISBN: 979-8-88697-034-0 (eBook)

Advances in Health and Disease. Volume 53
Lowell T. Duncan (Editor)
2022. ISBN: 978-1-68507-820-1 (Hardcover)
2022. ISBN: 978-1-68507-905-5 (eBook)

Advances in Health and Disease. Volume 52
Lowell T. Duncan (Editor)
2022. ISBN: 978-1-68507-665-8 (Hardcover)
2022. ISBN: 978-1-68507-673-3 (eBook)

Advances in Health and Disease. Volume 51
Lowell T. Duncan (Editor)
2022. ISBN: 978-1-68507-657-3 (Hardcover)
2022. ISBN: 978-1-68507-772-3 (eBook)

Advances in Health and Disease. Volume 49
Lowell T. Duncan (Editor)
2021. ISBN: 978-1-68507-495-1 (Hardcover)
2022. ISBN: 978-1-68507-504-0 (eBook)

Advances in Health and Disease. Volume 48
Lowell T. Duncan (Editor)
2021. ISBN: 978-1-68507-375-6 (Hardcover)
2021. ISBN: N/A (eBook)

More information about this series can be found at
https://novapublishers.com/product-category/series/advances-in-health-and-disease/

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Editor

Advances in Health and Disease

Volume 57

NOTICE TO THE READER

Library of Congress Cataloging-in-Publication Data

ISBN: 979-8-88697-098-2
ISSN: 2770-7385

Published by Nova Science Publishers, Inc. † New York

Contents

Preface

This volume includes ten chapters that present recent research advances in health and disease. Chapter One reviews the importance of 5alpha-reductase inhibitors, with focus on oxidized steroidal derivatives, for treatment of prostatic diseases. Chapter Two examines inflammation in chronic obstructive pulmonary disease. Chapter Three indicates plausible mechanistic insights of binding of vitamin B12 and its precursor cobinamide. Chapter Four summarizes fluid therapy for treatment of traumatic brain injury. Chapter Five explores the healthcare response to COVID-19 in Bhutan and Nepal. Chapter Six examines changes in immune function associated with aging. Chapter Seven discusses the epidemiology, clinical features, investigations, and management of pyogenic spondylodiscitis.

Chapter 1 - This chapter reviews the importance of 5α-reductase inhibitors (5ARIs), with focus on oxidized steroidal derivatives, with potential interest in the treatment of prostatic diseases, such as benign prostatic hyperplasia (BPH) and prostatic cancer (PCa). In humans, the high activity of 5α-reductase (5AR) enzymes results in excessive 5α-dihydrotestosterone (DHT) levels in peripheral tissues. DHT is the most potent androgen and acts by binding to androgen receptors, promoting gene transcription and leading to the biosynthesis of specific proteins and cell proliferation and, consequently, to the development and progression of BPH. Hence, inhibition of androgen action through the inhibition of the 5AR pathway constitutes a valid approach for BPH management. In addition, 5ARIs demonstrated potential as PCa chemopreventive and therapeutic agents, although some studies have reported the possibility of 5ARIs increasing the risk of high-grade PCa in men. Currently, the 4-azasteroids finasteride and dutasteride are the principal 5ARIs approved for BPH treatment and are deeply studied. However, in the last years, high attention has been given to non-azasteroids as 5ARIs, particularly oxidized steroids, and interesting results were observed. In this context, an overview of the main achievements obtained will be presented herein and a

special focus will be made on oxidized steroids, such as progesterone, 16-dehydropregnenolone, and dehydroepiandrosterone derivatives.

Chapter 2 - Inflammation plays a central role in the development of chronic obstructive pulmonary disease (COPD). COPD, an irreversible and progressive pulmonary disorder, is characterized by chronic bronchitis, airflow obstruction, and emphysema. The onset of COPD is a result of aberrant immune responses induced by various genetic and environmental factors. The presence of interleukin-1 (IL-1)- like cytokines in the sputum and bronchoalveolar lavage fluid (BALF) of COPD patients indicates the involvement of inflammasomal complex in COPD development and progression. Among inflammasomal complex, NLR family pyrin domain containing 3 (NLRP3) inflammasome induces caspase-1-mediated proteolytic activation and the secretion of IL-1-like cytokines. The release of these cytokines is responsible for pyroptosis-mediated cell death. Recent studies suggest the role of NLRP3 inflammasome in airway inflammation, especially in COPD progression. Here, the authors have focused on the current progress made in the field of research describing the involvement of NLRP3 inflammasome in the pathogenesis of COPD.

Chapter 3 - Vitamin B12 or cobalamin, the most complex B-type vitamin, has rare organometallic bonds in its biologically active forms. These bonds are used by several enzymes involved in different rearrangement and transmethylation reactions in central metabolic pathways of bacteria and archaea. However, of all the sequenced bacterial genomes which depend on Vitamin B12 for growth, only 50% encode the ability to synthesize an active form de novo, a process that requires over 30 gene products, making it a highly energy-consuming phenomenon. Hence, B12 is salvaged from the environment by bacteria that are incapable of synthesizing the same. Type II ABC importers are present only in prokaryotes and are responsible for the uptake of metal chelates including heme and vitamin B12; the ABC importer inE. coli, for example, can also bind and uptake cobinamide, a precursor of cobalamin. The authors solved the crystal structure of the periplasmic B12 binding protein of the cholera-causing pathogenic bacteria, Vibrio cholerae, VcBtuF in cyanocobalamin bound state and established binding of cobinamide and heme with VcBtuF as well. However, the mechanism of B12 and precursor uptake by ABC importer is still elusive in V. cholerae, and other biofilm-forming and/or pathogenicVibriospecies. Furthermore, sequence analysis of genome databases has revealed that a majority ofVibriospecies, includingVibrio cholerae,lack the genes forde novovitamin B12 synthesis while containing the genes required for salvaging cobalamin or cobinamide.

This chapter indicates plausible mechanistic insights of binding of vitamin B12 and its precursor cobinamide by VcBtuF ofVibrio cholerae and similar species from structural and bioinformatics point of view.

Chapter 4 - Traumatic brain injury (TBI) is a major public health problem and a significant cause of death and disability. The damage may be divided into two phases: a) a primary acute injury because of the traumatic event; and b) a secondary injury due to the hypotension and hypoxia generated by the previous lesion, which leads to ischemia and necrosis of neural cells. In TBI, the development of cerebral edema is a crucial prognosis marker. In the early stages of TBI, minimal changes in intracranial pressure are observed because of cerebral edema due to a compensatory effect of the cerebrospinal fluid. However, if edema increases, this mechanism fails, increasing intracranial pressure. To avoid this chain effect, several treatments are applied in the clinical practice, including elevation of the head of the bed, maintenance of normothermia, pain and sedation drugs, mechanical ventilation, neuromuscular blockade, controlled hyperventilation, and fluid therapy (FT).

The goal of FT is to improve the circulatory system to avoid the lack of oxygen to organs. Therefore, rapid and early infusion of large volumes of crystalloids is performed in clinical practice to restore blood volume and blood pressure. Despite the relevance of FT in the early management of TBI, there are few clinical trials regarding which solution is better to apply.

This chapter aims to summarize the actual knowledge about the different types of FT used in clinical practice. A physiopathological approach to TBI will be performed to achieve this goal, explaining why the different types of FT are used.

Chapter 5 - While the world was caught off guard by the 2019-2020 COVID-19 pandemic, healthcare systems in Nepal, like other South Asian low-or-middle-income countries experienced drastic consequences. As the overall system came to a halt, Nepal saw crippled general healthcare delivery, poor maternal and neonatal health planning, rising mental health issues, further socioeconomic stratification and other spillovers particularly as a result of prioritizing COVID-19 over other services, whereas its neighbor Bhutan stood out as a meaningful case of contrast despite a similar national lockdown in place. This chapter explores the features of healthcare delivery and resource allocations in the two countries to explain their differential responses to the crisis, and by doing so, looks to inspire discussions of better action plans for South Asia to prepare for similar disasters in the future.

Chapter 6 - There is evidence of declining effectiveness of the immune system with aging. This must reflect a diminution of immune surveillance

mechanisms, and gradually leads to the emergence of several harmful tendencies. The ability of the aged to mount an effective defense against micro-organisms and to respond well to vaccination are both impaired. These account for the increased susceptibility of the aged to bacterial and viral disease. Such diminished immune function with aging takes place despite increasing levels of inflammatory activity. The ensuing elevation of inflammation with no readily recognizable antigenic trigger, leads to compromised tissue function. The increased presence of irrelevant inflammatory activity can result in elevation of oxidative stress and these events together can proceed to adversely effects including organ damage. Additionally, failure of the immune system in the elderly is also reflected by a growing incidence of autoimmune disease. It seems that the defective immune system of age is not just increasingly quiescent but progresses to a different form of action that, while being ineffective in performing its classical role, is expressed in a new faulty configuration leading to harmful rather than favorable outcomes. This article describes some possible mechanisms underlying such a transition. Finding means of impeding of this shift is also discussed since this could alleviate many of the diseases associated with senescence.

Chapter 7 - Infection of the spine can involve the vertebral body, intervertebral disc, spinal canal, or adjacent soft tissues. The most common mode of infection is hematogenous. Contiguous spread from the nearby focus of infection and direct inoculation are other routes of infection. It constitutes approximately 2 to 7% of infections of the musculoskeletal system. Pyogenic spondylodiscitis is common in young children and the elderly. Diabetes mellitus, immunodeficiency, HIV infection, and intravenous drug abuse are some of the predisposing features. Staphylococcus aureus is the most common pathogen, and Staphylococcus epidermidis, E. coli, and Pseudomonas are other organisms causing pyogenic spondylodiscitis. The infection can be acute, subacute, or chronic. Pyogenic spondylodiscitis has an indolent course. Most of the cases present with low back pain, which is not related to the activity. Rest pain and night pain are common. Constitutional symptoms are less common. Abscess formation and neurological symptoms are rare. The most common investigations include routine blood examination, ESR, and CRP. Blood cultures are positive in 25% of cases. Radiographs, CT scans, MRI scans, and PET scans are useful imaging modalities. The majority of cases can be treated nonoperatively using antibiotic therapy. However, surgery is indicated in cases with instability and neurological deficits and in cases that do not respond to antibiotic therapy. In this chapter, the authors discuss the

epidemiology, clinical features, investigations, and management of pyogenic spondylodiscitis.

Chapter 8 - Vitamin B12 cannot be synthesized by animals or plants as it is only produced by some archaea and bacteria, some of which can also synthesize various vitamin B12-associated compounds bearing distinct base moieties in their lower ligand. Animal-derived foods, such as meat, milk, and fish are major dietary sources of vitamin B12. Various inactive vitamin B12 compounds have been identified in foods using liquid chromatography/electrospray ionization-tandem mass spectrometry analysis approaches. In this chapter, the authors describe the latest information on the characterization of vitamin B12 compounds discovered in dietary sources.

Chapter 9 - Tryptophan, a naturally occurring aromatic amino acid, is a well-reported versatile molecule in literature. Its importance as an antibiofilm agent against several biofilm-forming organisms has been studied comprehensively. In this study, the antibiofilm effect of tryptophan has been presented against strong biofilm-forming organisms such as Staphylococcus aureus (a Gram-positive bacterium) and Pseudomonas aeruginosa (a Gram-negative bacterium) as the mentioned organisms have been reported to form a plethora of infections including urinary tract infections, gastrointestinal infections, skin infections, etc. on a human host by exploiting biofilm. Towards this direction, tryptophan has shown promising characteristics in inhibiting the microbial biofilm formation of these two organisms. On exploring the underlying mechanisms, it was found that the compound, tryptophan, inhibited biofilm formation by targeting the quorum-sensing property by downregulating their respective quorum-sensing linked genes. Thus, it can be stated that the biofilm-forming ability of both these organisms can be compromised by targeting their quorum-sensing property. Apart from this, tryptophan could also reduce the cell surface hydrophobicity resulting in the inhibition of biofilm development. It was also reported that tryptophan could be used in combination with other antibiofilm molecules for the sustainable management of biofilm threats. Hence, tryptophan could be recommended as a potential antibiofilm agent to manage the biofilm-associated infections caused by S. aureus as well as P. aeruginosa.

Chapter 10 - It has been proposed that the endogenous antioxidant defense mechanisms in MS are deficient and therefore do not prevent the progression of these lesions. In this sense, some of the current therapeutic approaches (for example, fumarate treatment) aim to upregulate the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) as an essential regulator of antioxidant protection. Nrf2 is a critical regulatory factor for many

cytoprotective molecules to counter oxidative stress and detoxification gene expression. Therefore, targeting the Nrf2 factor may be a suitable strategy for studying of diseases in whose pathogenesis oxidative stress is implicated. In pre-clinical studies, compounds such as melatonin, curcumin, resveratrol, and sulforaphane have been shown to reduce the symptoms of MS by activating the Nrf2 signaling pathway. Some of the chemical drugs and medicinal plants have been indicated to have beneficial effects in pre-clinical researches and clinical trials. In this chapter, the authors will discuss targeting Nrf2 by synthetic and natural agents and their impact on MS.

Chapter 1

Oxidized Steroids as 5α-Reductase Inhibitors with Potential Interest in Prostatic Diseases: A Review

V. Brito, G. Alves, P. Almeida and S. Silvestre*
Health Sciences Research Center, University of Beira Interior, Covilhã, Portugal

Abstract

This chapter reviews the importance of 5α-reductase inhibitors (5ARIs), with focus on oxidized steroidal derivatives, with potential interest in the treatment of prostatic diseases, such as benign prostatic hyperplasia (BPH) and prostatic cancer (PCa). In humans, the high activity of 5α-reductase (5AR) enzymes results in excessive 5α-dihydrotestosterone (DHT) levels in peripheral tissues. DHT is the most potent androgen and acts by binding to androgen receptors, promoting gene transcription and leading to the biosynthesis of specific proteins and cell proliferation and, consequently, to the development and progression of BPH. Hence, inhibition of androgen action through the inhibition of the 5AR pathway constitutes a valid approach for BPH management. In addition, 5ARIs demonstrated potential as PCa chemopreventive and therapeutic agents, although some studies have reported the possibility of 5ARIs increasing the risk of high-grade PCa in men. Currently, the 4-azasteroids finasteride and dutasteride are the principal 5ARIs approved for BPH treatment and are deeply studied. However, in the last years, high attention has been given to non-azasteroids as 5ARIs, particularly oxidized steroids, and interesting results were observed. In this context, an overview of the main achievements obtained will be presented herein

* Corresponding Author's Email: sms@ubi.pt.

In: Advances in Health and Disease. Volume 57
Editor: Lowell T. Duncan
ISBN: 979-8-88697-098-2

and a special focus will be made on oxidized steroids, such as progesterone, 16-dehydropregnenolone, and dehydroepiandrosterone derivatives.

Keywords: oxidized steroids, 5α-reductase inhibitors, benign prostatic hyperplasia, prostate cancer

Introduction

The prostate is walnut-shaped and sized, weighing about 15-20 g at the end of adolescence. Moreover, the prostate is a male-specific hormone-responsive gland, requiring androgenic hormones and an androgen receptor (AR) for normal growth and development (S. Clark and Nabity 2020; Patel 2011). This gland is located between the bladder and penis surrounding the urethra, in the retroperitoneal space (Reeves et al. 2016). Physiologically, the prostate has a relevant role in the male reproductive system, whose main function is the secretion of prostate fluid, one of the components of semen (Hedayat and Lapraz 2019). This organ can be mainly affected by three distinct pathologies: prostatitis (inflammation), benign prostatic hyperplasia (BPH), and malignant prostate cancer (PCa) (Coleman 2018).

BPH is one of the most common urological male age-related diseases and affects the majority of elderly men worldwide (Thorpe and Neal 2003; Pagano et al. 2014; Lim 2017). It is well established that the prevalence of this disorder increases significantly with increased age (Guess et al. 1990; Berry et al. 1984). The BPH comprises nodular hyperplasia caused by an abnormal proliferative process of the prostate elements (stromal and epithelial cells) resulting in an enlarged prostate. In consequence, BPH originates bladder outlet obstruction and compression, resulting in a series of lower urinary tract symptoms (LUTS) (McNeal 1988; Lepor 2005; Lee, Kozlowski, and Grayhack 1997; Yu et al. 2020; McNeal Burroughs Wellcome Company, 1983). These symptoms include principally voiding symptoms such as incomplete bladder emptying sensation, straining to void, urinary hesitancy, and a weak urinary flow rate (Homma et al. 2017; Langan 2019). BPH is not a lethal disease, however, it affects significantly the quality of life of patients, causing anxiety, sleep disorders, and sexual conflicts (Yu et al. 2020). The cause of this pathology remains unclear, but it has been associated with high plasma levels of the androgen 5α-dihydrotestosterone (DHT), the most potent androgen (Carson and Rittmaster 2003). DHT binds to the ligand-binding

pocket of AR, promoting the dissociation of heat-shock proteins from the AR. Then, the AR translocates into the nucleus, dimerizes, and binds in the promoter region to the AR target genes, leading to the synthesis of specific proteins, such as prostate-specific antigen (PSA), and cell proliferation (Salvador, Pinto, and Silvestre 2013; Wen et al. 2015). PSA is clinically used for the detection and monitoring of PCa recurrence and progression, being a well-established biomarker in this context (Rice and Stoyanova 2018). Consequently, the reduction of DHT levels by inhibiting its biosynthesis comprises a valid strategy for the management of BPH (E. Kim, Larson, and Andriole 2016). In this context, the enzyme 5α-reductase (5AR) emerged as a valuable therapeutic target, since it is responsible for the conversion of testosterone in DHT (Schmidt and Tindall 2011). Then, efforts to develop 5α-reductase inhibitors (5ARIs) have been made and, currently, finasteride and dutasteride are largely used in clinical practice (Aggarwal et al. 2010; Sudduth and Koronkowski 1993; Clark et al. 2004). This therapeutic approach leads to a decrease in prostatic volume and total serum PSA levels, improving the symptomatology associated with BPH (Azzouni and Mohler 2012). Currently, there are other therapeutic strategies to reduce LUTS, such as the use of α_1-adrenergic receptor antagonists (e.g., alfuzosin and tamsulosin), phytotherapy (e.g., *Serenoa repens* extract), and combination therapies (Cohen and Parsons 2012; Asseldonk et al. 2014).

PCa is the most diagnosed type of cancer worldwide among men and is the fifth leading cause of cancer-related death (Sung et al. 2021; Siegel, Miller, and Jemal 2020). In addition, the incidence and mortality are correlated with increasing age and it comprises a well-established risk factor, similarly to BPH (Sung et al. 2021). Despite the etiology of PCa remains also unclear, it is well known that the AR, a ligand-dependent nuclear transcription factor and member of the steroid hormone nuclear receptor family, is crucial in the proliferation of PCa cells (Chatterjee 2003; Davey and Grossmann 2016). Considering that the growth and progress are dependent on circulating androgens at the initial stages of PCa, initially, surgical castration and/or chemical castration emerged as a useful strategy for decreasing systemic androgen levels. However, this approach presents a relevant disadvantage since it does not affect the androgen biosynthesis in the adrenal glands (Dahl, Nadler, and Zietman 2009). In consequence, other agents more efficient in the context of androgen deprivation therapy (ADT) for the treatment of PCa were investigated. Currently, ADT includes the use of gonadotropin-releasing hormone (GnRH) agonists and antagonists, antiandrogens, and androgen synthesis inhibitors (Nelson, De Marzo, and Isaacs 2020). The GnRH agonists

and antagonists (e.g., degarelix) exhibited great efficacy in the ablation of circulating testosterone levels, leading to a substantial change in the disease management option from surgical castration to drug therapy (Dreicer et al. 2011). Nevertheless, even for patients responding favorably to hormonal therapy initially, PCa progresses to a castration-insensitive stage, known as castration-resistant PCa (CRPC) (Nelson, De Marzo, and Isaacs 2020; Chi et al. 2009). Despite this stage being castration-insensitive, it is well established that the AR axis remains active, existing activation of downstream genes (Ang, Olmos, and de Bono 2009; Attard et al. 2009). Interestingly, it was described that PCa is able to produce intracrine androgenic steroids, becoming hypersensitive to low steroidal levels, a crucial condition for disease progression (Aragon-Chin and Dahut 2010; Attard et al. 2013). Considering this knowledge, the ADT approach comprising androgen biosynthesis inhibition and AR blockage remains a valid strategy in all PCa phases, including in CRPC. Additionally, there are other pharmacological approaches in the treatment of PCa, such as the use of chemotherapeutic agents (ex.: docetaxel and cabazitaxel) (Nelson, De Marzo, and Isaacs 2020).

After a presentation of the structure and function of 5AR, this chapter will review the main achievements obtained in the context of steroid 5ARIs potentially useful in the treatment of prostatic disorders. A special focus will be given to oxidized steroids, such as progesterone, 16-dehydropregnenolone, and dehydroepiandrosterone derivatives, as well as carboxysteroids, and heterocyclic steroidal compounds.

Human Steroid 5α-Reductase Enzyme

The 5α-Reductase Isozyme Family

Steroid-5-reductases (5AR and 5β-reductase (5BR)) were first discovered, purified, and characterized in rat liver homogenates by Dorfman and Forchiell (1956). These early experiments demonstrated that these enzymes were capable of irreversibly reducing the double bond between carbons 4 and 5 of C-19 and C-21 steroids to 5α- and 5β-stereoisomers (Dorfman and Forchielli 1956).

In total, the 5AR family is composed bythree subfamilies and five members (isozymes). Isozymes are different proteins that perform the same function. The 3 subfamilies are 5AR type 1 and 5AR type 2, 5AR type 3, and

glycoprotein synaptic 2 (GPSN2) and glycoprotein synaptic 2-like (GPSN2L) (Azzouni et al. 2012). 5AR type 1 is mainly present in the skin, liver, kidney, brain, and lung. Moreover, it has been evidenced that type 1 activity is several times higher in PCa than BHP. On the other hand, type 2 isozyme predominates in the prostate and other genital tissues, playing a major role in BHP. In addition, it was observed that testosterone has a higher affinity to this isozyme than to type 1 isoform (Salvador, Pinto, and Silvestre 2013; Sun et al. 2011; Aggarwal, Thareja, Verma, et al. 2010). More recently, 5AR type 3 was identified in castration-resistant PCa cells as well as in other tissues such pancreas, brain, skin, and adipose tissues (Uemura et al. 2008). It is also known that this isozyme is expressed in peripheral tissues at higher levels than types 1 and 2 (Yamana et al. 2010). In addition to its potential role in synthesizing DHT in both androgen-stimulated and androgen-deprived human PCa, this isozyme is also considered a biomarker of malignancy in some tumors (Salvador, Pinto, and Silvestre 2013; Titus et al. 2014).

Gene Location, Structure, Biochemical Properties, and Function

5AR type 1 and type 2 are NADPH-dependent, membrane-associated (microsomal) enzymes, composed of 259 and 254 amino acids, and have molecular weights of 29.5 and 28.4 kDa, respectively. These isozymes contain a high content of hydrophobic amino acids distributed throughout their sequences, which suggests that they are intrinsic membrane proteins deeply embedded in the lipid bilayer (Azzouni et al. 2012).

These two isozymes are intrinsic membrane proteins and catalyze the same reaction. Nevertheless, they only share a limited degree of homology in protein sequence, they are located in distinct chromosomes and possess different biochemical properties. The average sequence identity between these two isozymes within given species is approximately 47%, while the sequence identity between the same isozyme across species is 60% for 5AR type 1 and 77% for 5AR type 2. The genes responsible for encoding these isozymes (SRD5A1, SRD5A2, and SRD5A3) are different but have similar structures, with five coding exons separated by four introns. The positions of the introns are essentially identical in the two genes. However, SRD5A1 is located on chromosome 5p15 whereas SRD5A2 is on 2p23 (Azzouni et al. 2012; Russell and Wilson 1994). Gene polymorphisms exist for the two genes and are more common for 5AR2. More than 850 and more than 550 single nucleotide polymorphisms (SNPs) have been reported for 5AR2 e 5AR1 genes,

respectively. Only a few polymorphisms affect the activity of the enzymes, decreasing (as V89L SRD5A2 variant) or increasing (as A49T SRD5A2) activity (Azzouni et al. 2012).

When examined in lysates of transfected cells, 5AR type 1 exhibits a wide optimum pH, ranging between 6.0 and 8.5, while 5AR type 2 shows a limited acid optimum pH (pH 5.0-5.5). However, there is evidence suggesting that inside intact human cells, 5AR type 2 isozyme functions optimally at a more neutral pH range (6.0-7.0). 5AR type 1 has a larger turnover number, as indicated by its K_{cat} value and a lower substrate affinity for testosterone, k_m = 1-5 μM. 5AR2 has a lower K_{cat} and a higher substrate affinity, as indicated by k_m = 0.004-1 μm for testosterone. Under optimal conditions, 5AR type 2 has a higher 5α-reducing activity than 5AR type 1 as indicated by its high V_{max}/k_m ratio. Both isozymes contain an NH_2-terminal steroid/ligand-binding domain and COOH-terminal NADPH binding domain. The apparent dissociation constant for NADPH cofactor is similar for both isozymes (3-10 μM). Until now, such comparisons do not exist for 5AR type 3 except that it appears to be efficient at pH 6.5-6.9 (Azzouni et al. 2012).

The 5AR isozyme family has several functions, being the most understood the 5α- reduction. However, these isozymes can carry out other functions. One of these functions is the *N*-glycosylation of proteins by 5AR type 3 isozyme (Cantagrel et al. 2010). 5α-C19 steroids increase the production of erythropoietin hormone in the kidneys and 5β-C19 steroids are important for heme synthesis in the liver. Both 5AR and 5BR are involved in bile acids biosynthesis: these enzymes catalyze the conversion of 7α,12α-dihydroxy-4-cholesten-3-one into 7α,12α-dihydroxy-5α-cholestan-3-one and 7α,12α-dihydroxy-5β-cholestan-3-one, respectively (Azzouni et al. 2012). GPSN2 subfamily seems to be related to the fourth reaction of fatty acid elongation by reducing a fatty chain double bond in mammals. Although the substrate of GPSN2 is structurally different from the other two 5AR subfamilies, all subfamilies of 5AR share a similar biochemical ability to reduce a double bond of the respective substrates (Moon and Horton 2003).

Mainly due to the instability during purification, the determination of crystal structures of 5AR isozymes by X-ray diffraction has been only achieved very recently for 5AR type 2and. In fact, only in 2020 Xiao et al. (2020) elucidated the crystal structure and coordinates of human 5AR type 2, in the presence of NADPH and finasteride (a 5ARI used in the clinical practice). This structure (Figure 1) revealed a topology of seven transmembrane α-helices (Xiao et al. 2020). This great advance allows to fully understand the molecular mechanisms underlying the function of 5AR,

particularly, its catalytic mechanism and the action of 5ARIs. In addition, this new data can boost the application of structure-based design of new more potent, and selective 5AR inhibitors.

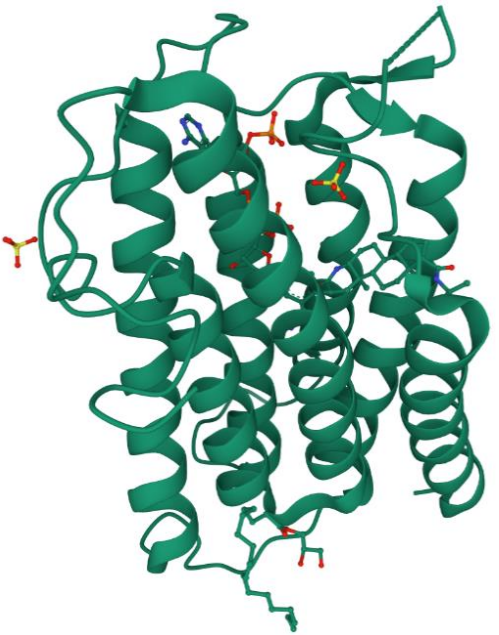


Figure 1. Crystal structure of 5α-reductase type 2 in complex with finasteride (PDB ID: 7BW1) (Xiao et al. 2020).

The Mechanism of Action

5AR enzymes catalyze the irreversible reduction of the double bond between C-4 and C-5 of the steroidal ligand, in the presence of the cofactor NADPH (Salvador, Pinto, and Silvestre 2013). The substrates for 5AR are 3-oxo (3-keto), $\Delta^{4,5}$ C-19/C-21 steroids, which include testosterone, progesterone, androstenedione, epi-testosterone, cortisol, aldosterone, and deoxycorticosterone (Azzouni et al. 2012). As previously referred, in the case of testosterone, is converted by 5AR in DHT, the most potent androgen (Figure 2).

The molecular mechanism comprises the binding of NADPH and substrate, forming a ternary complex. Then, an electrophilic residue in the

active site of the enzyme actives the Δ^4-3-ketone systems of the steroidal substrate, originating a delocalized carbocation at C-5. Afterward, a hydride (H^-) transfer from the cofactor NADPH to the α-face of this carbocation leads to the formation of an enolate of DHT, which is protonated at C-4 on the β-face to afford DHT (Figure 3) (Salvador, Pinto, and Silvestre 2013; Russell and Wilson 1994; Bull et al. 1996).

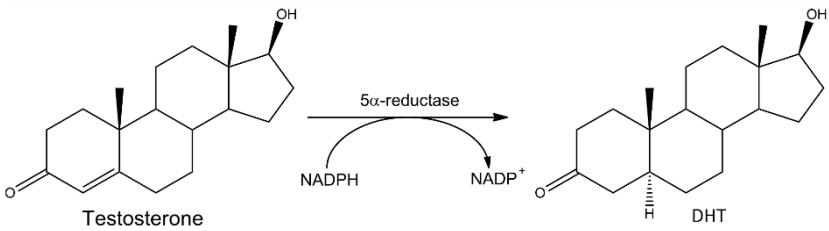


Figure 2. Conversion of testosterone into 5α-dihydrotestosterone catalyzed by the 5α-reductase enzyme, in the presence of the cofactor. Created in ChemDraw.

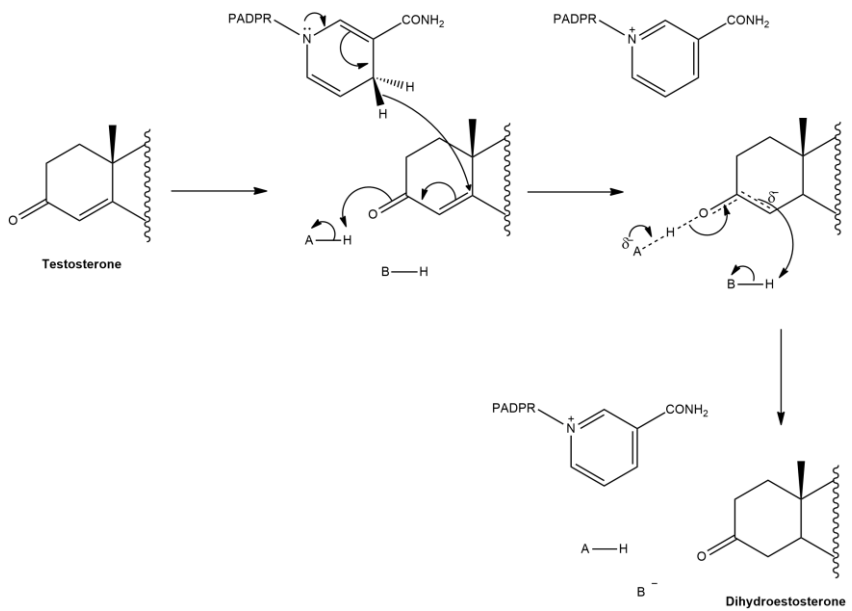


Figure 3. Mechanism of action of 5α-reductase enzyme considering testosterone as the substrate (PADPR = 2'- phosphoadenosine-5"- diphosphoribose). Adapted from Bull et al. (Bull et al. 1996). Created in ChemDraw.

Steroid 5α-Reductase Inhibitors

The 5AR overexpression increases serum DHT levels, which is implicated in some androgen-dependent disorders, including BPH, PCa progression, and also male androgenic alopecia, which tend to increase with age (Swerdloff et al. 2017; Egan 2016; Hamilton 1951). Consequently, lowering DHT levels through the inhibition of 5AR comprises an interesting and rational approach in the management of these conditions to mainly reduce low urinary tract symptoms (LUTS) that are associated. Thus, researchers focused their work on the discovery and development of 5ARIs.

One of the goals of 5ARIs development is to obtain molecules capable of binding to 5AR with low or no affinity to the AR or other steroid receptors or enzymes (Aggarwal, Thareja, Verma, et al. 2010; Azzouni and Mohler 2012; Azzouni et al. 2012). In this scope, usually biological pharmacological studies include *in vitro* enzymatic activity experiments, *in vivo* assessment of effects on testosterone serum levels, and evaluation of the AR binding and activation capacities of the developed molecules. The first inhibitors were steroids that mimicked testosterone and, in many cases, were substrates themselves, not being true inhibitors (Azzouni et al. 2012). Structurally, 5ARIs can be broadly grouped as steroidal and non-steroidal, with the steroidal class being larger than the non-steroidal class (Salvador, Pinto, and Silvestre 2013; Aggarwal, Thareja, Verma, et al. 2010).

Although this chapter is focused on steroidal inhibitors, it is important to highlight that several pharmaceutical industries and academia have also pursued the synthesis of non-steroidal 5ARIs due to the undesired hormonal side effects of steroidal compounds. Non-steroidal inhibitors are classified according to their structure, and most of them have been derived from azasteroidal inhibitors by removing one or more rings (Azzouni et al. 2012; Kumar and Kumar 2012). In this context, the most potent and selective non-steroidal inhibitors of human 5AR type 1 include, namely, piperidones, quinolinones, benzoquinolinones, benzoquinolizinones, nonsteroidal aryl acids, butanoic acid derivatives, polyunsaturated fatty acids, as well as some cations (especially zinc) (Jones et al. 1993).

Azasteroid 5α-Reductase Inhibitors

Currently, the most extensively studied class of 5ARIs is the azasteroid with a special interest in 4-azasteroids (Azzouni and Mohler 2012; Azzouni et al.

2012). Azasteroids were developed to mimic the enzyme-bond enolate intermediate by the isosteric change between carbon and nitrogen. The only two azasteroid 5ARIs that are approved for clinical use in the management of BHP are finasteride and dutasteride (Figure 4), which have the 4-azasteroid skeleton (Salvador, Pinto, and Silvestre 2013).

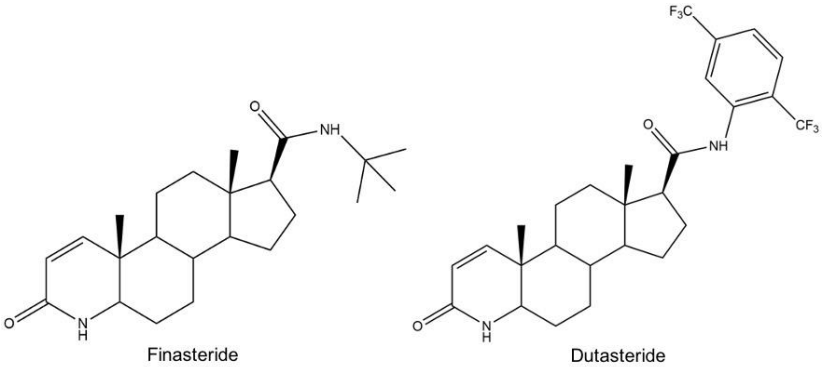


Figure 4. Molecular structures of steroid 5α-reductase inhibitors approved for clinical use in the management of benign prostatic hyperplasia: finasteride (Proscar®) and dutasteride (Avodart®). Created in ChemDraw.

In terms of mechanism of action, to allow inhibitor binding, there is a hydride transfer from the NADPH cofactor to the Δ^1-double bond of finasteride. The intermediate enolate tautomerizes at the enzyme active site to form a bisubstrate analog in which dihydrofinasteride is covalently bound to $NADP^+$ (Figure 5). Thus, this drug forms an extremely stable enzyme-bound NADP-dihydrofinasteride adduct, which is ultimately processed to dihydrofinasteride (Bull et al. 1996; Xiao et al. 2020; Aggarwal, Thareja, Bhardwaj, et al. 2010; Drury et al. 2009).

Finasteride was the first drug of this group that entered the market and the first approved by the US Food and Drug Administration (FDA) for BPH management (Schmidt and Tindall 2011). Finasteride is a relatively potent competitive inhibitor of 5AR type 2 (IC_{50} = 69 nM), but it inhibits less effectively 5AR type 1 (IC_{50} = 360 nM) (Tian et al. 1994). Finasteride was also shown *in vitro* to inhibit 5AR type 3 in a potency similar to 5AR type 2 (IC_{50} = 17.4 nM, 14.3 nM, respectively), in transfected HEK-293 cells (Azzouni et al. 2012). In addition, this drug was associated with a reduction in PCa incidence. However, finasteride may also increase the high-grade Gleason (score of aggressiveness) prostate tumors risk (I. Thompson et al. 2003;

Kaplan et al. 2009). Considering these contradictory findings, finasteride is not labeled for PCa treatment and it was rarely used for PCa prevention (I. M. Thompson et al. 2009). More recently, Diviccaro et al. (2019) performed *in vivo* studies to assess the effects of finasteride on the nervous system (Diviccaro et al. 2019). This research was based on the appearance of several side effects after discontinuation of the treatment with this drug. In fact, several studies reported impaired sexual function and high depression scores in patients, and this condition was named post-finasteride syndrome (Basaria et al. 2016; Melcangi et al. 2017). Diviccaro's research suggested that treatment and subsequent withdrawal of finasteride cause changes in the hippocampus, corresponding with the appearance of this depressive-like behavior (Diviccaro et al. 2019).

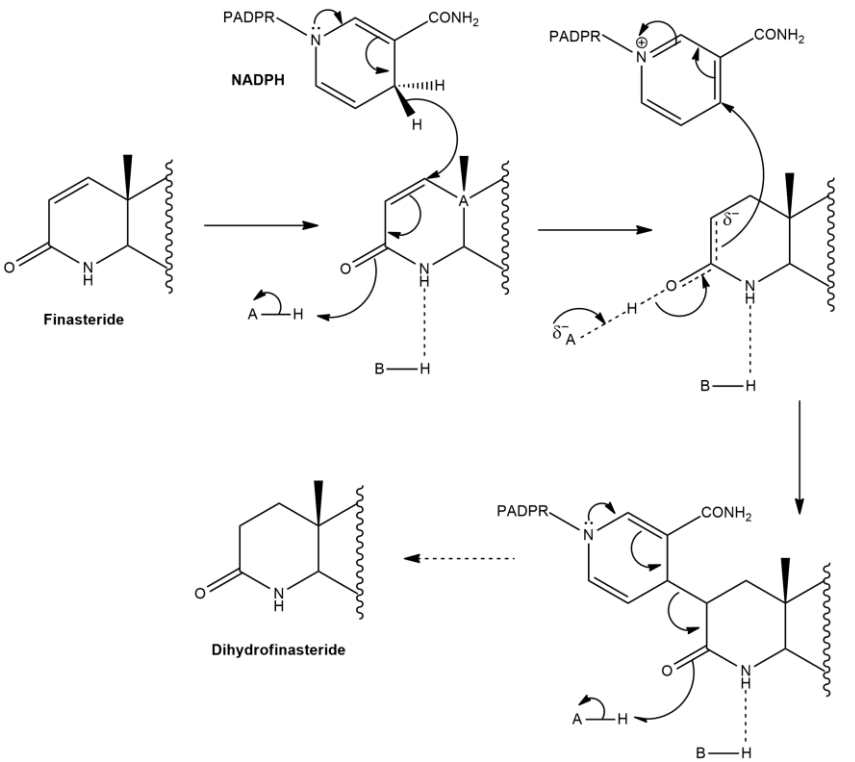


Figure 5. Mechanism of inhibition of 5α-reductase type 2 by finasteride (PADPR = 2'-phosphoadenosine-5"- diphosphoribose). Adapted from Bull et al. and Drury et al. (Bull et al. 1996; Drury et al. 2009). Created in ChemDraw.

In 2001, another molecule, dutasteride, was approved by FDA as monotherapy or in combination with tamsulosin for the treatment of BPH to improve symptoms by reducing LUTS and reducing the need for BPH-related surgery (C. Wu and Kapoor 2013). Dutasteride emerged as a new generation of 5ARIs, being capable of inhibiting 5ARs type 1 and 2 more effectively than finasteride. The IC_{50} for 5AR type 1 inhibition is 7 nM and for 5AR type 2 is 6 nM (Azzouni et al. 2012). Interestingly, a study reported by Yamana et al. (2010) also demonstrated the potential inhibition of 5AR type 3 by dutasteride (Yamana et al. 2010). Moreover, dutasteride provides almost complete suppression of DHT (94.7 - 98.4%), compared with finasteride which only reduces the DHT by 70.8% in the same administration conditions (R. V. Clark et al. 2004). A meta-analysis of randomized clinical trials revealed that dutasteride is highly effective in mitigating BPH-associated symptoms. In addition, it was observed an efficient reduction in enlarged prostate size, a reduction in risks of acute urinary retention, and a reduction in surgical intervention needs. However, medical therapy with dutasteride seems to be related to an increased rate of sexual dysfunction (X.-J. Wu et al. 2013). Interestingly, some studies also reported that dutasteride can improve the prognosis of PCa patients, particularly in low-risk PCa (Fleshner et al. 2012; Schröder et al. 2013).

Despite finasteride and dutasteride improving the symptom score, retarding the BPH progression, sexual adverse effects arise as the most common drug-related adverse effects that, usually, occur during the first year of treatment. The most common side effects include decreased libido, erectile dysfunction, and gynecomastia. Usually, these adverse effects seriously compromise the patients' quality of life, justifying the research for the discovery of new drugs more effective and with fewer side effects on patients' sexual life (Yu et al. 2020; Aggarwal, Thareja, Verma, et al. 2010; Montorsi and Moncada 2006). Regarding the chemical structures of these two most successful 4-azasteroids, finasteride and dutasteride, they just diverge in the respective lateral chain bond to C-17 (Figure 4). Consequently and considering the side effects of finasteride and dutasteride and the structural difference referred, a variety of modifications mainly in this part of the structure of 4-azasteroids have been extensively explored in an effort to overcome these problems. In this context, and as an example, other 4-azasteroids such as 20-oxime-4-azasteroidal derivatives and 16*E*-arylidene-4-azaandrostenes were obtained and some motivating bioactivity results were achieved (Brito et al. 2018; S. Kim and Ma 2009; S. Kim, Kim, and Ma 2012). In addition, other promising compounds with 5AR inhibitory activity were

obtained and investigations in this field are still in development. Moreover, the preparation of azasteroids with nitrogen atoms in other positions, such as 6-azasteroids, 17-azasteroids, and 19-nor-10-azasteroids was also accomplished, and good values for 5AR inhibition were obtained (Haffner et al. 1994; Scarpi et al. 2002; Guarna et al. 1997; Malhotra et al. 2013). Some reviews on this subject have been published and the structure-activity relationship of this class of steroidal compounds has been studied (Salvador, Pinto, and Silvestre 2013; Haffner et al. 1994; Aggarwal, Thareja, Bhardwaj, et al. 2010; Birudukota and Mudgal 2019; Malhotra et al. 2013; Kumar and Kumar 2012).

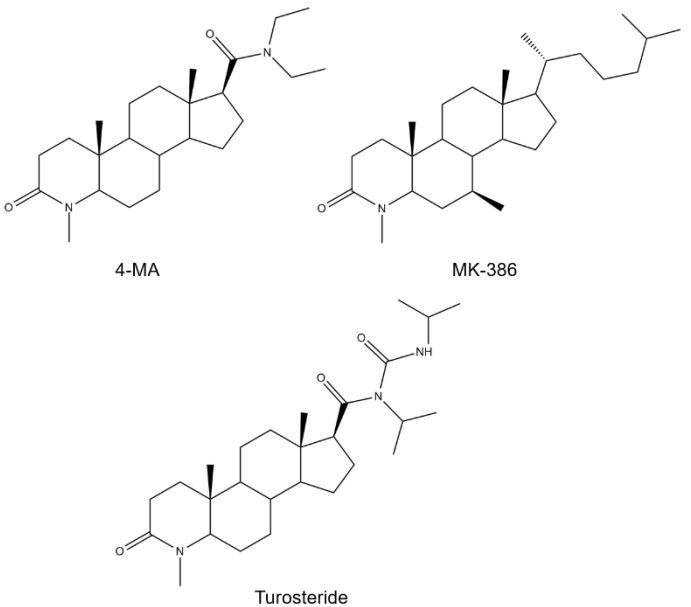


Figure 6. Molecular structures of some representative 4-azasteroidal 5α-reductase inhibitors: 4-MA, MK-386, and turosteride. Created in ChemDraw.

Some of the most representative examples of other 4-azasteroids known for inhibiting 5AR are 4-MA, turosteride, and MK-386 (Figure 6) (Birudukota and Mudgal 2019). MK-386 is a selective 5AR type 1 inhibitor, while 4-MA is a potential dual inhibitor of 5ARs type 1 and 2. Despite its potential and very low affinity to AR, 4-MA was withdrawn from clinical development after it was shown to be an inhibitor of 3β-hydroxysteroid dehydrogenase (3β-HSD) and to cause hepatotoxicity (Azzouni et al. 2012; Kumar and Kumar 2012). In

addition, turosteride (FCE 26073), an analog of 4-MA, is also a potent selective 5AR type 1 inhibitor (IC_{50} in human 5AR type 1 = 55 nM). Contrary to 4-MA, turosteride is a weak 3β-HSD inhibitor (Di Salle et al. 1994).

Oxidized Steroids with 5α-Reductase Inhibitory Activity

In the past few years, high attention has been given to non-azasteroids as 5ARIs, particularly to oxidized steroids. In this context, interesting results were observed, and progesterone, 16-dehydropregnenolone, and dehydroepiandrosterone (DHEA) derivatives emerged as the main steroidal compounds with relevant 5AR inhibitory activity and thus potentially useful in prostatic diseases. Moreover, carboxysteroids and heterocyclic steroidal derivatives also comprise important classes of non-azasteroids as potential 5ARIs.

Progesterone Derivatives

Progesterone and deoxycorticosterone compete with testosterone for the 5AR enzyme. Considering this fact, the pregnane skeleton emerged as an interesting template for the design and development of 5ARIs. In relation to the oxidized derivatives, the main modifications reported comprise stereoselective epoxidation of α,β-unsaturated ketones at C-4 or C-6, which is commonly combined with D-ring functionalizations, such as the introduction of a C-17 ester (Figure 7).

Primarily, Bratoeff and coworkers prepared aromatic esters of progesterone, including 4α,5α-expoxyprogesterone derivatives, and these progesterones were evaluated as 5AR and prostate growth inhibitors. The *in vitro* 5AR activity studies were performed on human prostate homogenates that were centrifuged to obtain prostatic enzyme fractions. Then, the enzymatic fractions were incubated with [^{3}H]-testosterone, and the activity of human 5AR was measured through thin layer chromatography analysis. The epoxysteroids 1 and 2 inhibited the 5AR enzyme with IC_{50} values of 13 and 4.9 nM, respectively (Table 1). In addition, *in vivo* studies revealed that these steroids significantly decreased the prostate weight of gonadectomized hamsters treated with testosterone. This effect was similar to the observed for finasteride, used as the positive control. Interestingly, when comparing the

biological activities of epoxysteroidal derivatives and the corresponding non-epoxidized steroids, it was observed that the introduction of the epoxide led to a higher 5AR inhibitory activity. In fact, the determined IC_{50} values for non-epoxidized derivatives were 360 and 370 nM, respectively. Additionally, competitive studies to determine the capacity of these steroids to bind to the AR present in the rat prostate cytosol, using labeled mibolerone as a tracer, were also performed. The results showed that the epoxysteroids did not significantly bind to AR (Bratoeff et al. 2009).

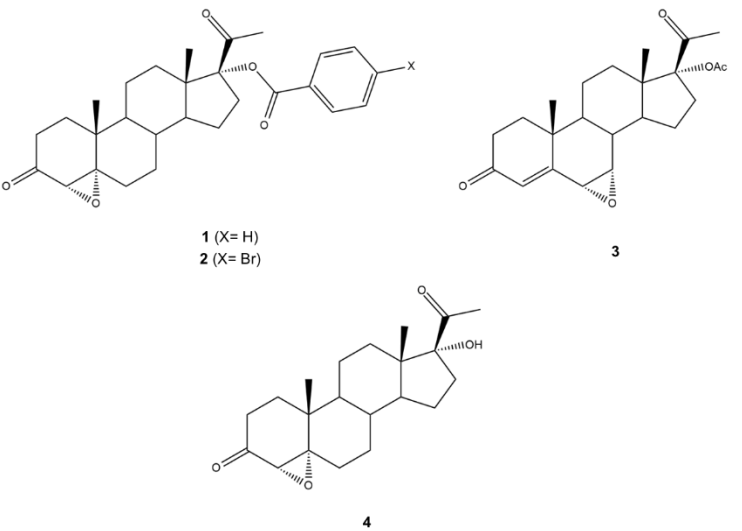


Figure 7. Oxidized progesterone derivatives with relevant 5α-reductase inhibitory activity.

Table 1. IC_{50} values of progesterone derivatives against 5α-reductase (human, prostatic)

Progesterones	**5AR IC_{50} (nM)**	**Reference**
1	13.0	(Bratoeff et al. 2009)
2	4.9	
3	22.0	(Bratoeff, Zambrano, et al. 2010)

Later, the same group conducted similar studies with new series of 6- and 17-substituted progesterone derivatives synthesized from 17α-acetoxyprogesterone. The synthetic intermediates, including a 6α,7α-epoxyprogesterone derivative (3, Figure 7), were also biologically evaluated.

Concerning the *in vitro* inhibitory activity of the 5AR enzyme, the determined IC_{50} value for epoxysteroid 3 was 22 nM (Table 1). Moreover, the lowest prostate weight in the *in vivo* experiments was observed for the hamsters treated with steroid 3, indicating the highest effect among these new derivatives (Bratoeff, Zambrano, et al. 2010). These authors also reported the synthesis of 17α-hydroxyprogesterone, 17α-hydroxy-4α,5α-epoxypregnan-3,20-dione (4, Figure 7), and 4-chloro- and 4-bromopregn-4-ene-3,20-diones from 17α-acetoxyprogesterone and their bioactivity evaluation. The 4α,5α-epoxyprogesterone derivative 4 showed selective inhibition of 5AR type 1 (IC_{50} = 0.32 μM), being the most potent of this group of steroids against this isozyme. Concerning the activity of 5AR type 2, the determined IC_{50} value was 40 μM. In contrast with previous studies, this *in vitro* experiment assessed the activities of 5AR type 1 (from rat liver microsomes) and 2 separately. However, when hamsters were treated with epoxysteroid 4, the reduction of the weight of the prostate gland was not significant. Moreover, derivatives 3 and 4 did not show affinity to AR in competitive studies (Bratoeff, García, et al. 2010).

16-Dehydropregnenolone Derivatives

Another important group of 5ARIs based on the pregnane skeleton includes oxidized 16-dehidropregnenolone derivatives, mainly functionalized at C-3, C-5, and C-6. These modifications considered that a nucleophilic portion of the 5AR attacks the double bond of the steroid through Michael's addition reaction, after the formation of the steroid-enzyme complex. The representative examples of oxidized 16-dehidropregnenolone derivatives with relevant 5AR inhibitory activity are shown in Figure 8.

Of these, oxidized steroids 5, 7, 9, and 11 exhibited an efficient inhibition of the enzyme 5AR. On contrary, steroids 6 and 8 seemed not to possess relevant inhibitory properties (Table 2). According to the authors, the weak Michael acceptor reactivity of steroid 6 and low solubility of steroid 8 are probably the main reasons for the lack of biological activity (Pérez-Ornelas et al. 2005). Bratoeff and coworkers also synthesized 3-substituted pregna-4,16-diene-6,20-dione derivatives (12-15, Figure 8). After biological evaluation, it was observed that these 3β-(3-halopropanoyl) pregnanes were capable of decreasing the prostate weight in castrated animals treated with testosterone. In addition, the steroids 13-15 inhibited significantly the activity of the 5AR (Table 2) and, on the other hand, these steroids did not bind to AR (Bratoeff et al. 2008).

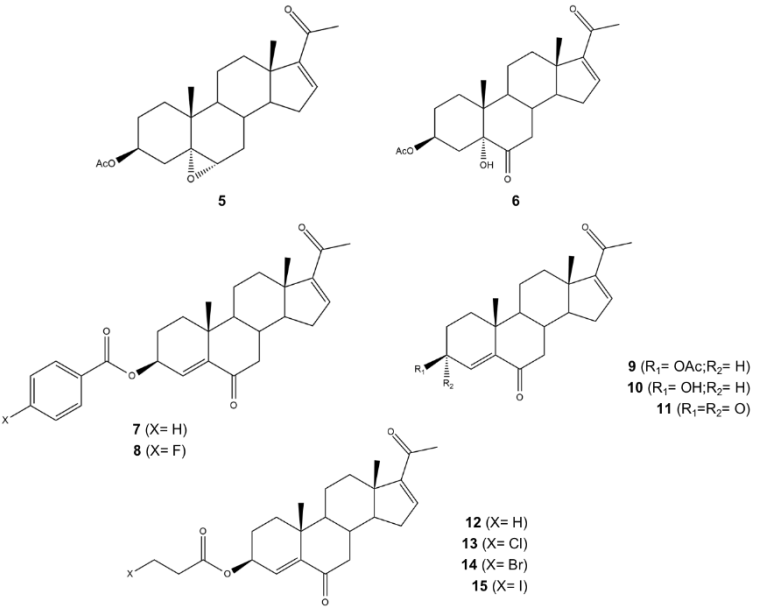


Figure 8. Representative oxidized 16-dehydropregnenolone derivatives evaluated as 5α-reductase inhibitors.

Table 2. IC_{50} values of 16-dehydropregnenolone derivatives against 5α-reductase (human, prostatic)

Steroids	**5AR IC_{50} (nM)**	**Reference**
5	0.063	(Pérez-Ornelas et al. 2005)
6	200,000	
7	0.070	
8	350,000	
9	0.065	
10	Not active	
11	0.850	
12	40,000	(Bratoeff et al. 2008)
13	14.0	
14	1.8	
15	10.0	

More recently, four new oxidized (*p*-fluoro)benzoyloxy-21-esters of pregnenolone were obtained and their effects as inhibitors of 5AR type and 2 were assessed through *in vitro* and *in vivo* experiments (Figure 9, Table 3). In general, the *in vitro* results showed that steroids 16, 17, and 18 selectively

inhibit the 5AR type 2 (Table 2). In contrast, the 21(*p*-fluoro)benzoyloxypregna-4,16-diene-3,6,20-trione derivative 19 has the capacity to *in vitro* inhibit the activity of both isozymes, being a non-selective 5ARI. Moreover, steroid 19 also displayed *in vivo* activity, since, when administrated in combination with testosterone to castrated hamsters, it significantly decreased the weight of the prostate and seminal vesicles. Nevertheless, oxidized derivatives 16–18 did not exhibit statistically significant effects (Chávez-Riveros et al. 2015).

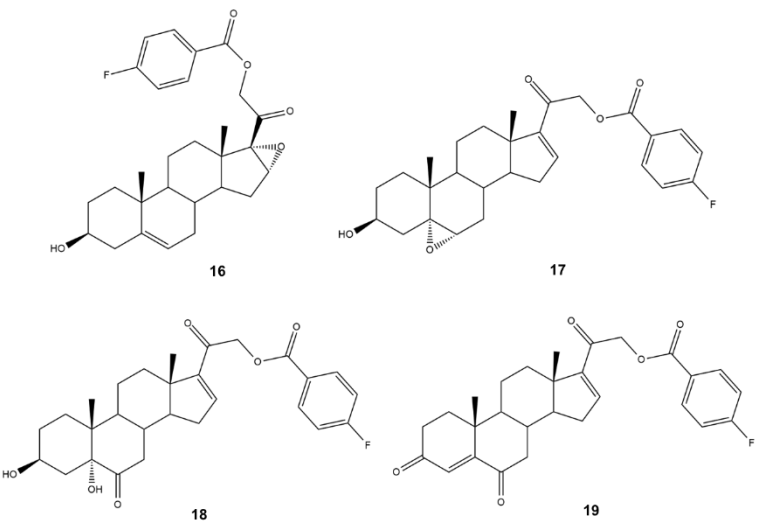


Figure 9. Principal (p-fluoro)benzoyloxy-21-esters of pregnenolone as principally type 2 5α-reductase inhibitors.

Table 3. IC_{50} values of (p-fluoro)benzoyloxy 21-esters of pregnenolone against 5 α-reductase type 1 (rat, liver) and type 2 (human, prostatic)

Steroids	5AR type 1 IC_{50} (μM)	5AR type 2 IC_{50} (nM)	Reference
16	100	34.000	(Chávez-Riveros et al. 2015)
17	100	179.000	
18	Not active	33.000	
19	1	0.179	

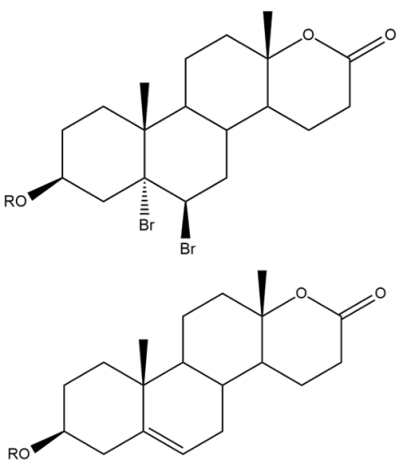
According to the authors, the increase in pharmacological effects of the steroid 19 may be related to the presence of a ketone group at C-6, since this group reduces the possibility of steroid metabolization by hepatic enzymes.

Additionally, they hypothesized that the improved inhibitory activity of this steroid could be related to the double bond at C-4, while the C-21 ester moiety increased its lipophilicity (Chávez-Riveros et al. 2015).

More recently, Cortéz-Benítez reported the synthesis of new androst-4-ene-3-one derivatives with different arylcarbamoyl groups at C-17 from pregnenolone and their anti-proliferative effect on human androgen-sensitive LNCaP cells. Some of these androstenones exhibited a higher growth inhibitory effect than finasteride, flutamide and ketoconazole on LNCaP cells, in the presence and absence of androgens (Cortés-Benítez et al. 2016). Later, the same research group evaluated the inhibitory activity of these androstenones against 5AR type 1. The most potent one was the derivative 17β-*N*-(3-fluorophenylcarbamoyl)androst-4-ene-3-one, which presented an IC_{50} value of 0.35 µM (Bratoeff et al. 2018).

Dehydroepiandrosterone Derivatives

The androstane nucleus also provides a relevant starting point for the preparation of potential 5ARIs. In this context, some new lactones derived from DHEA were obtained and evaluated as 5AIRs, showing promising results (Figure 10, Table 4).



20 (R= $COCH_2CH_3$)
21 (R= $CO(CH_2)_2CH_3$)
22 (R= $CO(CH_2)_3CH_3$)
23 (R= $CO(CH_2)_4CH_3$)
24 (R= $COCH_2OCH_2CH_3$)

25 (R= $COCH_2CH_3$)
26 (R= $CO(CH_2)_2CH_3$)
27 (R= $CO(CH_2)_3CH_3$)
28 (R= $CO(CH_2)_4CH_3$)
29 (R= $COCH_2OCH_2CH_3$)

Figure 10. Examples of dehydroepiandrosterone derivatives with 5α-reductase inhibitory activity.

Table 4. IC_{50} values of dehydroepiandrosterone derivatives against 5α-reductase (human, prostatic)

Steroids	5AR IC_{50} (nM)	Reference
20	1.200	(Garrido et al. 2011)
21	0.028	
22	0.069	
23	110.000	
24	1.200	
25	780.000	
26	1,300	
27	4.200	
28	0.025	
29	36.000	

Garrido et al. (2011) prepared and evaluated these new D-ring steroidal lactones, which bear a 5α,6β-dibromo moiety (20-24, Figure 10) or a double bond at C-5 (25-29, Figure 10) and linear esters attached to the hydroxyl group at C-3. In general, *in vitro* studies evidenced that these steroids inhibited the 5AR enzyme. Steroids 21, 22, and 28 presented the best values for inhibitory activity with determined IC_{50} values of 0.028, 0.069, and 0.025 nM, respectively (Table 4). Moreover, *in vivo* studies revealed that all of these steroids decreased the weight of the prostate and seminal vesicles. Competitive studies to assess the binding of these steroids to AR were also accomplished, and only steroids 23, 25, and 29 showed to bind to this receptor (Garrido et al. 2011).

Carboxysteroids

Several steroidal carboxylic acid derivatives and analogs were designed to mimic the presumed enzyme-bond enolate intermediate and consequently inhibit the 5AR enzyme. The preparation of these steroids was accomplished by inserting sp^2-hybridized centers at C-3 and C-4, and an anionic carboxylic acid at C-3 to substitute the enolate oxyanion. Furthermore, it demonstrated the crucial role of Δ^3- and Δ^5-double bonds, and di-*iso*-propyl and *tert*-butyl amides at C-17 for increased enzyme inhibitory activity. In this context, the most relevant examples of steroidal carboxylic acid derivatives with important 5AR inhibitory activity are represented in Figure 11. Considering the literature, the most representative carboxysteroid is the epristeride (32), which

potently inhibits selectively the 5AR type 2, presenting a weak inhibition of the type 1 isozyme (Yamashita et al. 1996). Two decades ago, epristeride was subjected to clinical trials, and currently, it is used in the treatment of BPH in China (Ju et al. 2002). Analogs of epristeride, namely steroids 30, 31, 33-35 were also reported with interesting effects against 5AR (Table 5) (D. Holt et al. 1990; D. A. Holt et al. 1990).

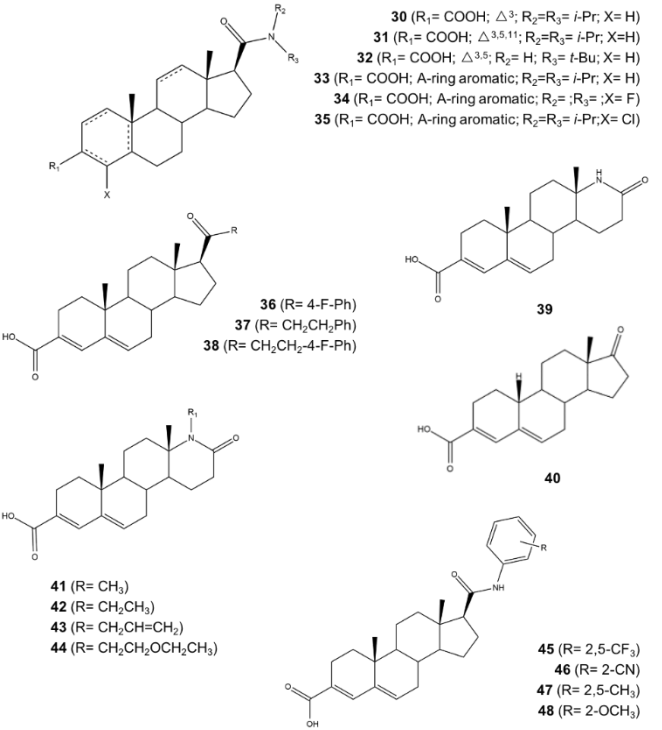


Figure 11. Representative 3-carboxysteroids with 5α-reductase inhibitory activity.

Table 5. Carboxysteroids and respective Ki values relative to 5α-reductase inhibitory activity (human, prostatic)

Carboxysteroids	5AR K_i (nM)	Reference
30	30	(D. A. Holt et al. 1990)
31	7	
32	30-36	
33	20	(D. Holt et al. 1990)
34	10	
35	120	

Table 6. 3-carboxysteroids and respective *in vitro* percentages of 5α-reductase inhibitory activity (human, recombinant)

Carboxysteroids	5AR type 1% of inhibition at 10 μM	5AR type 2% of inhibition at 10 μM	5AR type 2 IC_{50} (nM)	Reference
40	82.1	100	212.9	(Aggarwal et al. 2012)
41	18.9	100	54.1	
42	32.3	100	22.1	
43	0.4	100	72.8	
44	8.5	100	26.5	

Table 7. IC_{50} values of androst-3,5-diene-3-carboxysteroid derivatives against 5α-reductase type 1 (rat, liver) and type 2 (human, prostatic)

Carboxysteroids	5AR type 1 IC_{50} (μM)	5AR type 2 IC_{50} (μM)	Reference
45	0.25	0.13	(Lao, Sun, Wang, Lyu, et al. 2017)
46	0.40	0.29	
47	0.58	0.98	
48	0.48	0.41	

The steroidal carboxylic acids 36-38 (Figure 11) were synthesized and evaluated against the two isozymes. When compared with epristeride (IC_{50} = 412 nM), the determined IC_{50} values for these carboxysteroids revealed a major capacity of inhibition of 5AR type 1 (IC_{50} 36 = 85 nM; IC_{50} 37 = 4-6 nM; IC_{50} 38 = 7 nM). Relative to inhibition of 5AR type 2, the values for steroids 36-38 were 2, 1-2, and 6 nM, respectively, while for epristeride an IC_{50} value of 0.12 nM was determined (Yamashita et al. 1996).

Later, an interesting study explored the combination of important structural features of finasteride and epristeride (32). This study resulted in the obtention of a hybrid steroidal derivative, the steroid 39 (Figure 11). *In vitro* experiments using human prostatic 5AR, evidenced that steroid 39 is a potent 5ARI (IC_{50} = 71 nM), however, it is slightly less potent than finasteride (IC_{50} = 35 nM) (Yao et al. 2011).

Aggarwal and coworkers also synthesized novel steroidal 17a-substituted 17-oxo-17a-aza-D-homo-3,5-androstadien-3-oic acids (41–44) and 17-oxo-19-nor-3,5-androstadien-3-oic acid (40) (Figure 11). Moreover, their inhibitory activities against 5AR were assessed by *in vitro* and *in vivo* experiments. In general, the results showed that the carboxysteroids 40–44 had a higher capacity to inhibit 5AR type 2 (Table 6). On the other hand, in *in vivo*

studies, it was observed that these steroids can significantly reduce the weight of the rat prostate gland (Aggarwal et al. 2012).

More recently, Lao et. al prepared a series of new steroidal androst-3,5-diene-3-carboxylic acids (45-48, Figure 11) bearing substituents in C17 similar to the existent in dutasteride. Biological evaluation of their effects comprised both *in vitro* enzyme inhibition assay of 5AR types 1 and 2 and *in vivo* prostate gland weighing. Interestingly, most of these steroids displayed a potent 5AR inhibitory activity, exhibiting slightly higher type 2 isozyme inhibition (Table 7). Carboxysteroid 45 was found to be the most potent inhibitor (IC_{50} 5AR type 1 = 0.25 μM; IC_{50} 5AR type 2 = 0.13 μM). In addition, *in vivo* experiments showed that carboxysteroid 45 also exhibited a more significant reduction effect in rat prostate weight than epristeride. This research also comprised *in silico* ADME studies, which revealed that carboxysteroid 45 can have favorable pharmacokinetic properties (Lao, Sun, Wang, Lyu, et al. 2017).

Oxidized Heterocyclic Steroidal Derivatives

Another approach to developing potential 5ARIs is based on the introduction of heterocyclic moieties in the steroidal D-ring. In this ambit, Wölfling and coworkers described several steroidal tetrahydrooxazin-2-ones (steroids 49–52, Figure 12, Table 8), with a very reasonable 5AR inhibitory effect, however, inferior to finasteride (IC_{50} = 55 nM) (Wölfling et al. 2004). Posteriorly, the same research group also synthesized new steroidal oxazolines containing various phenyl substituents coupled to the heterocyclic moiety (structures 53–56, Figure 12). These steroids exhibited moderate 5AR inhibitory activities, with IC_{50} values ranging from 720 to 2750 nM (5AR type 1 isolated from rat liver) (Szécsi et al. 2010). More recently, Al-Mohizea and coworkers prepared several steroids with cyanopyridone and cyanothiopyridone heterocycles fused with the D-ring (e.g., pyridones 57–61, Figure 12). Then, a set of pharmacological properties was assessed, comprising the 5AR inhibitory activities and anti-tumor properties (e.g., in LNCaP and PC-3 PC cell lines). In these studies, it was observed that all these steroids exhibited potent 5AR inhibitory activities (*in vivo* assay with Sprague-Dawley rats). Interestingly, the bioactivity seems to be related not only to the functionalized pyridone ring but also to the steroidal A and B-ring modifications (Figure 12, Table 9). Additionally, the anti-prostate cancer activity of these steroids was assessed against two human PCa cell lines,

LNCaP and PC-3. All the tested steroids displayed good cytotoxicity in both cell lines (Al-mohizea et al. 2012). In addition, several heterocyclic steroidal derivatives, with potential 5AR inhibitory activity were synthesized from oxidized intermediate products (Lao et al. 2019; Kiss et al. 2021). For example, Lao and coworkers reported the preparation and biological evaluation of novel androst[3,2-*c*]pyrazole derivatives with interesting IC_{50} values (lower than 1 μM) for inhibition of 5AR type 1 and 2. The synthesis of these steroidal pyrazoles comprised several steps, including the formation of an α,β-unsaturated ketone by Oppenauer oxidation (Lao et al. 2019). Interestingly, the same research group also described the synthesis of novel 3-oxo-4-oxa-5α-androst-17β-amide derivatives. Most of the prepared androstanamides displayed good 5AR inhibitory activities. However, the IC_{50} values determined were higher than the control, finasteride. Their anti-proliferation activities in PC-3 and LNCaP cells were also assessed, and the results indicated that most of the synthesized derivatives exhibited potent anti-proliferative activities (Lao, Sun, Wang, Wang, et al. 2017).

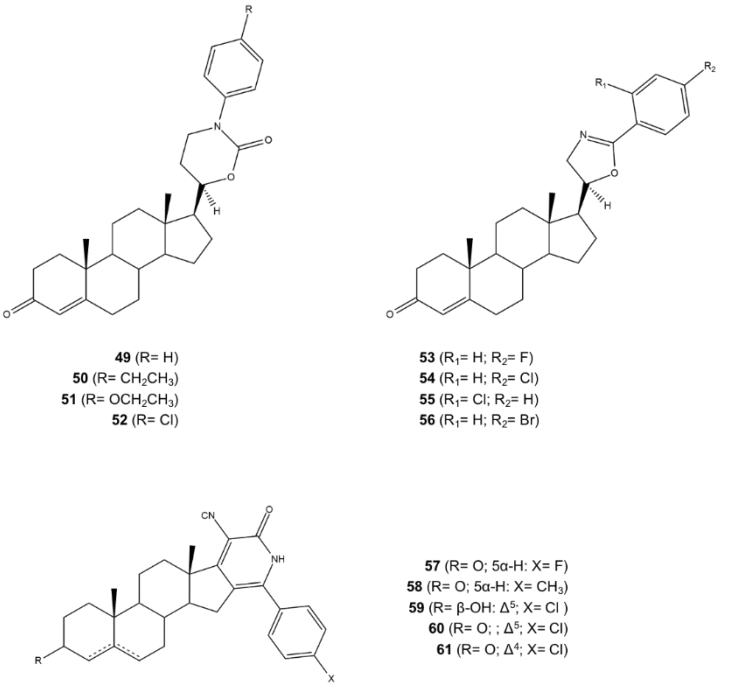
Table 8. IC_{50} values against 5α-reductase (rat, liver) of several new steroidal tetrahydrooxazin-2-ones

Steroids	**5AR IC_{50} (nM)**	**Reference**
49	270	(Wölfling et al. 2004)
50	260	
51	245	
52	420	

Table 9. *In vivo* 5α-reductase inhibitor activities (Sprague-Dawley rats) of some new steroids with cyanopyridone and cyanothiopyridone heterocycles fused with the D-ring

Steroids	**ED_{50} (μM)[a]**	**Reference**
57	300	(Al-mohizea et al. 2012)
58	330	
59	290	
60	390	
61	370	

[an] ED_{50} is the effective dose for 50% of the population.



49 (R= H)
50 (R= CH_2CH_3)
51 (R= OCH_2CH_3)
52 (R= Cl)

53 (R_1= H; R_2= F)
54 (R_1= H; R_2= Cl)
55 (R_1= Cl; R_2= H)
56 (R_1= H; R_2= Br)

57 (R= O; 5α-H: X= F)
58 (R= O; 5α-H: X= CH_3)
59 (R= β-OH: Δ^5; X= Cl)
60 (R= O; ; Δ^5; X= Cl)
61 (R= O; Δ^4; X= Cl)

Figure 12. Relevant examples of oxidized steroidal derivatives bearing a heterocycle at the D-ring with 5α-reductase inhibitory activity.

Conclusion

The importance of the enzyme 5AR as a biological target in prostatic diseases, such as BPH and PCa, has been strongly demonstrated, as it was explained in the present chapter. In the last few decades, numerous compounds have been developed as 5ARIs, and for some of them, very promising results have been observed. Nevertheless, the only 5ARIs being marketed and approved for clinical use are the two 4-azasteroids finasteride and dutasteride, as well as the carboxysteroid epristeride. Currently, these steroids are mainly used for the management of BPH, principally the 4-azasteroids. However, these drugs also have become a popular treatment option in androgenic alopecia in men, since a good response was observed in several randomized control studies. In addition, 5ARIs may be useful in the chemoprevention of PCa, however, this clinical application remains a controversial topic and a subject of discussion. This is due to the fact that 5ARIs can increase the risk of high-grade PCa.

Consequently, the research for new 5ARIs more effective and safer remains an important topic for the scientific community.

In this context, high attention has been given to non-azasteroids in the last years. This chapter described the main achievements in this context with a special focus on oxidized steroids, such as progesterone, 16-dehydropregnenolone, and dehydroepiandrosterone derivatives, which presented interesting and promising results relative to their 5AR inhibitory activity. In consequence, these compounds may be considered a relevant class of steroidal derivatives potentially useful in prostatic diseases. Despite these interesting results, it is still crucial to perform further investigations to find more potent and selective inhibitors in order to improve the efficacy and safety of the approved drugs. Furthermore, more complete studies in the early phases of drug discovery and development should be performed using the three-dimensional structure of active sites of the correspondent target. However, concerning the 5AR enzymes, merely the X-ray crystal structure of 5AR type 2 was unveiled and published very recently (2020). This could compromise the investigation pipeline of the inhibitors described until this time, requiring a new look at these molecules. Thus, the release of the referred three-dimensional structure will certainly be an important step for the implementation of new research pathways and methods for 5ARIs design, and, consequently, new achievements in this context are expected in the next years.

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Chapter 2

NLRP3 Inflammasome-Assisted Pathogenesis in Chronic Obstructive Pulmonary Disorder

Sushweta Mahalanobish, Noyel Ghosh and Parames C. Sil*

Division of Molecular Medicine, Bose Institute, Calcutta, West Bengal, India

Abstract

Inflammation plays a central role in the development of chronic obstructive pulmonary disease (COPD). COPD, an irreversible and progressive pulmonary disorder, is characterized by chronic bronchitis, airflow obstruction, and emphysema. The onset of COPD is a result of aberrant immune responses induced by various genetic and environmental factors. The presence of interleukin-1 (IL-1)- like cytokines in the sputum and bronchoalveolar lavage fluid (BALF) of COPD patients indicates the involvement of inflammasomal complex in COPD development and progression. Among inflammasomal complex, NLR family pyrin domain containing 3 (NLRP3) inflammasome induces caspase-1-mediated proteolytic activation and the secretion of IL-1-like cytokines. The release of these cytokines is responsible for pyroptosis-mediated cell death. Recent studies suggest the role of NLRP3 inflammasome in airway inflammation, especially in COPD progression. Here, we have focused on the current progress made in the field of research describing the involvement of NLRP3 inflammasome in the pathogenesis of COPD.

Keywords: cigarette, inflammasome, interleukin, lung, NLRP3

* Corresponding Author's Email: parames@jcbose.ac.in/parames_95@yahoo.co.in.

In: Advances in Health and Disease. Volume 57
Editor: Lowell T. Duncan
ISBN: 979-8-88697-098-2

Abbreviations

ASC	Apoptosis-associated speck-like protein containing a CARD
BALF	Broncho alveolar lavage fluid
CARD	Caspase recruitment domain
COPD	Chronic obstructive pulmonary disease
CS	Cigarette smoking
CSE	Cigarette smoke extract
DEP	Diesel exhaust particles
EC	Epicatechin
ECs	Endothelial cells
eATP	Extracellular ATP
IPF	Idiopathic pulmonary fibrosis
LRR	Leucine-rich repeat domain
MMP	Matrix metalloproteinases
NLRP3	NLR family pyrin domain containing 3
NACHT	Nucleotide binding domain
PM	Particulate matter
PBMC	Peripheral blood mononuclear cells
PKR	Protein kinase R
PYD	Pyrin domain;
RNS	Reactive Nitrogen Species;
ROS	Reactive Oxygen Species;
SIRT1	Silent information regulator 1
TAK1	TGF-β-activated kinase 1
TAM	Tyrosine-based activation motif

1. Introduction

The immune system of our body provides protection against various microbial infections, removes cancerous cells, and evokes immune responses against cellular damage. In the innate immune system, germline-encoded signaling receptors (pattern recognition receptors) can interact with microbial molecules [pathogen-associated molecular patterns (PAMP)] or molecules released by damaged host cells [danger-associated molecular patterns (DAMP)]. These interactions trigger the onset of inflammatory responses against the microbial pathogen or damaged cell and restore cellular homeostasis. Interestingly, due

to the presence of commensal microbes in various organs (like lung, and gut), tissues develop a specific mechanism to selectively distinguish such local microflora from foreign pathogens and therefore maintain a balanced immune response.

During inhalation, various exogenous particulates and infectious agents come inside our respiratory tract. To protect the pulmonary system from these obnoxious particles, innate immunity plays a significant role. Specialized PRRs like Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs) in the lung initiate the inflammatory responses. The release of various cytokines like tumor necrosis factor (TNF) and chemokines (e.g., IL-8) from immune cells induces the recruitment of other immune cells (like neutrophils, lymphocytes, etc.). Cytokines like IL-1β, and IL-18 induce lung inflammation. The proteolytic activities of IL-1β and IL-18 are regulated by various innate immune receptors which can form a large multiprotein complex known as inflammasome (Martinon et al., 2002). Activation of inflammasome not only depends upon the pathogen but also metabolic dysregulation or tissue damage can stimulate inflammasome-mediated immune response. Emerging evidence indicates that although the activation of NLRP3 inflammasome is necessary against bacterial or viral infections, continuous activation of this signalling molecule is responsible for developing several respiratory diseases like asthma, idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD), etc.

COPD refers to obstructive lung disease which leads to airway obstruction, chronic bronchitis, airway remodelling along with decreased pulmonary activity. It has been considered the fourth leading cause of worldwide death. COPD is responsible for affecting 2.4% of the global population and it causes more than 5 million deaths annually (Burney et al. 2015). Although cessation of smoking or inhalation of corticosteroids can lessen the disease symptoms, proper treatment for a complete cure of this disease is still lacking. Although the main reason behind COPD is smoking (Barnes et al. 2015), several other factors like passive smoking, age, pollution, infections, and genetic factors like alpha-1 antitrypsin deficiency are also associated with COPD development (Brode et al., 2012). The release of toxic irritants from tobacco smoke or polluted air causes the activation of myeloid and epithelial cells and subsequent chronic inflammatory responses which are the key factors featuring COPD. Here, first, we have discussed our understanding of NLRP3 inflammasome activation and followed by the involvement of NLRP3 in COPD pathogenesis.

2. NLRP3 Inflammasome

NLR family pyrin domain-containing 3 (NLRP3) inflammasome refers to a multiprotein complex that leads to the activation and maturation of pro-inflammatory cytokines: IL-1β and IL-18 (Wen et al., 2011). It belongs to NOD-like receptor family and possesses three domains: C-terminal leucine-rich repeat domain (LRR), central nucleotide-binding domain (NACHT), and N-terminal pyrin domain (PYD). After sensing a PAMP or a DAMP by the LRR domain, oligomerization takes place in the NLRP3 NACHT domain, and then its PYD domain connects with the PYD domain of adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC). Consequently, the interaction of caspase recruitment domain (CARD) domain of ASC with CARD domain of pro-caspase-1 leads to the auto-activation and cleavage of caspase-1. The active caspase-1 causes the cleavage of pro-IL-1β and pro- IL-18 and stimulates the release of active IL-1β and IL-18 cytokines and resulting in inflammatory outbursts. The activated caspase-1 causes gasdermin D activation also and its release which leads to the onset of programmed cell death, and pyroptosis (Swanson et al., 2019).

3. Activation of the NLRP3 Inflammasome

Activation of NLRP3 inflammasome requires a diverse range of stimuli like exogenous activators and endogenous activators. Exogenous activators include various microbial components or environmental particulates (asbestos fibers, silica crystals, etc.). Whereas, the accumulation of various endogenous molecules under metabolic dysfunctions or altered tissue homeostasis leads to the activation of the NLRP3 inflammasome. Under normal physiological circumstances, uric acid exists in a harmless soluble form. But high levels of uric acid in circulation form monosodium urate crystals that lead to the activation of NLRP3 inflammasome and result in IL-1β driven chronic inflammation. Similarly, intracellular ATP levels are essential for normal cellular homeostasis. But the release of extracellular ATP (eATP) due to tissue damage activates the NLRP3 inflammasome by interacting with P2X purino receptor 7 (P2X7) (Pelegrin and Surprenant, 2007). As the formation of NLRP3 inflammasome requires a broad array of signals, these stimuli do not directly associate with the receptor. Instead, NLRP3 can bind to a common upstream activation signal. It has been found that various intracellular events

like alteration of redox potential, lysosomal stability, and ion concentrations are responsible for the facilitation of NLRP3 activation.

3.1. Intracellular Reactive Oxygen Species (ROS)

Generation of ROS is widely associated with NLRP3 activation. Initially, it was believed that intracellular ROS produced by NADPH oxidase system was responsible for NLRP3 activation. However, in the absence of NADPH oxidase, both human and mouse cells are capable of NLRP3 activation (Latz, 2010; van Bruggen et al., 2010). Moreover, generation of mitochondrial ROS is also associated with NLRP3 activation (Zhou et al., 2011; Wen et al., 2011; Nakahira et al., 2011). ROS is required during transcriptional priming step rather than post translational modifications of NLRP3 (Bauernfeind et al., 2011).

3.2. Lysosomal Destabilization

Immune cell mediated ingestion of fibrillar protein aggregates (e.g., amyloid -beta) or crystalline structures (e.g., cholesterol crystals) and its degradation inside the lysosomal vesicle causes the release of cathepsins like proteases (Hornung et al., 2008; Halle et al., 2008; Duewell et al., 2010). The released proteases can trigger the activation of NLRP3 inflammasome. However, how the lysosomal damage is responsible for NLRP3 activation is poorly understood and further research is needed in this field.

3.3. Ion Flux

Any alternation in cytosolic ion concentration (an increase of Ca^{+2} or decrease of K^{+}) has been found to lead to NLRP3 activation (Fernandes-Alnemri et al., 2007; Pétrilli et al., 2007; Perregaux and Gabel, 1994). In a study, Muñoz-Planillo et al. verified several upstream activators of NLRP3 and concluded that K^{+} efflux is a common signal required for NLRP3 activation (Muñoz-Planillo et al., 2013). Interestingly, it has been found that signals emerging from damaged mitochondria can be a common mediator connecting these intracellular events. Initially, NLRP3 after activation is associated with mitochondria via mitochondrial antiviral signalling protein (MAVS) (Misawa

et al., 2013; Subramanian et al., 2013). K^+ efflux from mitochondria triggers ~~the~~ mitochondrial ROS production (Malinska et al., 2010). Moreover, rupture of phagolysosome can induce Ca^{2+} mobilization which leads to ~~the~~ mitochondrial damage and further NLRP3 activation (Shimada et al., 2012). Ca^{2+} mediated damage of mitochondria results in the production of mitochondrial ROS and the release of various mitochondrial derivatives (oxidized mt. DNA) which can be sensed by NLRP3. It has been found that NLRP3 activators trigger the release of cardiolipin, an important constituent of mitochondrial membrane lipid. Release of cardiolipin and its interaction with the LRR domain of NLRP3 along with K^+ efflux trigger the activation of NLRP3 in macrophages (Iyer et al., 2013). Altogether, these findings indicate the role of mitochondrial cardiolipin and mitochondrial dysfunction in NLRP3 activation.

4. Regulation of NLRP3 Inflammasome

Regulation of NLRP3 occurs at various levels, i.e., from the transcriptional stage to post-transcriptional modification.

4.1. Transcriptional Level

In macrophages, NLRP3 itself does not have enough potential to induce inflammasome activation; therefore, NF-κB-dependent transcriptional priming is required to activate NLRP3. In immune cells, the sensitivity towards NLRP3 induction is also controlled via other immune signalling receptors (like TLRs, TNFR, etc.) (Franklin et al., 2014; Bauernfeind et al., 2009; Franchi et al., 2009). It has been found that to get a sufficient amount of NLRP3 protein for activation of the inflammasome, macrophages require prolong stimulation.

4.2. Post-Transcriptional Level

Various studies reported that NLRP3 expression is regulated negatively at the post-transcriptional level by miRNA in cells of myeloid origin (CD11bþ) (Ferhani et al., 2010; Mortaz et al., 2010). When miR-223 interacts with the

untranslated region of NLRP3, this reduces NLRP3 translation and expression. Interestingly, miR-223 expression is not regulated by a specific pro-inflammatory signal, but its expression differs among myeloid cells: neutrophils with higher expression, macrophages with a moderate expression where dendritic cells (DCs) exhibit lower expression. Therefore, this differential expression profile of mi-223 allows a cell-specific sensitivity and requires additional transcriptional regulation to inhibit the abnormal activation of the NLRP3 inflammasome.

4.3. Post-Translational Modifications

Before the activation of the inflammasome, NLRP3 undergoes several post-translational modifications. When macrophages are exposed to lipopolysaccharide treatment for 10 mins, NLRP3-mediated caspase-1 activation occurs even under protein synthesis inhibition. Upregulation of mitochondrial ROS after LPS treatment leads to the deubiquitination of NLRP3 and therefore, its activation. BRCC3 enzyme is responsible for deubiquitinase activity upon NLRP3 (Py et al., 2013; Juliana et al., 2012).

Besides deubiquitination, different types of modifications are also responsible for active NLRP3 formation. Although there is no report about direct NLRP3 phosphorylation, various experimental studies suggest that kinase activity can promote NLRP3 activation. Tyrosine kinase Syk causes NLRP3 activation during *Candida albicans* infection. Tyrosine-based activation motif (ITAM)-coupled receptors recognize *C. albicans* and stimulate Syk activation and resulting in NLRP3 inflammasome formation (Gross et al., 2009). Protein kinase R (PKR) can directly bind with NLRP1, NLRP3, and NLRC4, and thus, removal of the kinase domain of PKR hampers inflammasome-mediated caspase-1 activity (Lu et al., 2012). Additionally, TGF-β-activated kinase 1 (TAK1) has been reported also to be responsible for NLRP3 activation as the use of TAK1 inhibitor (5Z-7-oxozeaenol) inhibits NLRP3 inflammasome formation (Gong et al., 2010). Interestingly, intracellular Ca^{2+} mobilization during cell swelling causes TAK1 mediated NLRP3 activation (Compan et al., 2012). These findings indicate that Syk, PKR, and TAK1 enhance the chances of NLRP3 activation to form a functional inflammasome. Regulation of NLRP3 inflammasome in transcriptional, post-transcriptional, and post-translational levels has been depicted in **Figure 1**.

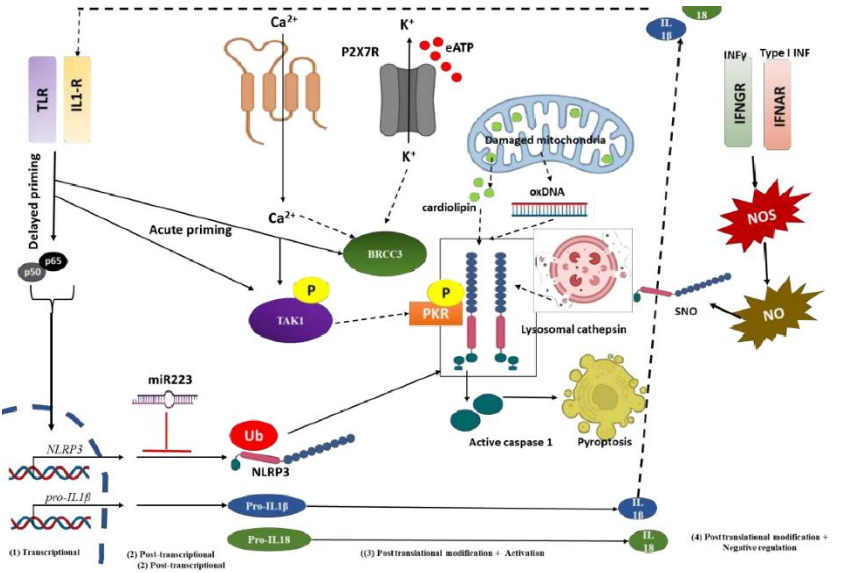

Figure 1. Activation and regulation of NLRP3 inflammasome formation in transcriptional, post- transcriptional, and post-translational levels.

5. COPD as an Inflammatory Disorder

COPD is a heterogeneous inflammatory disorder characterized by chronic bronchitis, obstruction in the airway, and emphysema. Symptoms include breathing shortness, increased sputum production, etc. in an irreversible manner (Decramer et al. 2012; Kosmider et al., 2011). In COPD patients, acute symptomatic exacerbations occur due to secondary pulmonary bacterial or viral infections which put on structural alterations in the airway. In COPD, increased numbers of neutrophils, macrophages, T lymphocytes, and B lymphocytes are found to be present in the airway lumen (Barnes, 2008; Cosio et al., 2009). These inflammatory responses involve mechanisms of both innate and adaptive immunity which are linked by dendritic cells (Van Pottelberge et al., 2009). In response to the notorious inhaled substances, different types of cells like alveolar macrophages, DCs, alveolar epithelial cells, etc. get activated and start to produce pro-inflammatory cytokines, and tissue-degrading enzymes leading to emphysema and chronic inflammation (Mortaz et al., 2010). Several inflammatory mediators released from inflammatory cells of the airways and lungs are elevated during COPD

pathogenesis (Barnes, 2004). Cigarette smoke leads to the alveolar epithelial cell injury and stimulates the transmigration of inflammatory cells into mucosa, submucosa, and glandular tissue to initiate the innate immune response. Moreover, the release of transforming growth factor β (TGF-β) also participates in tissue fibrosis (Barnes et al., 2003). A similar type of inflammation has been found in the lung of smokers, but in COPD this inflammation is amplified due to acute exacerbations by viral or bacterial infection. Though the molecular basis of exaggerated inflammation is not fully understood, various genetic and epigenetic factors have been reported to be responsible for this amplified inflammatory response. Inhalation of cigarette smoke and irritants causes the activation of alveolar macrophages and airway epithelial cells and subsequent release of various chemotactic molecules which attract monocytes, circulating neutrophils, and lymphocytes into the lungs (Lee et al., 2018). Even after quitting smoking, such inflammation does not resolve which suggests that this is a self-perpetuating mechanism (Kobayashi et al., 2013).

6. Animal Models of COPD

Various animal models have been utilized for experimental purposes for investigating the cellular and molecular pathway associated with COPD disease. Although different species like dogs, guinea pigs, monkeys, rodents, etc. have been used for COPD study, the use of the mouse model is more convenient due to the shared physiology between mice and humans.

6.1. Elastase Induction for COPD Model

Administration of specific kinds of proteases provokes inflammation and resulting tissue damage which is similar to COPD pathogenesis. Elastase administration in mice either oropharyngeal (single dose) or intratracheally (four times a day) can induce the infiltration of inflammatory cells and pulmonary emphysema within four weeks (Antunes and Rocco, 2011; Suki et al., 2017).

6.2. LPS Induction for COPD Model

LPS, an endotoxin, synthesized by gram-negative bacteria can induce the generation of various pro-inflammatory mediators and results ~~in~~ immune cells infiltration (like neutrophils, macrophages, etc.). Intranasal LPS administration in mice and rats can lead to the cellular damage and pulmonary dysfunction due to the accumulation of macrophages and neutrophils in bronchoalveolar lavage fluid (BALF) and increase the level of pro-inflammatory cytokines and chemokines (Wu et al., 2016; Lee et al., 2018). LPS along with elastase can cause severe lung inflammation, a situation like COPD pathogenesis (Kobayashi et al., 2013).

6.3. Ozone Induction for COPD Model

During urban air pollution, ozone is released due to the interaction of nitrogen oxide and organic compounds. Existing reports suggest that ozone has an adverse impact on the respiratory system. When ozone comes in contact with the cell membrane of airway macrophages or alveolar epithelial cells, it generates various bioactive mediators that trigger oxidative stress and alter innate immune responses (Mudway and Kelly, 2000). Ozone also affects the phagocytosis property of macrophages and induces the release of cytokine from alveolar epithelial cells and therefore, damages the lung tissue (Manzer et al., 2008). Exposure to ozone for a prolonged time span reduces the lung function and causes emphysema development in COPD patients (Halonen et al., 2008; Wang et al., 2019).

In mice, exposure to ozone for short time initiates hyper-reactivity and neutrophilic infiltration in the airway, where chronic ozone exposure causes the onset of bronchial inflammation. It has been found that ozone exposure (3ppm) for 3 hours reduced lung performance and airway hyper-responsiveness in C57BL/6 mice. Furthermore, increased infiltration of macrophages, neutrophils, and lymphocytes and upregulation of various pro-inflammatory cytokines like TNF-α, GM-CSF, and MIF have been found in BALF of ozone-treated mice.

6.4. CS exposure for COPD Model

The presence of free radicals in cigarette smoke increases the chance of oxidative stress and leads to the damage of lung tissue. Basically, two systems have been used to induce COPD in animal models by CS: a nose-only exposure system and whole-body exposure system, both of which are used widely to induce COPD pathogenesis. Although both systems can induce COPD onset, Wright et al. suggested that nose-only exposure to CS is responsible for many prominent structural alterations in the lung (Wright et al., 2008). Additionally, another important factor is the exposure time of CS. Study reports suggested that acute CS exposure (1 hour to 4 weeks) evokes lung tissue inflammation without emphysema or reduced lung function (Churg et al., 2003). Chronic CS exposure (up to six months) causes the onset of emphysema, airway remodeling, and reduced lung performance; though, long-term CS exposure limits the use of this model. So, for mechanistic studies of COPD, the CS model can be an appropriate experimental tool for future drug screening for therapeutic purposes. **Figure 2** represents various factors responsible for COPD development.

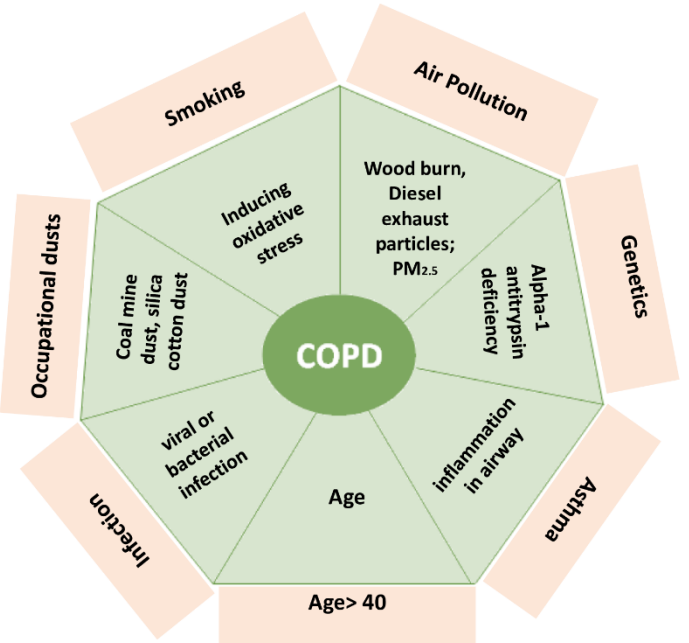

Figure 2. Role of various factors for COPD development.

7. NLRP3 Inflammasome in COPD

In the murine lungs, a mRNA-based study showed a higher expression level of NLRP3. Although alveolar macrophages and dendritic cells are the main sources of NLRP3 in the lung, alveolar epithelial cells also show the expression of NLRP3 and other inflammasome-associated genes expression (Hornung et al., 2008). Patients suffering from COPD show the overexpression of NLRP3 in the lung which is associated with airflow blockade (Duewell et al., 2010). In an *in vitro* study, it was found that CS extract exposure in alveolar epithelial cells (A549) induces the activation of NLRP3 (Finger et al. 2012). Wang et al. suggested the association between NLRP3 inflammasome and the development of acute exacerbation of COPD (AECOPD) (Wang et al., 2018). The study revealed that patients with AECOPD showed increased levels of NLRP3 inflammasome in peripheral blood mononuclear cells (PBMCs) and bronchial tissues compare to smokers without lung diseases. Additionally, molecules associated with NLRP3 inflammasome increase the disease susceptibility and pathogen load inside the lung tissue. Therefore, NLRP3 inflammasomal activity on both systemic as well as the local airway is associated with COPD exacerbation.

One of the principal cytokines associated with NLRP3 inflammasome activation is IL-1β which is upregulated in several COPD studies. Increased level of IL-1β in the sputum and serum of COPD patients is associated with disease severity (Murakami et al., 2012; Lee et al., 2012). Furthermore, Kuschner et al. revealed that smoking increased IL-1β concentration inside the lung of COPD patients (Kuschner et al., 1996). These findings indicate the association of inflammasome in COPD development. In IL-1β transgenic mice whose alveolar epithelial cell expresses human IL-1β, show inflammation inside the lung similar to COPD patients. IL-1β stimulates the release of various chemokines like MIP-2, KC, and matrix metalloproteases like MMP-9, and MMP-12. All of these factors increase the transmigrtaion of macrophages, neutrophils, and lymphocytes in the lung and therefore promote pulmonary inflammation. In a study, Doz et al. reported that adverse effects of tobacco are suppressed in IL-1R null mice (Doz et al., 2008). Therefore, IL-1β and IL-1R signaling play a significant role in lung inflammation development. However, Botelho et al. showed that IL-1α, but not IL-1β, is mainly involved in COPD pathogenesis (Botelho et al., 2011). In another study, Pauwels et al. showed that both IL-1α and IL-1β neutralization reduced the severity of smoke-induced lung inflammation (Pauwels et al., 2011).

Therefore, further investigation is required to find out the relative role of IL-1α and IL-1β in COPD pathogenesis.

IL-18 is another important cytokine that can regulate the NLRP3 inflammasome. An increased amount of IL-18 in the plasma and lung tissue of COPD individuals indicates its role in COPD pathogenesis (Kang et al., 2007; Petersen et al., 2007). Similarly, IL-18 transgenic mice exhibit COPD-like symptoms, wherein CS-induced COPD model, IL-18R deficit reduces disease severity (Hoshino et al., 2007; Kang et al., 2007). Therefore, the above data indicate the involvement of IL-18 in COPD lung inflammation.

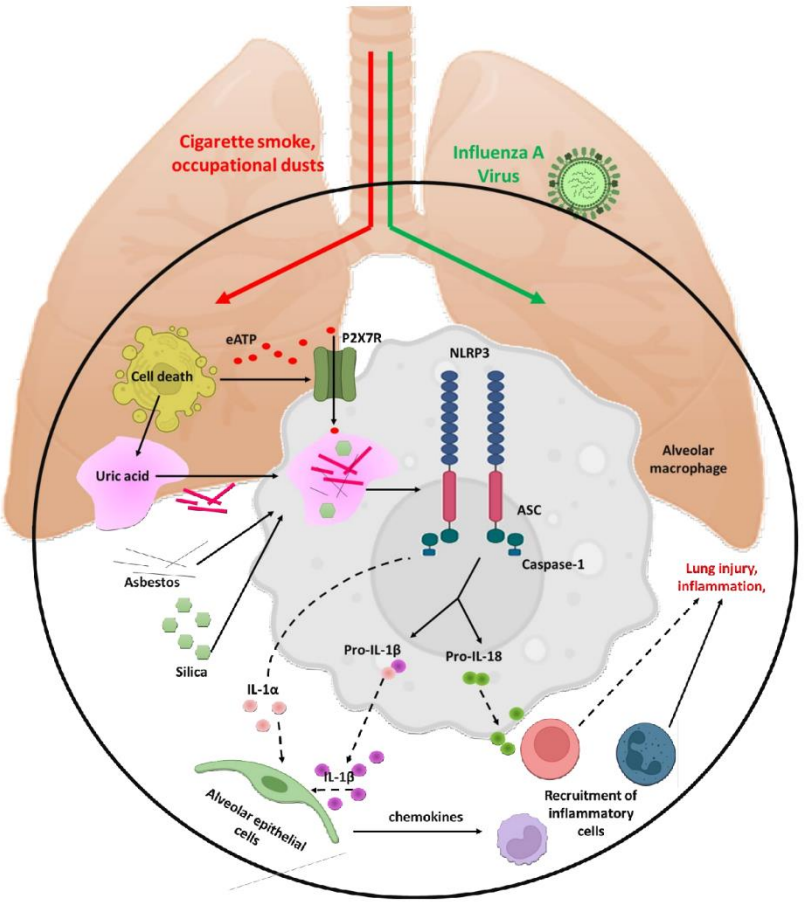

Figure 3. Role of NLRP3 inflammasome in COPD pathogenesis.

ASC mediates NLRP3 inflammasome assembly involves the interaction between NLRP3 and pro-caspase-1. Therefore, inflammasome activation causes ASC to form a disc-shaped structure- "ASC speck", which is an indicator of NLRP3 inflammasome formation. The presence of ASC speck in the BALF of COPD mice amplifies the inflammatory response (Franklin et al., 2014). The level of caspase-1 is increased in the lung of smokers whereas caspase 1 inhibition reduced the inflammation in the CS-induced emphysema model (Eltom et al., 2011; Churg et al., 2009). Therefore, caspase-1 activation is associated with lung inflammation. **Figure 3** represents the role of NLRP3 in lung inflammation.

8. Factors Affecting COPD by NLRP3 Activation

Various risk factors like CS, pollution, age, occupational dust, viral and bacterial infection, genetic deficiency, etc. contribute significantly to COPD development. Among these factors, some cause direct activation of NLRP3 inflammasome and therefore are associated with COPD exacerbation.

8.1. Cigarette Smoke

CS induces the priming as well as activation of NLRP3 inflammasome. Exposure to CS causes HMGB1 release, which in turn triggers TLR signaling and therefore elicits the priming signal of inflammasome activation (Ferhani et al., 2010). CS promotes the extracellular ATP (eATP) production in COPD lungs. The resulting eATP and its receptor P2X7 are associated with lung inflammation (Mortaz et al., 2010). Genetic ablation of P2X7 mitigates CS-mediated neutrophilia in mice model (Eltom et al., 2011). As eATP is associated with NLRP3 activation, Cs-mediated eATP release might be a serious factor for COPD development.

The presence of reactive nitrogen species (RNS) and reactive oxygen species (ROS) in CS also induces oxidative stress which leads to damage to lung tissue (Durham and Adcock, 2015). ROS acts as an upstream regulator of NLRP3 inflammasome (Harijith et al., 2014). Therefore, CS-mediated ROS generation triggers NLRP3 activation and evokes lung inflammation in COPD patients.

Moreover, in COPD patients, the level of uric acid is elevated in both plasma and lungs tissue (Wattanachayakul et al., 2020). Therefore, uric acid acts as a risk factor for COPD in smokers. Increased accumulation of uric acid leads to uric acid crystal formation which triggers the activation of NLRP3 inflammasome (Martinon et al., 2006; Braga et al., 2017). These results indicate that uric acid crystal-induced NLRP3 inflammasome activation causes COPD development (Wanderer, 2008).

8.2. Air Pollution

Air pollution is another important risk factor for COPD development. Air pollutants like particulate matter, gases, and other biological molecules increase the chance of COPD development by NLRP3 inflammasome regulation. Diesel exhaust particles (DEP) are one of the lead components of air pollution. It has been found that DEP induces the NLRP3 inflammasome activation in the elastase-induced emphysema model which can be blocked by using N-acetylcysteine, a potent antioxidant (Uh et al., 2017). Recent epidemiological studies suggested that another air pollutant component-particulate matter 2.5 (PM2.5) can increase the chance of morbidity and mortality in COPD (Zhao et al., 2019) Zheng et al. showed that PM2.5 mediated the release of Cathepsin B, and increased ROS generation and potassium efflux cause activation of NLRP3 inflammasome (Zheng et al., 2018). Furthermore, various other air pollutants like biomass fuel, and ground-level ozone can induce NLRP3 inflammasome activation and therefore increase the chance of COPD development ((Tian et al., 2021; Li et al., 2016). Interestingly sulfur dioxide exposure causes the development of COPD-like symptoms, but it does not induce NLRP3 inflammasome activation (Wagner et al., 2006). Yang et al. also reported that sulfur dioxide inhibited NLRP3 inflammasome activation (Yang et al., 2018). Therefore, further investigations are required to determine the role of sulfur dioxide-induced COPD and the NLRP3 inflammasome axis.

8.3. Genetic Factors

Besides CS, various genetic factors are associated with COPD progression (Sandford et al., 1997). The role of α1-antitrypsin and vitamin D-binding protein in COPD development is described elsewhere (Hall et al., 2019). Some

of these genes can control the activity of the NLRP3 inflammasome. It has been reported that in murine astrocytes, α1- antitrypsin causes inhibition of NLRP3 inflammasome activity (Ebrahimi et al., 2018). To find out similar observations in alveolar macrophages, further studies need to be carried out. Moreover, the analysis of other genetic factors in the aspect of NLRP3 inflammasome may help us for understanding the complex regulatory network in COPD pathogenesis.

8.4. Viral and Bacterial Inflammation

In COPD patients, the lungs are more susceptible to a bacterial or viral infection which can exaggerate the inflammation (Sethi, 2010). For instance, Mycobacterium tuberculosis infection in COPD lung can promote COPD development by stimulating the release of matrix metalloproteinases (MMPs) (Jain, 2017). MMPs along with other factors induce neutrophils activation and lead to structural alteration in COPD-affected lungs. Moreover, M. tuberculosis induces NLRP3 inflammasome activation and increases IL-1β and IL-18 generation from alveolar macrophages that in turn amplify the inflammatory response in COPD lung (Dorhoi et al., 2013).

In *in vitro* COPD model, LPS treatment stimulates the release of pro-inflammatory cytokines, IL-8, and MCP-1 from A549 cells (alveolar epithelial cell line) (Nachmias et al., 2019). Combined treatment of CS and LPS can amplify NLRP3 inflammasome expression along with increased IL-1β secretion from A549 cells. Therefore, these results suggest that the release of LPS due to bacterial infection can exacerbate inflammation in COPD individuals.

Viral infection is also responsible for COPD aggravation. Studies indicate that persistent viral infections like a respiratory syncytial virus, human rhinovirus, and influenza can induce COPD exacerbation by the NLRP3 inflammasomal pathway (Segovia et al., 2012; Kuriakose et al., 2016; Liu et al., 2019). Therefore, these shreds of evidence suggest the involvement of both bacterial and viral infection in COPD exacerbation by activating the NLRP3 inflammasome.

8.5. Occupational Exposure

Exposure to various occupational dust produced through mining, forestry, and agriculture purpose can enhance the chance of COPD development. The

release of silica dust during crystalline silica processing acts as an important respiratory toxic material. It causes the activation of NLRP3 inflammasome and also elevates plasma IL-18 and IL-1Ra level (Hedbrant et al., 2020). Other occupational dust like wooden particles is also associated with COPD pathogenesis and reduces lung functional capability ((Shamssain, 1992; Mandryk et al., 1999). However, whether this dust has any role in NLRP3 inflammasome activation, requires vigorous investigation.

8.6. Genetic Polymorphism of NLRP3 and Increased COPD Risk

Single nucleotide polymorphism (SNP) refers single nucleotide difference in genomic DNA sequence among the members of the same species. SNPs can alter the gene expression and are responsible for disease susceptibility and genome evolution. Many SNPs have been identified in COPD where these SNPs are directly associated with inflammatory pathway alterations and protease-antiprotease balance (Kumar et al., 2013). Research showed the occurrence of SNPs in inflammasome-related genes which are responsible for COPD development. Till now, no SNP in NLRP3, PYCARD, and CASP1 is associated with COPD severity although SNPs of IL-1β or IL-18 are associated with increased or decreased risk of COPD development.

The genetic polymorphism of the IL-1β gene and its association with COPD development is controversial. For instance, a study conducted on the Turkish population does not find any correlation between COPD and IL1β −51, +3954 gene polymorphisms. In line with this outcome, Ishii et al. also reported no correlation between IL-1β and IL1RN polymorphism and COPD severity (Ishii et al., 2000). Xie et al. reported that polymorphisms at the −511, −31 locus but not the +3954 locus of the IL1β gene enhance the risk of COPD development among East Asians (Xie et al., 2014). In another study, it has been found that polymorphism in IL1B promoter (−511C/T) suppresses the chance of COPD development in the Asian population (Wang et al., 2015). The presence of IL1RN*2/IL1RN* in males confers protection whereas this same genotype in females seems to be associated with COPD development (Shukla et al., 2012). In the Korean population, IL1β polymorphisms −511C–>T and −31T–>C increase the chance of COPD (Ji et al., 2012). However, individual with at least one copy of the IL1RN*2 allele has a minor chance of COPD development. A polymorphism in IL-18 was associated with COPD susceptibility (Wang et al. 2013). SNP in IL18 promoter (−607 C/A) causes

the advancement of the disease where SNP −137 G/C does not exhibit diverse outcomes in COPD patients over healthy individuals.

9. NLRP3 as a Therapeutic Target for COPD Prevention

9.1. Role of Synthetic Inhibitors

Evidence-based studies showed that IL-1β transgenic mice develop COPD-like features where the deficiency of IL-18 or IL-1R reduces the inflammation in lung tissue in the COPD model (Lappalainen et al., 2005). The use of anakinra (IL-1 receptor antagonist) can effectively reduce lung inflammation due to LPS stimulation (Hernandez et al., 2015). All of this evidence indicates the involvement of IL-1β, IL-18, and IL-1R signaling in COPD pathogenesis. However, the use of anti-human IL-1β monoclonal antibody (Canakinuma), or human IgG1 monoclonal antibody targeting IL-18 or human IgG2 monoclonal antibody against IL-1R in clinical trials do not represent any significant results in COPD patients (Kumar et al., 2013). These results indicate that inhibiting the function of single inflammasome-associated cytokine or IL-1R signalling is not efficient to provide protection against COPD. Use of IL-1β, IL-18, and IL-1R blockers altogether may be a possible therapeutic strategy to inhibit COPD progression in the future. It has been found that NLRP3 or caspase-1 deficiency reduces COPD exacerbation in the mice models. MCC950, a potent NLRP3 inhibitor can suppress LPS-induced lung inflammation (Wang et al., 2021). Therefore, directly inhibiting NLRP3 or caspase-1 activity in clinical trials may be a probable treatment option for COPD.

Additionally, these clinical trials indicate that upstream regulators of NLRP3 inflammasome may also participate in COPD development. Therefore, NLRP3 activation may be secondary in this mechanism. The use of combination therapy to block multiple signaling pathways associated with NLRP3 activation is required for COPD treatment. In the virus-induced COPD model, combined IL-1α and IL-1β neutralization can mitigate airway inflammation in comparison to individual cytokine blockage. Another study reported that inhibition of IL-1β or IL-17A can diminish influenza-induced COPD pathogenesis (Sichelstiel et al., 2014). Due to the redundancy of two inflammatory cascades, the combined neutralization of two cytokines efficiently suppresses the inflammation and improves lung performance. As

COPD is a combination of both genetic and environmental factors, therefore therapeutic responses vary from patient to patient. Taking this thing into consideration, in the future, various combination therapies should be applied clinically to get better outcomes against this disease.

9.2. Role of Natural Inhibitors

Various natural compounds can effectively downregulate the NLRP3 inflammosomal activity in COPD. Melatonin, a hormone synthesized in pineal gland, is associated with circadian rhythm and seasonal rhythmicity. It has been found that melatonin provided the protection against *in vitro* and *in vivo* models of COPD. Melatonin exhibits its anti-inflammatory potential by suppressing NLRP3 inflammasome formation. Melatonin is capable to downregulate the TXNIP activity and upregulate the level of intracellular antioxidant thioredoxin-1 and resulting suppression of TXNIP/NLRP3 pathway. It has been found to stimulate mitophagy by restoring PINK-1, Parkin, LC3B-II expression and thereby, suppresses mitochondrial dysfunction mediated NLRP3 inflammasome activation formation (Mahalanobish et al. 2020). Melatonin efficiently suppressed the production of ROS and NLRP3 inflammasome-mediated pyroptosis in CSE-treated endothelial cells (ECs). Melatonin administration can mitigate the oxidative stress and NLRP3 inflammasome in the carotid arteries of smoking rats and therefore, can suppress the onset of pyroptosis (Wang et al. 2019). Silent information regulator 1 (SIRT1) also plays a significant role in NLRP3 activity. Inhibition of SIRT1 can upregulate the expression of NLRP3. In COPD rats, the use of SIRT1 inhibitor EX527 along with melatonin can abolish the protective effect of melatonin. Administration of melatonin in COPD rats can restore the pulmonary function and the altered lung physiology and also reduce the inflammatory cells number. It suppressed the production of NLRP3, cleaved caspase-1 and ASC in the lung tissues of COPD. Moreover, melatonin upregulated SIRT1 level in lung tissues while SIRT1 inhibition diminished the protective nature of melatonin against COPD. These results indicated that melatonin can reduce airway inflammation in SIRT1 dependent manner and suppress NLRP3 inflammasome and IL-1β in COPD rats (Peng et al. 2018). (-)-Epicatechin (EC), a polyphenol, can alleviate CS-induced COPD by suppressing the intracellular ROS generation and restoring the viability of human bronchial epithelial cells after cigarette smoke extract (CSE) intoxication. EC inhibited NLRP3 inflammasome activation and

blocked the pyroptosis by reducing the lactate dehydrogenase release and caspase-1 activity. EC also upregulated tripartite motif-containing protein 25 expression and stimulated Nrf2 nuclear localization event (Tian et al. 2021).

Conclusion

PRRs act as an important component of lung immunity and are essential for inflammatory responses against invading pathogens in order to help in restoring tissue homeostasis. However, when an innate defense mechanism is activated in an uncontrolled way, it can lead to the onset of drastic adverse consequences inside the host body. Although several types of PRRs are responsible to trigger the inflammation inside the lung, studies indicate that the NLRP3 inflammasome has a central role in inflammatory outbursts. Acute NLRP3 activation is required to defend against viral and bacterial lung infections. However, NLRP3 activation for a prolonged time causes the onset of chronic and deleterious inflammatory impacts on the lung. The level of NLRP3 inflammasome is upregulated in the lung of both patients and animal models of COPD. SNPs in IL-1β and IL-18 are associated with COPD development. IL-1β or IL-18 can induce the inflammatory outbursts in COPD patients where blocking the activities of such cytokines can effectively alleviate COPD pathogenesis. Although the exact role of NLRP3 inflammasome in COPD development is not well understood, possible involvement of NLRP3 inflammasome in COPD progression can be marked as follows: cigarette smoke or particulate matter induces NLRP3 inflammasome activation in alveolar macrophages or dendritic cells that triggers the release of pro-inflammatory cytokines such as IL-1β and IL-18 to promote inflammation. Although clinical trials can block IL-1β, IL-18, or IL-1R activity to prevent the onset of pathophysiological consequences in COPD patients, various alternative strategies to target NLRP3, caspase-1, or blockage of multiple molecules associated with NLRP3 inflammasome to suppress the COPD progression is of utmost importance.

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Chapter 3

Structural Insights of Cobalamin and Cobinamide Uptake by ABC Importer of *Vibrio* Species

Arunima Bhattacharya[1,†], Samriddhi Bhattacharya[1,†], Shubhangi Agarwal[1,2] and Jhimli Dasgupta[1,*]

[1]Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, West Bengal, India
[2]Weill Cornell Medicine, Department of Anesthesiology, New York, USA

Abstract

Vitamin B12 or cobalamin, the most complex B-type vitamin, has rare organometallic bonds in its biologically active forms. These bonds are used by several enzymes involved in different rearrangement and transmethylation reactions in central metabolic pathways of bacteria and archaea. However, of all the sequenced bacterial genomes which depend on Vitamin B12 for growth, only 50% encode the ability to synthesize an active form *de novo*, a process that requires over 30 gene products, making it a highly energy-consuming phenomenon. Hence, B12 is salvaged from the environment by bacteria that are incapable of synthesizing the same. Type II ABC importers are present only in prokaryotes and are responsible for the uptake of metal chelates including heme and vitamin B12; the ABC importer in*E. coli*, for example, can also bind and uptake cobinamide, a precursor of cobalamin. We solved the crystal structure of the periplasmic B12 binding protein of the cholera-causing pathogenic bacteria, *Vibrio cholerae*, *Vc*BtuF in cyanocobalamin

[†] These authors contributed equally to this chapter.
[*] Corresponding Author's Email: jhimli@sxccal.edu.

In: Advances in Health and Disease. Volume 57
Editor: Lowell T. Duncan
ISBN: 979-8-88697-098-2

bound state and established binding of cobinamide and heme with *Vc*BtuF as well. However, the mechanism of B12 and precursor uptake by ABC importer is still elusive in *V. cholerae*, and other biofilm-forming and/or pathogenic *Vibrio* species. Furthermore, sequence analysis of genome databases has revealed that a majority of *Vibrio* species, including *Vibrio cholerae*, lack the genes for *de novo* vitamin B12 synthesis while containing the genes required for salvaging cobalamin or cobinamide. This chapter indicates plausible mechanistic insights of binding of vitamin B12 and its precursor cobinamide by *Vc*BtuF of *Vibrio cholerae and similar species from structural and* bioinformatics point of view.

Keywords: vitamin B12 uptake, cobinamide, ABC transporters, *vibrio cholerae*, VcBtuCD-F

1. Introduction

Vitamin B12, also known as cyanocobalamin, is a cyclic pyrroline/ tetrapyrrolidine that contains cobalt (Escalante-Semerena and Warren 2008). It is structurally complex and contains rare organometallic bonds formed by different ligands that are used in different rearrangement and transmethylation reactions in the central metabolic pathways of bacteria and archaea (Agarwal et al. 2019, Santos et al. 2018). Cobalamin contains a corrin ring in which a cobalt ion is bonded to the nitrogen atoms of the pyrroles through equatorial coordination bonds. It also contains an upper axial ligand at the β-axial position and a lower axial ligand at the α-axial position which is coordinated with the cobalt ion through a pH-dependent coordination bond. The upper axial ligand varies among different cobalamin derivatives and can include adenosine (adenosylcobalamin, AdoCbl), methyl group (Methylcobalamin, MetCbl), hydroxy group (Hydroxycobalamin, OH-Cbl), and cyano group (Cyanocobalamin, CN-Cbl) (Gruber, Puffer, and Kraeutler 2011; Roth et al. 1993; Santos et al. 2018). These ligands are responsible for the formation of the rare organometallic bonds, providing unique catalytic properties to the enzymes that use cobalamin as a cofactor (Gruber, Puffer, and Kraeutler 2011; Santos et al. 2018).MetCbl and AdoCbl are the two most biologically active forms of cobalamin. The lower axial ligand is 5,6-dimethylbenzimidazole (DMB). DMB is also tethered to the corrin ring via an α-N-glycosidic bond and a phosphodiester bond (Figure 1). In fact, cobalamin is one of the only three coenzymes known to have a phosphodiester bond, the other two being

coenzyme F_{420} and methanopterin (Gruber, Puffer, and Kraeutler 2011; Roth et al. 1996; Santos et al. 2018; Escalante-Semerena and Warren 2008).

Vitamin B12 is an essential nutrient for animals. Humans cannot synthesize MetCbl and AdoCbl. Instead, they depend on the dietary uptake of CNCbl and OH-Cbl which are in turn converted to the biologically active forms, or the coenzyme forms of cobalamin. It is thought that prokaryotes and eukaryotes have similar mechanisms for this conversion (Leal et al. 2004). Severe, and often fatal health problems can arise due to a cobalamin-deficient diet. For example, megaloblastic or pernicious anemia is caused due to impaired absorption of cobalamin. The inability to convert OH-Cbl to AdoCbl causes an inborn metabolic disorder called methylmalonic aciduria, leading to severe mental retardation and high infant mortality (Escalante-Semerena and Warren 2008).

So far, vitamin B12 biosynthesis has only been found in prokaryotes, including anaerobes, facultative anaerobes, aerobes, photosynthetic bacteria, and fermentative bacteria of the archaeal and bacterial domains (Escalante-Semerena and Warren 2008). Synthesis of vitamin B12 can occur both *de novo* as well as through a salvage pathway in microbes. *De novo* synthesis in turn can occur through aerobic or anaerobic pathways, each of which requires over 30 gene products, making them highly energy-consuming processes (Gruber, Puffer, and Kraeutler 2011.; Roth et al. 1996; Santos et al. 2018).Hence, a lot of the bacteria that require vitamin B12 for growth are dependent on its uptake from the environment for their survival. (Zhang et al. 2009; Escalante-Semerena 2007; Agarwal et al. 2019). Furthermore, even certain bacteria that possess the genes required for *de novo* synthesis also have the genes required for the salvage pathway (Fang, Kang, and Zhang 2017). The uptake of vitamin B12 occurs through type II ABC importers, which are only found in prokaryotes. These ABC importers are responsible for the uptake of metal chelates. We have solved the crystal structure of the periplasmic B12 binding protein of the cholera-causing pathogenic bacteria, *Vibrio cholerae*, *Vc*BtuF in cyanocobalamin bound state and established binding of cobinamide and heme with *Vc*BtuF as well. While BtuCD-F system is well studied in *E. coli* (Korkhov, Mireku, and Locher 2012), the mechanism of cobalamin uptake by *Vc*BtuCD-F remains elusive, not only in *V. cholerae* but also in other biofilm-forming and/or pathogenic*Vibrio*species. Our observations suggested that a majority of the *Vibrio* species (including *V. cholerae*) contain the genes required for the salvage pathway while lacking those required for *de novo* vitamin B12 synthesis (Agarwal et al. 2019).

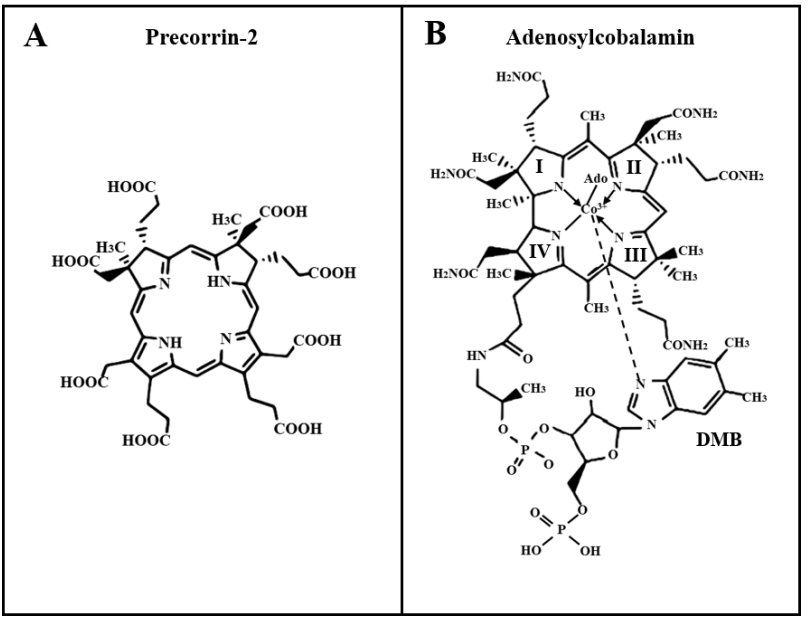

Figure 1. (A) Structure of precorrin-2. (B) Structure of adenosylcobalamin. The pyrrolic rings of the corrin ring are marked by roman numerals.Adapted from Fang, Kang, and Zhang, 2017.

The following section in this chapter discusses the details of both the *de novo* and the salvage pathway for cobalamin synthesis, as well as the interconnection between them. It also details different prokaryotic ABC transporters responsible for the uptake of vitamin B12 and its precursor cobinamide. Lastly, this chapter addresses the probable mechanism of uptake of vitamin B12 and cobinamide by *Vc*BtuF of*Vibrio cholerae and similar species from a structural and* bioinformatics point of view.

2. Synthesis of Vitamin B12 in Microbes

As mentioned earlier, vitamin B12 can be synthesized, both *de novo* as well as through a salvage pathway in microbes (Fang, Kang, and Zhang 2017). In this section, we discuss the *de novo* synthesis pathway of cobalamin (AdoCbl), the salvage pathway for cobalamin/cobinamide uptake, as well as the interconnection between the two.

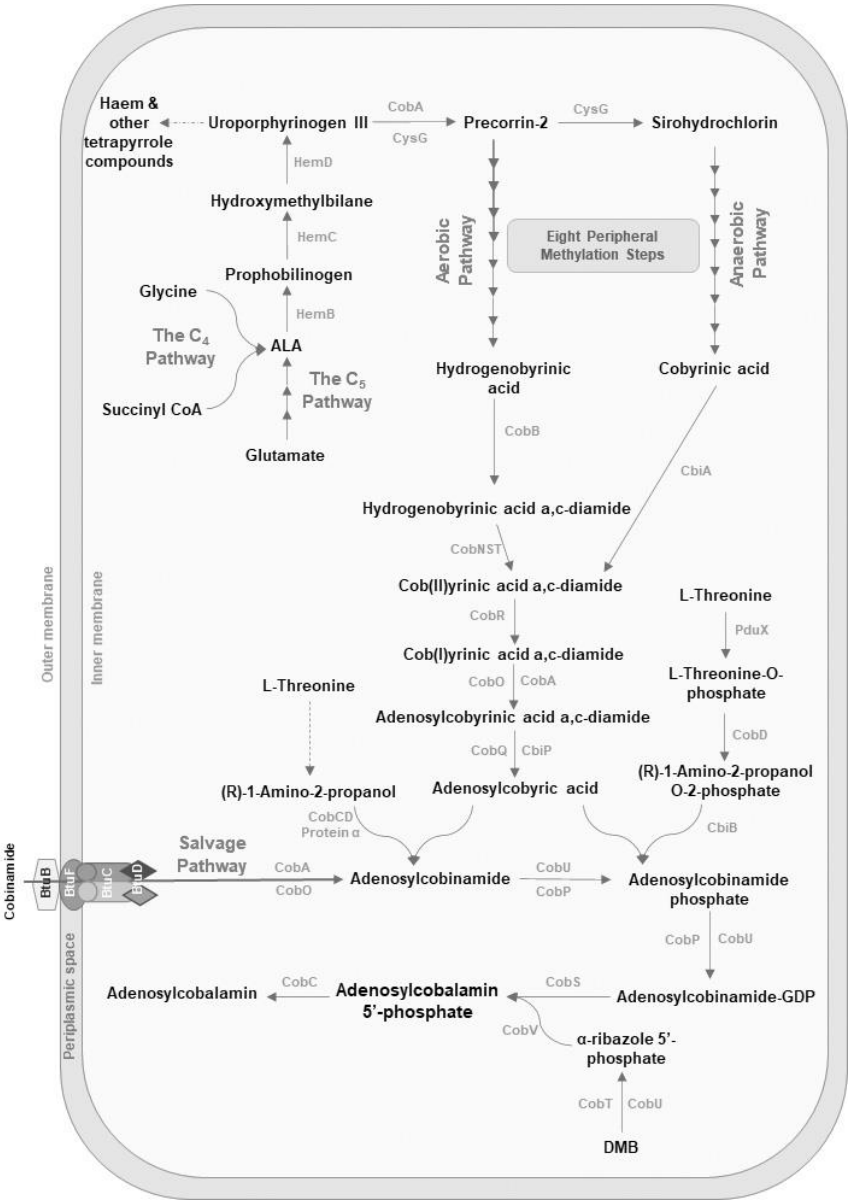

Figure 2. Overall schematic of the aerobic and anaerobic pathways of de novo synthesis of adenosylcobalamin as well as the salvage pathway for the synthesis of the same. Enzymes are represented in blue and the substrates and products are represented in black. Adapted and modified from Fang, Kang, and Zhang 2017.

2.1. *De Novo* Pathway

De novo biosynthesis of vitamin B12 in microbes occurs primarily through two alternate pathways; the aerobic and the anaerobic pathways in bacteria and archaea. The difference lies in the timing of the insertion of cobalt and the requirement of molecular oxygen (Fang, Kang, and Zhang 2017). The genes for corrin synthesis through the aerobic pathway are known as *cob* genes while those for the anaerobic pathway are called *cbi* genes (E. Raux, Schubert, and Warren 2000; Roth et al. 1993; Agarwal et al. 2019). In the aerobic pathway, cobalt chelation is catalyzed by the CobNST complex which converts hydrogenobyrinic a, c-diamide cob(II)yrinic acid a,c-diamide. Additionally, oxygen is required to promote ring contraction which occurs during the eight peripheral methylation steps that precorrin-2 undergoes in both the aerobic and anaerobic pathways (Figure 2). In this step, the corrin ring of vitamin B12 loses one of the bridging carbons joining rings I and IV (Figure 1) (Escalante-Semerena & Warren 2008). On the other hand, cobalt chelation occurs through precorrin-2, catalyzed by CbiK in the anaerobic pathway, and oxygen is not required for the ring contraction step (Fang, Kang, and Zhang 2017). The aerobic pathway is best studied in *Pseudomonas denitrificans* while the anaerobic pathway has been best studied in *Salmonella typhimurium*, *Bacillus megaterium*, and *Propionibacterium shermanii* (Moore and Warren 2012; Fang, Kang, and Zhang 2017).

Tetrapyrrole compounds like bacteriochlorophyll, heme, and vitamin B12 are derived from δ-aminolevulinate (ALA), which thus, is the first committed precursor of the tetrapyrrole synthesis pathway. ALA can be synthesized by either the C_4 or C_5 pathway (Fang, Kang, and Zhang 2017). In the C_5 pathway, glutamate undergoes three enzymatic reactions to form ALA. On the other hand, ALA is synthesized from glycine and succinyl-CoA in a reaction catalyzed by the enzyme ALA synthase in the C_4 pathway (Avissar, Ormerod, and Beale 1989; Fang, Kang, and Zhang 2017). This is followed by the formation of monopyrrole porphobilinogen by condensation of two ALA molecules, and this reaction is catalyzed by porphobilinogen synthase. Then, four porphobilinogen molecules are polymerized and cyclized to form uroporphyrinogen III, the reactions being catalyzed by the enzymes porphobilinogen deaminase and uroporphyrinogen III synthase, respectively.

This is followed by methylation of uroporphyrinogen III at C-2 and C-7 to form precorrin-2, siroheme, and coenzyme F_{430}. Precorrin-2 is a common precursor of cobalamin (Martens et al. 2002; Zappa, Li, and Bauer 2010; Fang, Kang, and Zhang 2017). This reaction is catalyzed by different enzymes in different organisms. For example, CysG (a fusion enzyme) catalyzes the formation of siroheme in *S. typhimurium* and *E. coli*. Its C-terminus has uroporphyrinogen III methyltransferase activity while its N-terminus has dehydrogenase/ferrochelatase activity. Thus, CysG also catalyzes the step for cobalamin synthesis in these organisms. This methylation step is catalyzed by the enzyme CobA in *P. denitrificans* and MET1p in *S. cerevisiae* genes (Evelyne Raux et al. 1999; Fang, Kang, and Zhang 2017).

The two pathways (aerobic and anaerobic) diverge after the synthesis of precorrin-2 (Figure 2). Eight peripheral methylation steps occur within identical spatial and temporal orders in both pathways. High degrees of sequence similarities are observed in many of the methyltransferases involved in this reaction (Escalante-Semerena and Warren 2008; Fang, Kang, and Zhang 2017). The pathways again converge at cob(II)yrinic acid a,c-diamide, where the former is converted to cob(I)yrinic acid a,c-diamide by the enzyme CobR, a corrin reductase. This is followed by the adenosylation of cob(I)yrinic acid a,c-diamide to form adenosyl cobyrinic acid a,c-diamide by cob(I)yrinic acid a,c-diamide adenosyltransferase. The carboxyl groups of adenosyl cobyrinic acid a,c-diamide at positions b, d, e, and g undergo stepwise amidations to form adenosyl cobyric acid. At this point, the two pathways again diverge to yield different products. This is followed by the attachment of either (R)-1-amino-2-propanol or (R)-1-amino-2-propanol phosphate the f position of the carboxyl group of adenosyl cobyric acid. For this, two different methods have evolved in the aerobic and anaerobic pathways (Figure 2). The linker between the corrinoid ring and the lower axial ligand is phosphorylated by an L-threonine kinase before attachment of the corrinoid ring in the anaerobic pathway (Fan and Bobik 2008; Fang, Kang, and Zhang 2017). This is followed by the decarboxylation of L-threonine O-3-phosphate to form (R)-1-amino-2-propanol O-2-phosphate. In *S. typhimurium* LT2, the enzyme which catalyzes this reaction is CobD (Brushaber, O'Toole, and Escalante-Semerena 1998; Fang, Kang, and Zhang 2017). In *P. denitrificans* (aerobic pathway), however, (R)-1-amino-2-propanol is most likely directly attached to the corrinoid ring via proteins α and β, although proof of this has not yet been published. Protein β is a complex of CobC and CobD, but protein α has not been identified as of now. The product is then phosphorylated by CobP, a bifunctional enzyme, possessing both ATP:AdoCbi(adenosylcobinamide)

kinase as well as GTP:AdoCbi-P guanylyltransferase activity (Cohen2016; Fang, Kang, and Zhang 2017). This is followed by the transfer of lower axial ligands onto AdoCbi-GDP to produce AdoCbl. There are two views as to how this is achieved. The first view is that cobalamin synthase catalyzes the addition of α-ribazole (a nucleotide loop that contains dimethylbenzimidazole attached to ribose), making it the last step in the reaction. The second view is based on the pathway followed by *S. typhimurium*, in which α-ribazole 5'-phosphate is added to AdoCbi-GDP. Therefore, the last reaction would involve the dephosphorylation of AdoCbl 5'-phosphate to form AdoCbl, catalyzed by CobC, an AdoCbl-5-P phosphatase (Zayas and Escalante-Semerena 2007; Fang, Kang, and Zhang 2017).

It is quite evident that *de novo* synthesis of vitamin B12 requires quite a high number of enzymes. The aerobic and anaerobic pathways each require approximately 30 different enzymes (Gruber, Puffer, and Kraeutler 2011; Roth, Lawrence, and Bobik 1996; Santos et al. 2018). This makes *de novo* synthesis a highly energy-consuming process and possibly explains why of all the sequenced bacterial genomes which depend on Vitamin B12 for growth, only 50% encode the ability to synthesize an active form. These bacteria depend on the uptake of B12 for survival (Zhang et al. 2009; Escalante-Semerena 2007; Agarwal et al. 2019). It also provides a plausible explanation as to why even bacteria such as *Pseudomonas denitrificans* and *Salmonella typhimurium*, which possess the genes for *de novo* synthesis, also have the genes required for the salvage pathway (Fang, Kang, and Zhang 2017). Furthermore, there is a demand for Cbl uptake among Cbl auxotrophs as Cbl production becomes a limiting factor for biomass production (Bertrand et al. 2011; Santos et al. 2018).

2.2. Salvage Pathway

The salvage pathway is an energy-efficient pathway for obtaining cobalamin for bacteria and archaea. In gram-negative bacteria, exogenous corrinoids (including cobalamin and cobinamide) are imported from the host into the bacterial cell through an ATP-binding cassette (ABC) transporter system (Escalante-Semerena 2007; Fang, Kang, and Zhang 2017). ABC importers, one of the major classes of transporters, bind and hydrolyze ATP to generate energy for the internalization of a wide array of substrates, ranging from ions to macromolecules (Rees, Johnson, and Lewinson 2009; Korkhov et al. 2014). ABC importers have been classified into type-I and type-II based on overall

topology and transport mechanism. Generally, type-I ABC importers possess fewer transmembrane helices than type-II and display an "up and down" topology (Rice, Park, and Pinkett 2014). The transmembrane helices of type-II importers are intricately packed around a translocation pathway (Locher, Lee, and Rees 2002; Amy L. Davidson 2002). The methionine transporter MetNI is an example of a type-I ABC importer (Kadaba et al. 2008) while examples of type-II ABC importers include BtuCD (Locher, Lee, and Rees 2002; Korkhov, Mireku, and Locher 2012) and the heme transporter HmuUV (Woo et al. 2012). The characteristic architecture of type-II ABC importers generally includes four subunits - two ATPase subunits, or nucleotide-binding domains (NBDs) located in the cytoplasm and two transmembrane domains (TMDs) or permeases embedded in the membrane bilayer. The NBDs contain a characteristic set of highly conserved motifs. The NBDs can be further divided into two domains: a structurally diverse α-helical domain, and a catalytic core domain. The α-helical domain contains the ABC signature motif. The catalytic core domain contains a conserved P-loop or Walker A motif, a Walker B motif, a Q-loop, and a switch region or an H-motif. On the other hand, the architectures, and sequences of the TMDs are variable (Hollenstein, Dawson, and Locher 2007; Rees, Johnson, and Lewinson 2009). This variability observed in the TMDs reflects the chemical diversity of substrates that are translocated. Additional regulatory elements can also bind to the TMDs and/or ABCs of the transporters. Although members of the ABC transporter family are present in organisms of all kingdoms of life, ABC importers seem to be exclusively in prokaryotes. In these importers, an additional high-affinity binding protein is also required for substrate translocation. This protein specifically binds to the ligand in the periplasm and delivers it to the appropriate ABC transporter (Rees, Johnson, and Lewinson 2009).

In *E. coli* and many other gram-negative bacteria such as *Vibrio cholerae*, the ABC transporter for vitamin B12 uptake consist of BtuC (membrane permease), BtuD (ATPase), and BtuF (periplasmic ligand-binding protein) (Korkhov, Mireku, and Locher 2012, Escalante-Semerena 2007; Fang, Kang, and Zhang 2017; Agarwal et al. 2019). Additionally, this system requires BtuB which is a TonB-dependent transporter located in the outer membrane. BtuB delivers the corrinoid to BtuF, which subsequently delivers it to the BtuCD

complex located in the inner membrane (Escalante-Semerena 2007; Fang, Kang, and Zhang 2017). Once inside the cytoplasm, cobinamide is adenosylated by ATP:co(I)rrinoid adenosyltransferases (ACATs) to form AdoCbi. PduO, EutT, and CobA are the three families of ACATs that exist (Moore and Warren 2012; Fang, Kang, and Zhang 2017). In bacteria, this AdoCbi follows the same pathway as discussed previously in the *de novo* synthesis section. However, it is converted to adenosylcobyric acid in archaea by an amidohydrolase encoded by the gene *cbiZ*. This adenosylcobyric acid is then condensed with 1-aminopropanol-O-2-phosphate to yield AdoCbi-P by CbiB, an AdoCbi-P synthase. This is followed by the transfer of guanylyl to AdoCbi-P by CobY, which has GTP:AdoCbi-P guanylyltransferase activity (Escalante-Semerena 2007; Newmister et al. 2011; Fang, Kang, and Zhang 2017). After this, two additional reactions transfer lower axial ligands onto AdoCbi-GDP to produce AdoCbl, just like in the *de novo* pathway. ABC transporters are also used by archaea for corrinoid uptake. For example, in *Halobacterium* sp. strain NRC-1, archaeal orthologs of the bacterial BtuC, BtuD, and BtuF have been found (Woodson, Reynolds, and Escalante-Semerena 2005; Fang, Kang, and Zhang 2017). A schematic of the aerobic and anaerobic *de novo* synthesis pathways of adenosylcobalamine and the salvage pathway for the synthesis of the same has been depicted in Figure 2. The BtuCD-F ABC transporter system along with other vitamin B12 ABC transporter systems have been discussed in the subsequent sections.

3. Prokaryotic ABC Transporters Responsible for Vitamin B12 Uptake

The two major transporter systems related to vitamin B12 uptake in bacteria, which have been characterized structurally and functionally, include the ECF (Energy-Coupling Factor) transporters and the BtuCD-F transporters (Korkhov, Mireku, and Locher 2012; Locher, Lee, and Rees 2002; Rodionov et al. 2009). Although both belong to the ATP-binding cassette (ABC) transporter family, which is associated with the uptake of essential micronutrients in many bacterial species, they are structurally distinct, indicating a functional convergence. In this section, we discuss both in detail.

3.1. Btu-Type ABC Transporters

As discussed in section 2.2, cobalamin uptake in bacteria such as *E. coli* and *V. cholerae* is mediated by BtuCD-F ABC importer (Korkhov, Mireku, and Locher 2012; Korkhov et al. 2014). The structure of *E. coli* BtuC comprises 20 transmembrane helices (10 in each subunit), packed intricately around a translocation pathway (Locher, Lee, and Rees 2002; Amy L. Davidson 2002). This pathway is closed to the cytoplasm by a gate region - cytoplasmic gates I and II (Locher, Lee, and Rees 2002; Korkhov et al. 2014). BtuC forms the contact region with BtuD through a prominent cytoplasmic loop (Locher, Lee, and Rees 2002). Structural analysis and biophysical studies of the ATP-bound state of *E. coli* BtuCD revealed that the nucleotide-bound BtuD dimer is present in a closed sandwich conformation. Furthermore, it was concluded from these studies that ATP promotes the docking of B12-bound BtuF, thereby accelerating transport (Korkhov et al. 2014).

A complete mechanism of vitamin B12 transport catalyzed by BtuCD has been established that could perhaps serve as a model for other type II ABC importers. The cycle starts with ATP-bound BtuCD (state 1) where ATP-binding triggers the closure of the BtuD dimer. This results in the simultaneous opening of the cytoplasmic gate I (towards the periplasm) and closing of cytoplasmic gate II (towards the cytoplasm). This is followed by the docking of B12-bound BtuF to BtuCD (state 2), which is an occluded conformation. Here, B12 can be trapped in a central, low-affinity cavity. Due to the insufficient space in the distorted binding pocket of BtuCD-bound BtuF, B12 release is invariably triggered upon binding of BtuF to BtuCD. Hydrolysis of ATP and the subsequent release of ADP and inorganic phosphate disrupts the closed sandwich dimer conformation of BtuD subunits, leading to the transition from state 2 to state 3. In this state, the cytoplasmic gate II is pulled open, leading to the formation of an inward-facing conformation that allows the release of B12 into the cytoplasm. It is currently unclear whether the substrate diffuses freely or is ejected into the cytoplasm due to peristaltic forces. This inward-facing conformation is probably transient and relaxes into asymmetric state 4 upon release of B12. The transition from state 3 to state 4 is not possible if the substrate is present in the central cavity. This is due to the large size of B12, which would not fit in the small cavity provided in state 4. Therefore, the pace for proceeding with the subsequent steps in the cycle is set by the release of B12. The small cavity size in state 4 may prevent non-specific transport of solutes or the back-reaction through the translocation pathway. Depending on the local concentration of nucleotides in the vicinity of BtuCD

in the *E. coli* inner membrane, there are two possible pathways from state 4. There is a transition from state 4 to state 5 upon dissociation of BtuF and rearrangement of the cytoplasmic gate I to form a symmetric outward-facing conformation. A high-intracellular ATP concentration causes state 5 to rapidly convert to state 1 where cytoplasmic gates I and II switch positions. It is assumed that BtuCD, with its two canonical and symmetrical ATPase sites, hydrolyzes two ATP molecules during each productive transport cycle (Korkhov et al. 2014). Non-productive transport cycles (in which B12 is lost on the outside) produce states 5 and '2-like' *in vitro*. The possibility that these states can also be formed *in vivo* cannot be excluded (Locher, Lee, and Rees 2002; Woo et al. 2012; Korkhov et al. 2014). The transitions into these states cause ATP hydrolysis without transport. This is an inherent inefficiency of the transporter that is consistent with the published data (Borths et al. 2005; Korkhov et al. 2014). Vitamin B12 transport through BtuCD-F shows high affinity (nanomolar K_d) and specificity, which can be exclusively attributed to BtuF. Its high-affinity pocket for B12 is distorted and occupied by residues of the periplasmic gate of BtuC upon binding to BtuCD (Korkhov et al. 2014).

As mentioned in Section 2, cobinamide is a precursor of cobalamin that lacks the DMB moiety and the sugar-phosphate linker. Thus, cobinamide is smaller than cobalamin. *In vivo* transport data suggested that the cobalamin-specific uptake system in *E. coli* also transports cobinamide (Di Girolamo & Bradbeer 1971; Mireku et al. 2017). It was also observed through *in vitro* binding assays that BtuF variants in *Thermotoga* species can bind cobalamin as well as cobinamide (Butzin et al. 2013; Mireku et al. 2017). It was later identified that a single tryptophan residue in BtuF (W66) adopts a distinct conformation depending on whether cobalamin or cobinamide is bound. Upon comparing the structures of cobalamin- and cobinamide-bound BtuF, the only major structural difference was observed for W66. Its side chain swung approximately 130° around the C_α-C_β bond towards the inside of the binding pocket in the cobinamide-bound structure. The side chain occupied the position which was occupied by the 5-methyl group of DMB in the cobalamin-bound state, and thus compensated for the missing DMB. Structural analysis of W66 mutant forms of BtuF revealed that a bulky, hydrophobic, and aromatic residue such as tryptophan or phenylalanine at position 66 is crucial for the strong binding of cobinamide. Other *in vitro* transport assays indicated that W66 is not involved in cobinamide transport during a productive transport cycle as the transport rate remained unaffected in W66 mutants (Mireku et al. 2017).

Apart from *E. coli*, a putative ABC transporter system, BtuCD-F, consisting of BtuF, BtuC, and BtuD has also been identified in *Vibrio cholerae*. This transporter system could be important for importing cobalamin. The mechanism of B12 and precursor uptake by ABC importer is still elusive in *V. cholerae*, and other biofilm-forming and/or pathogenic *Vibrio species*. Structural analysis and fluorescence quenching studies with *Vc*BtuF have revealed that it binds not only cyanocobalamin and dicyanocobinamide but also heme (Agarwal et al. 2019). The possible common residues involved in binding these substrates have been discussed in Section 4.

3.2. ECF-Type ABC Transporters

ECF transporters, first described in lactic acid bacteria (*Lactobacillus casei*) for uptake of thiamine and folate (Henderson and Zevely 1978; Henderson, Zevely, and Huennekens 1979), are only found in prokaryotes and are essential in organisms that cannot synthesize the micronutrient being transported (Rees, Johnson, and Lewinson 2009). The transported molecules range from large vitamins and their precursors to transition metal ions like Ni^{2+} or Co^{2+}. Broadly speaking, they are structurally composed of two parts – a common energizing unit or energy coupling factor or ECF module (approx. 93 kDa), and a smaller substrate or micronutrient-specific integral membrane protein, known as the S-component (approx. 19 kDa) (Thangaratnarajah et al. 2021). The ECF module is made of three proteins – EcfT or T component, which is an integral membrane protein with two characteristic conserved ARG (Ala-Arg-Gly) motifs, and EcfA and EcfA', collectively called the A components, which are cytosolic ATPases belonging to the NBD (Nucleotide Binding Domain) family of ATPases (Eitinger et al. 2011; ter Beek et al. 2011; Slotboom 2014). Depending on the location of the gene encoding the ECF modules and the S-components in the same operon (forming one dedicated complex of four proteins) or different operons (one ECF module of three proteins having the ability to combine with a diverse exchangeable array of S components), ECF transporters can be divided into two groups – I and II respectively (Rodionov et al. 2009). In the case of group II transporters, while the ECF module is constitutively expressed, the expression of S-components is mostly regulated by riboswitches, giving rise to a situation where there may be a stoichiometric excess of S-components compared to ECF modules. Such lone S-components are hypothesized to bind cognate micronutrient substrates with very low to sub-nanomolar dissociation constants, till they can associate

with an ECF module that can transport the substrate into the cytosol (Burgess et al. 2006; Rodionov et al. 2009). For example, eight different S-components have been predicted in *Lactobacillus delbrücki* by Rodionov et al. 2009, which interchangeably interact with a single ECF module. However, rare solitary functional S-components independent of an associated ECF module have also been reported, e.g., the S-component BioY, which binds biotin, is solitary in *Chlamydia* sp., different proteobacterial and cyanobacterial species (Fisher et al. 2012; Finkenwirth, Kirsch, and Eitinger 2013; Slotboom 2014; Santos et al. 2018).

Functionally speaking, like all ABC transporters, ATP binding and hydrolysis in ECF transporters occur at the interface of the ATPases. When ATP binds in the two cognate sites of EcfA and EcfA', they are brought into proximity (Smith et al. 2002; Rees, Johnson, and Lewinson 2009). This is hypothesized to pinch together two long X-shaped conserved alpha-helices of EcfT, X1 and X2, which do not span the membrane but are anchored to the two ATPases via their respective ARG motifs (Zhang, Wang, and Shi 2010; Wang et al. 2013; Xu et al. 2013). This orients the S-component to face the extracellular environment and binds its ligand. Thereafter, on ATP hydrolysis and release of ADP and inorganic phosphate, a unique toppling of the S-component occurs, which is not found in any other ABC transporter system yet - on toppling, the substrate-binding site of the S-component faces the cytosol, where the micronutrient is released due to alterations in the binding affinity caused by structural rearrangements while toppling (Wang et al. 2013; Xu et al. 2013; Slotboom 2014). The hydrophobic surface of EcfT is thought to provide a sliding scaffold for the hydrophobic S-component to enable toppling – helices X1 and X2 of EcfT are presumed to interact with a conserved AXXXA (X is any hydrophobic amino acid) motif in the first alpha-helix of the S-component (Rodionov et al. 2009; ter Beek et al. 2011; Berntsson et al. 2012; Slotboom 2014; Rempel, Stanek, and Slotboom 2019).

Both group I and group II ECF transporters specific for Vitamin B12 have been found in prokaryotes – the group I transporter is the CbrTUV system, while the group II transporter is the ECF-CbrT system, where CbrT is the S-component specific for cobalamin and its precursor cobinamide, and a 1/1/1/1 stoichiometry is observed for the four proteins (CbrT, EcfT, EcfA and EcfA') making up the transport system (Santos et al. 2018; Rempel, Stanek, and Slotboom 2019). The group I CbrTUV transporter has been identified in different Firmicutes, Actinobacteria and in the archaebacteria, *Methanosphaera stadtmanae*, accompanied by distinct hypothetical lipoprotein components such as CbrY-Z in *Moorella thermoacetica* and CbrX

in *Bacillus cereus* (Eitinger et al. 2011). CbrT of the group II transporter system has been shown to be promiscuous towards the β-ligand of cobalamin, which also allows the transport of compounds that are structurally similar to cobalamin, and not limited to its precursor cobinamide, such as – adenosyl-cobalamin, methyl-cobalamin, hydroxyl-cobalamin, and hemin (Santos et al. 2018). This broad substrate specificity can also be explained by the fact that the gene encoding CbrT lies in the *nrdJ–cbrS–cbrT–pduO* gene cluster, where *nrdJ* has been annotated to be an adenosylcobalamin-dependent ribonucleotide reductase which requires cobalamin as a co-factor, PduO is a cobalamin adenosyltransferase that converts cobalamin to adenosylcobalamin, making it accessible for NrdJ, and CbrS is a hypothetical lipoprotein (Eitinger et al. 2011; Santos et al. 2018). However, whether all the substrates that bind to CbrT are transported into the cell via the ECF module is still up to further structural and functional studies.

3.3. Other Transporters Involved in Vitamin B12 Import

There are plenty of other transporters associated with vitamin B12 transport apart from the ones discussed above. These transporters are new and not yet fully characterized. Characterization of these new cobalamin transporters could give us deep mechanistic insights into membrane protein transport. For example, humans possess a vitamin B12 ABC transporter, ABCD4, localized in the lysosomal membrane of the cell. It transports the cobalamin which has been liberated through proteolysis from its protein-chaperone inside the lysosomal compartment, into the cytosol (Wuerges et al. 2007; Coelho et al. 2012). This notion has serious implications for our current knowledge of ABC transporters. ABC-importers are thought to be exclusively present in prokaryotic organisms. However, ABCD4 could be a mammalian ABC importer as the lysosomal compartment is devoid of ATP, implying that the NBDs of ABCD4 must be in the cytosol. Therefore, it is not possible that the direction of transport is that of an ABC exporter.

Another vitamin B12 transporter is BtuM, which does not share any sequence identity with any known protein (Rodionov et al. 2003; Rempel et al. 2018). Surprisingly, the high-resolution crystal structure of BtuM from *Thiobacillus denitrificans* ($BtuM_{Td}$) revealed that it is structurally similar to S-components. However, it can be categorized as a solitary S-component as it does not make use of an ECF module and is capable of ECF module-independent transport. Furthermore, $BtuM_{Td}$ lacks an interaction motif that is

used to interact with ECF-T by non-solitary S components. Spectroscopic data and the high-resolution crystal structure revealed that there is an unusual thiolate coordination between a cysteine residue and the cobalt ion of cobalamin on the α-face. The intramolecular coordination of the cobalt ion that makes use of the DMB moiety is replaced by this interaction. As a result, cobalamin is converted into its base-off conformation. This thiolate coordination also results in the decyanation of cyano-cobalamin at the β-axial position. The cysteine residue is crucial for transport activity and thus, is conserved among BtuM homologs. This chemical modification of the substrate prior to transport is quite a rare phenomenon in membrane transporters. Binding assays carried out with cobinamide (which lacks the DMB moiety and thus, mimics the base-off conformation) and a mutant $BtuM_{Td}$ in which the cysteine is replaced with serine shows that the mutant still binds cobinamide. However, it is no longer capable of modifying the substrate. Furthermore, this mutant is unable to bind cobinamide, indicating that the cysteine residue is also involved in the conversion of cobalamin to its base-off conformation. The thiolate-mediated decyanation and cysteine-ligation indicate that a redox-mechanism is involved in the transport. Thus, a hypothesis arises that the reducing environment inside the cell causes the cysteine and cobalt bond between $BtuM_{Td}$ and cobalamin to break. This results in the substrate being 'pulled' inside the cell.

BtuN is another protein that was predicted to be a vitamin B12 transporter in the same study that predicted BtuM to be one (Rodionov et al. 2003). BtuN possesses large extracellular loops, four predicted transmembrane helices, and a mirrored architecture.It has sequence similarity with any other known protein. It is predicted to be a periplasm-spanning cobalamin transporter.

Rv1819c constitutes a cobalamin transporter in *Mycobacterium tuberculosis*, making it a desirable drug target as vitamin B12 uptake plays a role during *M. tuberculosis* pathogenesis (Gopinath, Moosa, et al. 2013). Rb1819c is also interesting from a structural point of view as it falls into the ABC-exporter fold as per its predicted secondary structure. Thus, Rv1819c could possibly be a novel type of ABC-importer with the exporter fold. Furthermore, there is no genetic co-localization of Rv1819c with any substrate-binding protein, which would make it the first importer that is independent of a substrate-binding protein (Gopinath, Venclovas, et al. 2013).

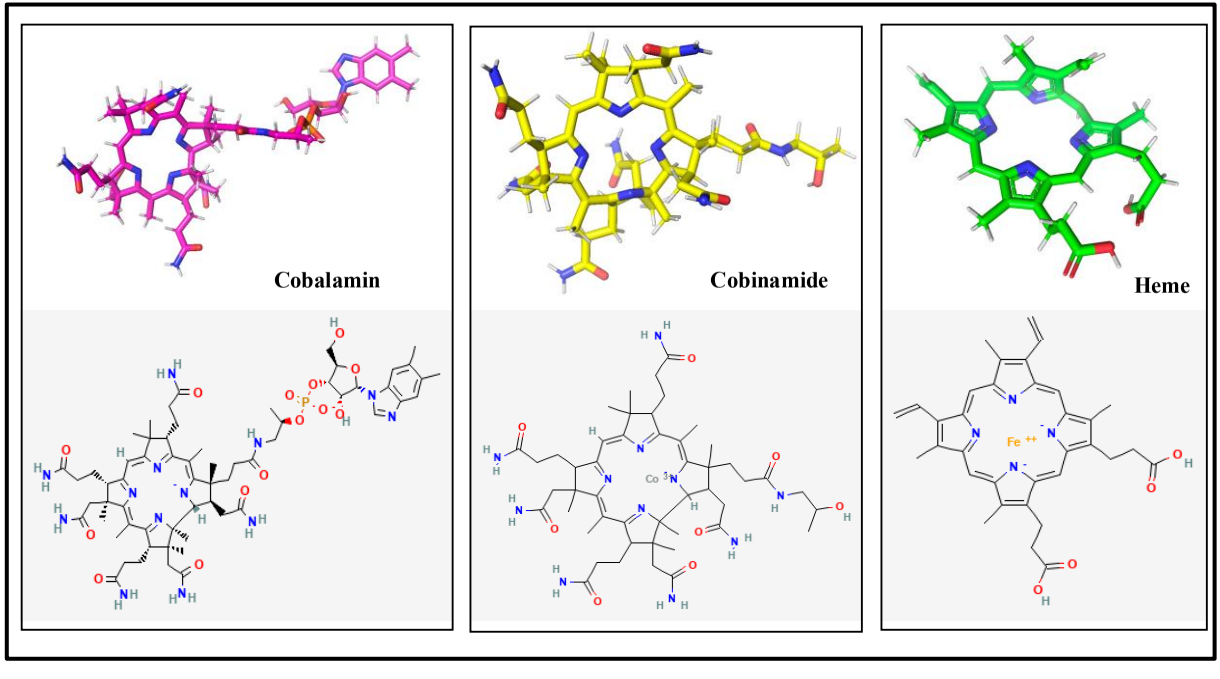

Figure 3. The three panels (L-R) show 3D (upper panels) and 2D (lower panels) structures of cobalamin (in magenta), cobinamide (in yellow) and heme (in green). Figures obtained using PubChem (Kim et al., 2021) and The PyMOL Molecular Graphics System, Version 2.5, Schrödinger, LLC.

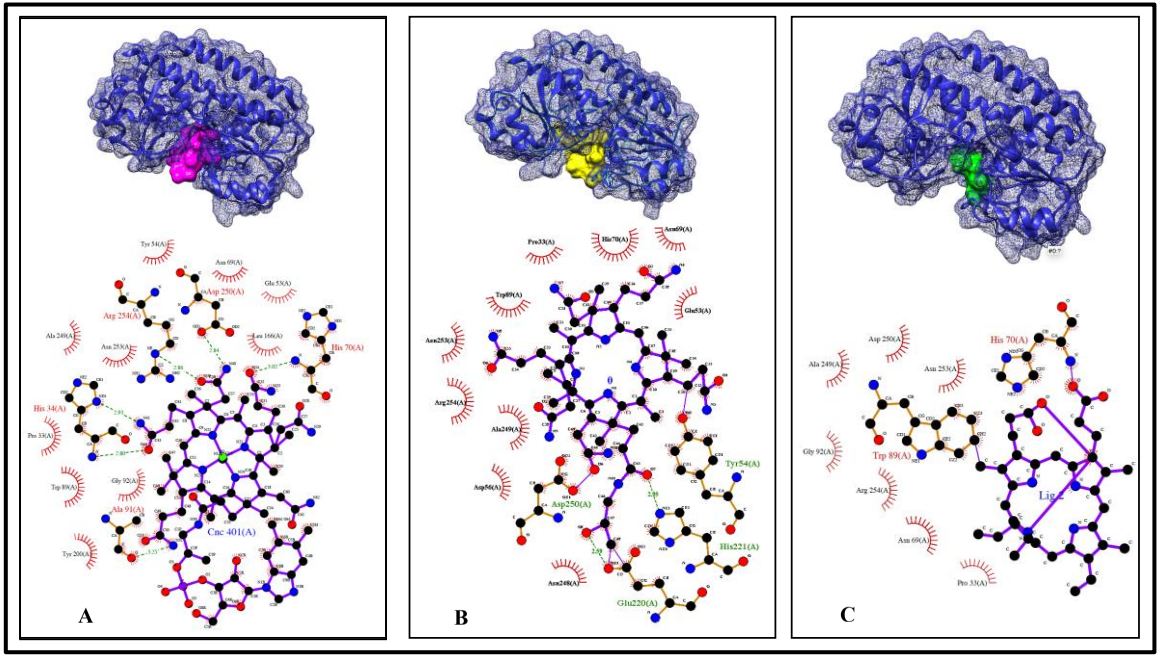

Figure 4. Molecular docking and protein-ligand interaction studies of VcBtuF (PDB ID 5YSC) with cobalamin (A), cobinamide (B) and heme (C). Upper panels show docked structures of VcBtuF (represented as a surface mesh on a ribbon structure in deep blue), with ligands (represented as solid surfaces) cobalamin (magenta), cobinamide (yellow) and heme (green). LigPlots generated from LigPlot+ are shown in lower panels for corresponding protein-ligand docked structure. Figures of docked structures have been generated using UCSF Chimera (Pettersen et al., 2004).

4. Heme, Cobalamin and Cobinamide – An *In-Silico* Comparative Account of Binding Interactions with *Vc*BtuF

As discussed in a previous section, the salvage pathway of cobalamin synthesis allows the transport of exogenous corrinoids via the BtuCD-F ATP-binding cassette (ABC) transport system (Escalante-Semerena 2007; Fang, Kang, and Zhang 2017). There have been findings that such ABC transport systems in different microbial systems can bind multiple substrates – for example, *Ec*BtuCD-F of *Escherichia coli* can transport both dicyanocobinamide and cyanocobalamin, and *Vc*FhuD of *Vibrio cholerae* binds siderophores like hydroxamate and catecholate (Agarwal et al. 2017; Mireku et al. 2017; Agarwal et al. 2019).

Fluorescence quenching studies of *Vc*BtuF with dicyanocobinamide, cyanocobalamin and heme by Agarwal et al. in 2019 showed that the three have comparable dissociation constant (K_d) values of 0.66 ± 0.015 μM, 0.95 ± 0.226 μM and 1.47 ± 0.226 μM respectively, indicating tightest binding with dicyanocobinamide and least with heme. This was an interesting result, indicative of the role of corrinoid binding to BtuF – cobinamide is an incomplete corrinoid, cobalamin has a corrin core, and although heme is not a direct precursor of Vitamin B12, it closely resembles a precorrin precursor or intermediate (illustrated in Figure 3).

Further studies by the same group also studied the stoichiometry of interaction between *Vc*BtuF and heme and found a 1:1 interaction, instead of the 2:1 heme interaction with the canonical heme transporter in *V. cholerae, Vc*HutB (Agarwal et al. 2019). *Vc*HutB binds to heme efficiently via a histidine residue (H164), and existing literature suggests that heme recognition and binding is generally initiated by a Histidine or a Tyrosine residue or a combination of both in trans, in the periplasmic binding proteins of ABC transport systems, as they form coordinate bonds with ferrous or ferric ions. Similarly, incremental titration of heme with free *Vc*BtuF showed a Soret band red shift characteristic of heme-binding with a histidine residue (Agarwal et al. 2017; Agarwal et al. 2019).

To represent a comparative picture of the binding of cobinamide, cobalamin and heme to *Vc*BtuF (PDB ID 5YSC, Agarwal et al., 2019), *in silico* docking was performed using PatchDock (Duhovny, Nussinov, and Wolfson 2002; Schneidman-Duhovny et al. 2005), protein-ligand interactions checked using LigPlot+ and PDBSum (Laskowski and Swindells 2011; Laskowski et

al. 2018), and structures visualized using PyMOL and UCSF Chimera (Pettersen et al. 2004). They have been illustrated in Figure 4.

The common residues of *Vc*BtuF involved in non-bonded interactions with the three ligands include Pro33, Glu53, Tyr54, Asn69, His70, Trp89, Ala249, Asp250, Asn253, and Arg254, all of which surround the ligand binding pocket of BtuF (Figure 5, Table 1). Since structural studies have indicated that Trp66 of *Ec*BtuF and its corresponding His70 of *Vc*BtuF act as 'gates' of the ligand binding pockets, H70 of *Vc*BtuF is also a hypothesized candidate residue that contributes to heme binding (Mireku et al. 2017; Agarwal et al. 2019).

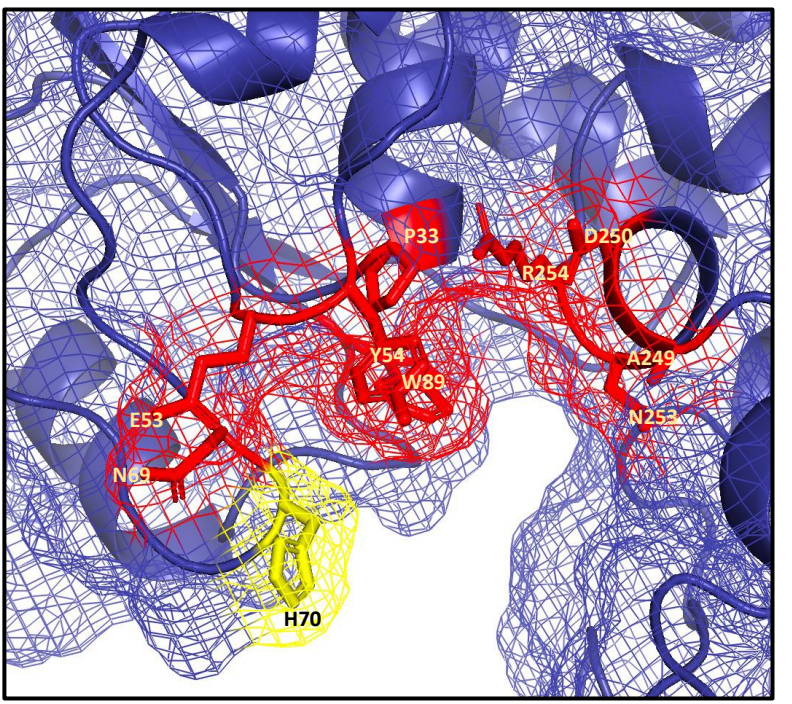

Figure 5. Residues of VcBtuF that interact with each of three ligands – cobinamide, cobalamin and heme. VcBtuF has been represented as a surface mesh on a ribbon structure in deep blue, with common residues represented with side chains as sticks in red. His70, the putative binding residue for the ligands has been shown in yellow. Figure generated using PyMOL (The PyMOL Molecular Graphics System, Version 2.5, Schrödinger, LLC).

Table 1. List of protein-ligand interactions between *Vc*BtuF and cobalamin, cobinamide and heme. Data obtained from PDBSum and LigPlot+ (Laskowski and Swindells 2011; Laskowski et al. 2018)

	Hydrogen Bonds formed with *Vc*BtuF	**Non-bonded contacts formed with *Vc*BtuF**
Cobalamin	His34, His70, Ala91, Asp250, Arg254	Pro33, His34, Glu53, Tyr54, Asn69, His70, Trp89, Ala91, Gly92, Leu166, Tyr200, Ala249, Asp250, Asn253, Arg254
Cobinamide	His221, Arg254	Pro33, Glu53, Tyr54, Asp56, Asn69, His70, Trp89, Glu220, His221, Asn248, Ala249, Asp250, Asn253, Arg254
Heme	His70	Pro33, Glu53, Tyr54, Asn69, His70, Gln71, Trp89, Gly92, Ala249, Asp250, Asn253, Arg254
Residues in common (cobalamin and cobinamide)	Arg254	Pro33, Glu53, Tyr54, Asn69, His70, Trp89, Ala249, Asp250, Asn253, Arg254
Residues in common (cobalamin and heme)	His70	Pro33, Glu53, Tyr54, Asn69, His70, Trp89, Gly 92, Ala249, Asp250, Asn253, Arg254
Residues in common (cobinamide and heme)	-	Pro33, Glu53, Tyr54, Asn69, His70, Trp89, Ala249, Asp250, Asn253, Arg254
Overall common interacting residues	-	**Pro33, Glu53, Tyr54, Asn69, His70, Trp89, Ala249, Asp250, Asn253, Arg254**

It is interesting that the same species shows ability to bind heme via periplasmic binding proteins belonging to different ABC transporter systems – *Vc*HutB and *Vc*BtuF. However, the rationale behind the evolution of such a system is not defined yet – it is probable that in situations of iron starvation, the pathogenic bacteria may take up heme as a source of the essential nutrient, iron, through both its canonical transporter, as also the BtuCD-F system; but whether heme can be transported after binding, inside the cell, is yet to be deciphered. Another perspective arising out of this finding is whether the relative abundance of heme and Vitamin B12 and/or its precursors define which nutrient is taken up by BtuF (Agarwal et al. 2019); a probable model based on competitive limitation of uptake of one nutrient by the other in the vicinity of the ligand-binding pocket of *Vc*BtuF, remains to be discovered.

Conclusion

In this chapter, we have discussed the importance of vitamin B12 as a nutrient in prokaryotes, whether it be synthesized *de novo* or acquired via a more energetically favorable salvage pathway. Although Type II ABC transporters like the BtuCD-F complex and ECF-type transporters have been structurally characterized in *E. coli*, *V. cholerae*, and lactic acid bacteria, others like $BtuM_{Td,}$ BtuN, Rv1819c, ABCD4, etc. are yet to be explored in further detail. An interesting aspect of such transporter assemblies is the ability of the substrate-binding components to show multiple substrate specificities. As outlined using bioinformatics-based comparisons, pathogenic bacteria can use the same ABC transporter to bind to cobalamin, its precursor cobinamide, as also structurally similar but functionally distant heme – whether binding ultimately leads to cellular transport is, however, yet to be deciphered. Several pathogenic bacteria like *Streptococcus pyogenes*, *Clostridium tetani*, etc. are auxotrophic for cobalamin, lack a BtuCD-F homolog, but carry the *cbrT* gene, making them specifically dependent on a particular transporter type for scavenging vitamin B12 or its precursors from the extracellular milieu (Santos et al. 2018). Given humans use endocytosis for cobalamin uptake (Quadros 2010), such specific transporters in vitamin B12-auxotrophic bacteria can act as prokaryote-specific drug targets, especially in the aggravating silent pandemic of antibiotic resistance when related membrane drug efflux proteins (reviewed by Greene et al. 2018) are being targeted as well. On the other hand, as Putnam et al. 2022 recently reported, novel classes of related vitamin B12 transporters are also being discovered in human gut commensal bacteria, which may complicate the target-specific drug development during pathogenic infections. Extensive structural and functional characterization of Vitamin B12 transporters both in pathogenic and non-pathogenic prokaryotes are thus required, including identification of shared substrates and common binding residues, for both academic and translational interests.

Acknowledgment

The authors would like to thank Dr. Sayak Ganguli, Post Graduate Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, for his valuable insights regarding bioinformatics analyses, and Rev. Fr. Dr. Dominic

Savio SJ, Principal, St. Xavier's College (Autonomous), Kolkata, for his active encouragement.

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Chapter 4

The Role of Fluid Therapy in Traumatic Brain Injury

Eduardo Esteban-Zubero[1,*], PhD,
Cristina García-Muro[2], MD
and Moisés Alejandro Alatorre-Jiménez[3], PhD

[1]Emergency Department, Hospital San Pedro, Logroño, Spain
[2]Department of Pediatrics, Hospital San Pedro, Logroño, Spain
[3]Department of Pediatric Gastroenterology, Children's Mercy Hospital, Kansas City, USA

Abstract

Traumatic brain injury (TBI) is a major public health problem and a significant cause of death and disability. The damage may be divided into two phases: a) a primary acute injury because of the traumatic event; and b) a secondary injury due to the hypotension and hypoxia generated by the previous lesion, which leads to ischemia and necrosis of neural cells. In TBI, the development of cerebral edema is a crucial prognosis marker. In the early stages of TBI, minimal changes in intracranial pressure are observed because of cerebral edema due to a compensatory effect of the cerebrospinal fluid. However, if edema increases, this mechanism fails, increasing intracranial pressure. To avoid this chain effect, several treatments are applied in the clinical practice, including elevation of the head of the bed, maintenance of normothermia, pain and sedation drugs, mechanical ventilation, neuromuscular blockade, controlled hyperventilation, and fluid therapy (FT).

* Corresponding Author's Email: eezubero@gmail.com.

In: Advances in Health and Disease. Volume 57
Editor: Lowell T. Duncan
ISBN: 979-8-88697-098-2

The goal of FT is to improve the circulatory system to avoid the lack of oxygen to organs. Therefore, rapid and early infusion of large volumes of crystalloids is performed in clinical practice to restore blood volume and blood pressure. Despite the relevance of FT in the early management of TBI, there are few clinical trials regarding which solution is better to apply.

This chapter aims to summarize the actual knowledge about the different types of FT used in clinical practice. A physiopathological approach to TBI will be performed to achieve this goal, explaining why the different types of FT are used.

Keywords: traumatic brain injury, fluid therapy, intracranial hypertension, osmotherapy

Introduction

Traumatic brain injury (TBI) is a major public health problem and a significant cause of death and disability (Langlois et al., 2006). The damage may be divided into two phases: a) a primary acute injury because of the traumatic event; and b) a secondary injury due to the hypotension and hypoxia generated by the previous lesion, which leads to ischemia and necrosis of neural cells (Kaur and Sharma 2018).

In middle-aged patients, trauma is the leading cause of death, with TBI responsible for most of these (Langlois et al., 2006, Stocchetti and Maas, 2014). In the United States, this pathology causes 275,000 hospitalizations and 52,000 deaths per year as a related factor in more than 30% of all injury-related deaths (Whitaker-Lea and Valadka 2017). The relevance of this pathology is also related to the sequelae, generating an economic impact of over $80 billion in the United States. This data is also associated with the clinical stratification of TBI, beginning from 10% (mild TBI), 60% (moderate TBI), and 100% (severe TBI), according to Glasgow Coma Scale Score (GCS) (Vella et al., 2017).

According to the literature, TBI studies are mainly categorized based on physical examination instead of the underlying cause. This way of stratification catalogs TBI as a neurological condition without a relationship with a pathology. This association is the key to understanding why several clinical trials have not achieved significant results. This situation is not observed in general guidelines, such as “chest pain”. This symptomatology results from different pathologies with well-known management guidelines,

such as myocardial infarction, pneumonia, and aortic dissection. Attending to TBI, the underlying pathology may be unclear, without a clear treatment (diffuse swelling, ischemia, blossoming contusion, etc).

Therefore, the patient evaluation and management of TBI in the Emergency Department and Urgent Cares is critical (Marehbian et al., 2017, Geeraerts et al., 2018). The first approach's main goal should be to avoid secondary brain injury. It has been observed that secondary insults such as systolic blood pressure lower < 90 mmHg or SpO2 < 92% in moderate and severe TBI patients increase mortality (Chi et al., 2006). Due to the relevance of maintaining correct brain oxygenation, some authors discuss the benefits of delayed patient transfer to a hospital due to complicated intubation. In this line, a study observed that prehospital rapid sequence intubation performed by paramedics in head-injured patients with GCS < 9 was associated with an increase in mortality. This result may be related to the transient hypoxia during the prehospital procedures, excessive over-ventilation causing hypocapnia, vasoconstriction, impaired cerebral blood flow (CBF), and longer scene times (Davis et al., 2003). This study concludes that rapid transfer and more basic airway strategies to maintain oxygenation in head-injured patients improve the results.

Fluid therapy (FT) also plays an essential role in the early management of TBI. This therapy is required to a) Normal maintenance; b) Blood or fluid loss due to wounds, drains, induced diuresis, etc., c) Third space losses called fluid sequestration in tissue edema or ileus; and d) Increased systemic requirements resulting from fever and hypermetabolic state (Dick et al., 2013). Consequently, rapid infusion as quickly as possible of large volumes of crystalloids is performed in daily practice, usually an empirical approach due to the lack of studies (Dick et al., 2013, Alvis-Miranda et al., 2014).

Pathophysiology of Truamtic Brain Injury

Brain parenchyma (80%), cerebrospinal fluid (CSF) (10%), and cerebral blood volume (CBV) compose the three compartments of the cranium (Allen and Ward 1998). The main characteristic is the equilibrium among them, regulating the intracranial pressure (ICP) in adults (10 mmHg) and children (7 mmHg) (Yanko and Mitcho 2001).

As we commented previously, the pathophysiology of TBI involves a complex cascade of events that could be divided into two different phases. The first one is a primary acute injury because of the traumatic event. Secondary

damage occurs after the first lesion when alterations in CBF, cerebral oxygen delivery, inflammation, and cellular metabolism lead to ischemia and necrosis of neural cells (Dutton and McCunn 2003, Kaur and Sharma 2018). An inflammatory response is generated after the acute injury. However, if this situation is maintained, it develops cerebral edema, leak of oxygen delivery, ischemia, and necrosis of cells (Adamides et al., 2006). Therefore, cerebral edema is a marker of evolving injury in TBI and is the consequence of disrupting the blood-brain barrier (BBB) and lymphatic drainage disruption (Allen and Ward 1998, Yanko and Mitcho 2001).

Cytotoxic and vasogenic edema are the main types of edema observed in TBI. The first one promotes the accumulation of intracellular water in cerebral cells, hypoxia, and ischemia (Qureshi and Suarez 2000). On the other hand, vasogenic edema results from cerebral blood vessels disruption, causing a breakdown of the BBB and increasing leakage into the extravascular interstitial space (Allen and Ward 1998, Qureshi and Suarez 2000). As a response to cerebral edema, intracranial volume increases. However, ICP suffers minimal variations due to vasoconstriction and the shunting of CSF (compensatory phase). If edema persists, these regulatory mechanisms fail, promoting intracranial hypertension (Changa et al., 2019). This state results from a reduction in CBF of oxygen, glucose, and essential substrates (Freeman 2015).

TBI is not only defined as the increase of intracranial pressure. In severe TBI, other pathophysiologic states could be observed, including hypovolemia and hypotension. Therefore, it is essential to measure the blood flow gradient, defined as cerebral perfusion pressure (CPP) (Carteron et al., 2017). CPP is defined as the difference between mean arterial pressure (MAP) and ICP (CPP = MAP – ICP). It could be concluded that systemic hypotension implies a decrease of CPP value—hemorrhage, third-space fluid losses, and vasoplegia that can develop hypotension (Pigott and Rudloff 2021). It is recommended to maintain CPP values > 70 mmHg in adult patients. In contrast, in the pediatric age group, due to the broad age range, it is recommended to aim CPP > 40-65 mmHg as an age-related continuum for the optimal treatment threshold (Mitchell et al., 2015, Figaji 2017).

Support treatment in TBI aims to enhance CPP, improve cerebral perfusion, and reduce the degree of brain injury. To achieve these results, non-invasive techniques could be performed, including the elevation of the head of the bed with the head in midline position and maintenance of normothermia. If this treatment fails, pain and sedation medications, mechanical ventilation, neuromuscular blockade, and controlled hyperventilation should be initiated.

Instead of this, euvolemia is the goal during resuscitation of TBI, being necessary to administrate FT and inotropic medications (Mangat and Härtl 2015, Schizodimos et al., 2020).

Fluid Therapy and Traumatic Brain Injury

The Brain Trauma Foundation (BTF) and the Lund Concept are the primary organisms that develop guidelines recommendations for TBI treatment (Carney et al., 2017, Grände 2017). Both documents have in common the lack of a strong recommendation about which FT is recommended to use in TBI (Muzevic and Splavski 2013, Caplan and Cox 2019).

Prior to the review of the different FT types, it is important to remark the relevance of vigorous resuscitation to achieve the goal of systolic blood pressure between 90-110 mmHg (Carney et al., 2017, Leibner et al., 2020). The dose-dependent relation between hypotension and irreversible brain damage has been observed (Chatrath et al., 2015, Dickson et al., 2018). It is known that TBI is usually related to hemorrhage, usually observed after a delay in bleeding control after normotensive resuscitation was not successful (Vrettos et al., 2016). The authors conclude that hypotension is not recommended in TBI patients. However, hemorrhage control usually does not provide the patient's survival, being necessary to explore this field to improve the outcomes.

Crystalloids

This type of FT contains small water-soluble molecules, being easier to cross the semi-permeable membranes. The osmolarity is similar to plasma, and its sodium levels affect the distribution among the body compartments (Fantoni and Shih 2017). The extracellular fluid compartment (ECF) contains 75% of interstitial fluid. It implies that 3-4 liters of crystalloids are required to replace 1 liter of blood loss (Hahn 2017). These values are affected by the patient's status (normovolemic or hypovolaemic) (Gondos et al., 2010).

Frequently used in prehospital admission, no benefits in survival outcomes have been observed, including aggressive resuscitation in hemorrhagic patients (Bickwell et al., 1994, Santry and Alam 2010, Kwan et al., 2014). As a result, recent studies suggested that transfusion of red blood

cells, plasma, and platelets (ratio 1:1:1) is better than crystalloids due to the diminished risk of hemodilution, brain edema, and inflammation secondary to a large volume of fluids (Grände 2017, Dekker et al., 2018, Leibner et al., 2020). Ko et al., (Ko et al., 2017) agree with observing an increase in mortality in patients that received ≥ 2 L during resuscitation compared to those who received less.

The osmolarity of crystalloids is possible to divide this solution into isotonic, hypotonic, and hypertonic. Isotonic solutions include normal saline, Ringer's solution, or plasmalyte. These three types of fluids do not affect the brain water content, being distributed easier in the ECF and intracellular fluid compartment (ICF). However, the most frequently used solution (Ringer) has a lower osmolarity (254 mOsm/L compared to 300 mOsm/L of the gold standard isotonic solution) (Tommasino and Picozzi 2007). This difference explains why large volumes of Ringer could generate brain edema due to increased ICP (Tommasino and Picozzi 2007, Grände 2017, Dekker et al., 2018, Leibner et al., 2020). Consequently, the use of hypotonic solutions does not have sense and must be avoided (Tommasino and Picozzi 2007).

A different type of crystalloid fluid is hypertonic saline (HTS). This treatment is primarily used in patients with elevated ICP due to TBI due to its effect in a small volume during resuscitation (Hashiguchi et al., 2007, Elliott et al., 2009). It has been suggested that the beneficial effects of HTS are due to its capability to modulate the innate immune response, especially the neutrophil burst activity. Therefore, an improvement in cardiovascular output and cerebral oxygenation is observed, reducing cerebral edema (Deitch et al., 2003, Powers et al., 2003, Homma et al., 2005, Deree et al., 2007). However, in clinical practice, these theories are not entirely supported in patients affected by TBI or hemorrhagic shock (Younes et al., 1992, Jousi et al., 2010). One of the largest clinical trials evaluating the neurological outcomes after six months of TBI and mortality rate after 28 days of the event did not observe any benefits (Bulger et al., 2010). These results agree with a later study of the same group, being necessary to stop the study due to an increase of mortality in a subgroup of patients treated with HTS but not blood transfusion in the first 24 h (Bulger et al., 2011). However, in the literature are also observed positive results using hypertonic saline-dextran solution (HSD) in patients presented with hypotension (Wade et al., 1997). These authors observed an increase in survival compared to regular treatment. Rockswold et al. (Rockswold et al., 2009) also agree with these results, monitoring a decrease of ICP and increase of CPP and brain oxygenation in patients affected by severe TBI, especially those affected with higher baseline ICP and lower CPP

levels. In conclusion, HTS is recommended in TBI patients without conferring a survival benefit in a general manner. However, in patients with intracranial hypertension, its benefits are higher than isotonic crystalloid solutions.

Synthetic Colloids

Gelatins

This semi-synthetic colloid is not used in daily practice since it has a high risk of anaphylactic reactions, especially in rapid infusions (Ertmer et al., 2009, Hahn 2017). Gelatin preparations have a low molecular mass range and a mean molecular weight of 30-35 kDa. One of their most important characteristics is their rapid renal excretion (80% molecules < 20 kDa), increasing the risk of dehydration if the adequate crystalloid infusion is not administered. In addition, their intravascular persistence is short (2-3 hours), especially in the urea-linked gelatins. Due to the negative charges contained in their molecules, chloride concentrations are lower compared to other colloids. Consequently, intracellular edema could be increased if large amounts of fluids are provided due to its hyposmolality (Ertmer et al., 2009).

Dextranes

Derived from the action of the bacterium Leuconostoc mesenteroides and mediate via the dextran sucrose enzyme, they are neutral, high-molecular-weight glucopolysaccharides based on glucose monomers. Its excretion is mainly via the kidneys (70%). Different molecules are produced in the hydrolysis grade, being the main characteristic of its capacity as a plasma expander (Alvis-Miranda et al., 2014). Blood flow improvement results from a reduction in blood viscosity. In addition, dextrans inhibit platelet adhesiveness, enhances fibrinolysis and reduces factor VIII activity (Alvis-Miranda et al., 2014).

Modern solutions do not affect blood crossmatching or cause rouleaux formation as previously. However, they may generate renal dysfunction via tubular obstruction, especially in renal insufficiency and hypovolaemia patients. As gelatins, severe anaphylactic reactions like immune complex type III can result from prior cross-immunization against bacterial antigens forming dextran reactive antibodies. However, the incidence is low, especially if monovalent hapten pre-treatment is administered (injection of 3 g dextran 1) (Hahn 2017).

Hydroaxyethyl Starch (HES)

HES is a semi-synthetic colloid prepared from amylopectin, a glucose polymer derivative. Its viscosity is lower than dextran or gelatin but does not reach the low viscosity of albumin. The mean molecular weight of the different HES preparations ranges from 70 and 670 kDa (Alvis-Miranda et al., 2014).

The kinetics of this degradation is determined by the molar substitution and the C2/ C6 ratio representing the quotient of the numbers of glucose residues hydroxyethylated at positions 2 and 6, respectively (Alvis-Miranda et al., 2014). Consequently, its intravascular half-life is observed if a high molar substitution and a high C2/C6 ratio are generated, making the HES molecule less susceptible to plasma amylase (Alvis-Miranda et al., 2014).

HES activity is also characterized by its capacity to decrease plug capillary induced by sepsis and major trauma and restore macrophage function after hemorrhagic shock. Compared with 20% albumin in these patients, 10% HES significantly improves hemodynamic parameters in the systemic and microcirculation (Naisbitt et al., 2016).

Like previously commented synthetic colloids, the main problem of HES is its increased risk of acute kidney injury. In the literature are few studies of its benefits in TBI. In a single-center retrospective cohort study of 171 people with severe TBI, 78% of patients received 6% HES 200/0.5 during hospitalization. There was no association with mortality, change in serum creatinine, or establishment of renal injury (Sekhon et al., 2011). Another study performed in 7000 patients admitted in the Intensive Care Unit revealed no significant difference in 90-day mortality between patients resuscitated with 6% HES (130/0.4) or saline. However, an increased risk of renal-replacement therapy was observed in patients who received HES treatment (Myburgh et al., 2012).

Natural Colloids

The main characteristic of colloids is their difficulty crossing semi-permeable membranes due to their larger and more insoluble molecules. The molecular weight, shape, ionic charge, and capillary permeability determine their movement out of the intravascular space and their duration of action (Wood 2021). Due to its higher osmolality, colloids increase plasma volume in a higher ratio than the volume infused (Naisbitt et al., 2016).

Albumin

Albumin is one of the most used colloids, being an effective volume expander without allergic-type reactions and no intrinsic effects on clotting (Tommasino and Picozzi 2007). The literature reveals contradictory results comparing albumin with different fluid therapies. A study compared 4% albumin with 0.9% sodium chloride for resuscitation in patients affected by hemorrhagic shock. In the subpopulation of TBI patients, higher mortality was observed in patients treated with albumin (SAFE Study Investigators et al., 2007). This result can be supported by the increased risk of brain edema (Van Aken et al., 2012). However, these results do not agree with a previous study performed by Tomita et al., (Tomita et al., 1994). Compared to synthetic colloids, increased survival has not been observed, dismissing its use in clinical practice due to its higher costs (Schortgen et al., 2008). The Lund Concept recommendations continue to support the use of 4% albumin (Grände 2017) in spite of the evidence of harm (SAFE Study Investigators et al., 2007, Van Aken et al., 2012).

Plasma Products

It is observed that high ratios of fresh frozen plasma (FFP) added to packed red blood cells results in an increased ratio of survival compared to massive transfusion (Kasotakis et al., 2013). It could be due to the complications associated with the large volume of crystalloid required during resuscitation and its protective effect on the endothelium and endothelial glycocalyx layer and BBB (Kozar et al., 2011) McDaniel et al., 2017, Nikolian et al., 2018). In the literature, there are few studies about the empirical use of FFP in patients affected by severe TBI. However, their results revealed an increased risk of delayed traumatic intracerebral hematoma formation than 0.9% sodium chloride (Etemadrezaie et al., 2007; Zhang et al., 2019). Regarding mortality, Zhang et al., did not observe significant differences, observing an increased rate in blood transfusions and coagulopathy in patients treated with FFP (Zhang et al., 2019). However, the study performed by Etemadrezaie et al., revealed contradictory results, observing a decreased ratio of surveillance without differences in coagulopathy in patients treated with FFP (Etemadrezaie et al., 2007).

The administration of plasma has also been studied in a ratio of 1:1:1 (FFP: packed red blood cells: platelet) compared to non-ratio (Jokar et al., 2016). Patients treated with a ratio-based resuscitation had significantly lower mortality than those who did not, and crystalloid administration was

associated with increased odds of death. In addition, it was not observed an increased risk of neurosurgical intervention and intracranial hemorrhage.

Chang et al., (Chang et al., 2017) evaluated the benefits of early plasma transfusion during resuscitation in patients affected by TBI without polytrauma or intracranial hemorrhage. The authors observed that early plasma transfusion increased survival in patients affected by multifocal intracranial hemorrhage. However, this study divided the patients into different subgroups attending the brain lesion, observing significant differences between them, making it difficult to achieve a conclusion.

The benefits of FFP added to standard care have also been observed in patients affected by TBI and transferred by air from the accident scene to the Emergency Department (Gruen et al., 2020). Their results revealed an improvement of 30-day survival in patients treated with FFP. In addition, these patients received less crystalloid fluid, vasopressors, and packed red blood cells in the first 24 h, had lower international normalized ratios, lower 24 h mortality, and lower 30-day mortality. These benefits were mainly observed in severe patients. In addition, these results were also increased if the treatment was initiated early, suggesting that minimizing the time from injury to administration may be necessary.

Hyperosomolar Fluids

The use of hyperosmolar fluids is not discussed in clinical guidelines (Grände 2017, Carney et al., 2017). This fluids group contains agents such as HTS (a crystalloid solution) and mannitol, used in patients affected by TBI with cerebral edema and raised ICP (Elliott et al., 2009). Its benefits are mainly based on its activity after administering a small fluid volume during resuscitation (Hashiguchi et al., 2007). One of the main characteristics of HTS is its capacity to improve cardiovascular output and cerebral oxygenation while reducing cerebral edema. In addition, innate immune-cell functions seem to be modulated by hypertonicity, specifically neutrophil burst activity, probably beneficial for modulation of the inflammatory response to trauma (Powers et al., 2003, Homma et al., 2005, Deree et al., 2007).

HTS and/or mannitol could play an essential role in mitigating the pathophysiological consequences observed in the secondary injury of the brain. In the brain, injured areas promote leukocytes congregation, causing vasodilation and peroxidase/protease-mediated cell death (Tan et al., 2016). In

addition, cell-mediated immunity could be altered, being moderated by HTS (Coimbra et al., 1996, Junger et al., 1997).

Hypoxemia results in the depletion of ATP, cellular membrane ion pump dysfunction, increased intracellular sodium levels, and endothelial cell swelling. These disturbs promote narrowing of the vascular lumen, hindering the red blood cells passing through vessels, leading to premature apoptosis of neuronal cells. In addition, a decrease of extracellular sodium reversing the direction of the Na-glutamate cotransporter could be observed due to neuronal depolarization induced by brain injury. As a consequence, an increase in extracellular glutamate is observed, increasing the neurotoxicity (Vespa et al., 1998, Koura et al., 1998). The potential benefits of HTS during resuscitation are based due to its capacity to improve alveolar gas exchange by reducing extravascular lung volume, reversing endothelial and red blood cell swelling, improving blood flow and oxygen delivery and restores extracellular sodium and cellular action potential, moderating glutamate toxicity in the brain (Shackford et al., 1994, Rabinovici et al., 1996).

During reperfusion of hypoxemic tissue, the production of radical oxygen species can propagate tissue injury. On the other hand, mannitol may limit the secondary oxidative damage in the brain due to its activity as a scavenger of radical oxygen species (Mizoi et al., 1986).

Despite these arguments, the literature did not conclude the role of HTS in patients affected by TBI. Cooper et al., (Cooper et al., 2004) did not observe statistical differences in survival outcomes comparing HTS and saline solution. Bulger et al., (Bulger et al., 2010) compared HTS, HTS/dextran, and normal saline in patients affected by TBI, evaluating the neurological outcome at six months after TBI. The study was finished early due to futility, as the interim analysis could not prove neurological status improvement or mortality at six months. However, in patients with increased ICP, HTS and mannitol effectively decreased it (Gantner et al., 2014, Rickard et al., 2014).

The literature also compared the beneficial effects of HTS and mannitol. Mangat et al., (Mangat et al., 2020) observed that HTS bolus therapy appears to be superior to mannitol in reducing the combined burden of intracranial hypertension and associated hypoperfusion in severe TBI patients. These results agree with two recent meta-analysis (Shi et al., 2020, Schhwimmbeck et al., 2021).

On the other hand, Wade et al., (Wade et al., 1997) observed that hypertonic saline-dextran solution (HSD) in patients who presented with hypotension increased survival outcomes compared to standard care. Rockswold et al., (Rockswold et al., 2009) also agree with these results,

observing a decrease of ICP and the increase of CPP and brain oxygenation in patients affected by severe TBI, especially those with higher baseline ICP and lower CPP levels. In conclusion, HTS is recommended in TBI patients without conferring a survival benefit in a general manner. However, in patients with intracranial hypertension, its benefits are higher than isotonic crystalloid solutions.

Conclusion

The literature does not observe a "gold standard" of fluid therapy on TBI treatment. In addition, the presence of acute hemorrhage or hemorrhagic shock difficult for the outcomes is frequently observed in these patients. Crystalloids and hyperosmolar fluids (especially in patients with increased ICP) could be the most beneficial treatments, being Ringer less desirable than other isotonic crystalloids. In addition, the use of plasma products during resuscitation may convey an improved outcome, especially in the out-of-hospital environment. Future clinical studies should focus on the effect of specific fluid prescriptions and osmotic agents on short- and long-term outcomes.

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Chapter 5

Effectiveness of the Healthcare Delivery Systems in Prioritizing COVID-19: Case Studies of Bhutan and Nepal

Chu Qian*
Weatherhead East Asian Institute, Columbia University, NYC, USA

Abstract

While the world was caught off guard by the 2019-2020 COVID-19 pandemic, healthcare systems in Nepal, like other South Asian low-or-middle-income countries experienced drastic consequences. As the overall system came to a halt, Nepal saw crippled general healthcare delivery, poor maternal and neonatal health planning, rising mental health issues, further socioeconomic stratification and other spillovers particularly as a result of prioritizing COVID-19 over other services, whereas its neighbor Bhutan stood out as a meaningful case of contrast despite a similar national lockdown in place. This chapter explores the features of healthcare delivery and resource allocations in the two countries to explain their differential responses to the crisis, and by doing so, looks to inspire discussions of better action plans for South Asia to prepare for similar disasters in the future.

Keywords: comparative study, Nepal, Bhutan, COVID-19, healthcare

* Chu Qian, MPH; Corresponding Author's Email: qianchu31@gmail.com.

In: Advances in Health and Disease. Volume 57
Editor: Lowell T. Duncan
ISBN: 979-8-88697-098-2

Introduction

Comparative study has long been done to draw parallels and differences between countries, and is categorized into two: 'Most Similar Systems Design' (MSSD) and the 'Most Different Systems Design' (MDSD)(Anckar, 2008). This study in particular, employs MSSD methodology as it facilitates the investigation of independent variables of systems to establish potential causality (San et al., 2021). Comparison of South Asian countries' response to this pandemic has been conducted for Bhutan, Sri Lanka and Bangladesh in the past (Bhaduri, 2020), while this study focuses on the case of Bhutan and Nepal as both countries, not far from each other, form the Himalayan mountain ranges; both of them border India and China, two regional giants that served the critical sources of transmission as well as of pandemic diplomacy during the crisis. Historically, Bhutan has a shadow cast on it by India, a country that long regarded itself as Bhutan's diplomatic guardian (Mitra & Thalitakkattil, 2018). While some studies discredited Bhutan's foreign policy as largely "band-wagoning" with India (Joseph, 2012), other studies taken a different angle say otherwise. Recognizing India's perception of China to be a major rival in the Himalayan region, as showcased in the constant geopolitical skirmishes and economic tug-of-war over nearby smaller nations, Bhutan has spent great effort in balancing its relation with both, and this outward projection of sovereignty and independence was particularly noteworthy in the Doklam standoff between the Chinese and Indian border soldiers in 2017 (Mitra & Thalitakkattil, 2018). Such awareness is also shared by Nepal, which has long been "playing" both sides for power checks (Joseph, 2012). That is to say, the geopolitical factor is comparable, despite a few limitations to be discussed in the final section.

Second, to expand on the topic of political determinant, Bhutan, for a century ruled by royal families, under the leadership of the fourth dragon king Jigme Singye Wangchuk officially adopted democratic rule in 2008 to become a "constitutional monarchy", the governance of which is complemented by Buddhist spiritual and religious guidance (Meier & Chakrabarti). Nepal, on the other hand, made the transition from King Birendra's monarchical rule to a multi-party democracy in the 1992 constitution, despite criticisms of the transition being taken advantage of by neoliberal capitalism (Shakya, 2020). As both countries conduct elections to select capable government leaders, differential nature of issues can be seen as Nepal's governing party is a constant subject of reproach of corruption and fraud (Shakya, 2020), while Bhutan a patriarchal homogenous nation whose 13% population, Nepali-

speaking Lhotshampas are excluded from general elections, together with other factors that led to their mass exodus by 2007 (Evans, 2010). Nonetheless, the level of political freedom showcased in the pandemic period is comparable among the two as The Economist's Democracy Index post-COVID-19 analysis ranked Bhutan and Nepal close to each other, (81 versus 102), both as "hybrid regime" (Economist, 2021). And in the specific context of healthcare funding, both countries rely on foreign aid, although to different extent and nature. India's early investment in Bhutan meant that it was reluctant to open the gate for international aids from other countries or organizations in the 70s despite the smallpox calamity (Bhattacharya, 2013). However, Bhutan was very sharp in recognizing the ramifications as early as the 60s when Indian funds started rolling in, first for the 5-year plan and then the hydropower station, that foreign aid dependence would subject itself to political manipulations, and thus, adjusting itself to be more aware and selective in accepting future aids (Kaul, 2021a). In comparison, Nepal's health logistic was virtually nonexistent before the 90s, and beginning in 1993, foreign aids including USAIDS of America, KfW of Germany, SDC of Switzerland etc. poured in large quantities, helping the country in establishing the infrastructure as well as of supply chain & logistics upkeep significantly, and studies found that this reality ushers in considerable risks unless the government is able to take accountability of the effective financing for its healthcare functions in the long-run (Bhuvan et al., 2016). Economically, Nepal and Bhutan belong to SAARC (South Asian Association for Regional Cooperation), a league of South Asian countries established in 1985. Heavily reliant on tourism export as a component of the national GDP, both countries' economies were hit hard by the COVID-19 crisis, and more specifically, Nepal saw a sharp drop in international tourism revenue from 2.34% to 0.71% of GDP and Bhutan from 4.37% to 3.63% (Valev, 2020). As all SAARC countries experienced capacity shortage of critical care (Bhutta et al., 2020), with beds estimated to be 0.7-2.8 per 100,000 population (Phua et al., 2020), it would be interesting to explore the differential reactionary measures taken by both countries under this common constraint.

Cultural similarities between the two are noteworthy, especially in the religious context. 80% of the Bhutanese population are Buddhists, with the main language spoken being Dzongkha (Meier & Chakrabarti). As for Nepal, the Ministry of Health's 2016 Demographic Health Survey shows that 85% of the population are Hindu, followed by Tibetans (6%) and Muslims (5%) (NDHS). The animistic feature in both religions, Buddhism and Hinduism, foretells that an anthropological aspect to their pandemic response is not to be

overlooked, as exemplified by the use of mantra prayers in deciding the auspicious day of vaccine roll-out in Bhutan's case (Rocha, 2021), and the population stronghold of folk medicine-based health-seeking behavior (80%) employing Ayurveda herbs, homeopathy, Tibetan traditional medicine and so forth in Nepal's case (Khadka et al., 2021).

Bhutan's Case Study

Bhutan adopts a Beveridge single-payer model (Tobgay et al., 2011). As it has long been promoting the idea of Gross National Happiness (GNH) as a holistic measure of well-being instead of the GDP, the government generally spends 7-12% of its budget on healthcare, and prohibits the profit-oriented privatization (Sithey et al., 2015). As the government single-handedly supplies the funding and service, removes all competitions and out-of-pocket cost for the general Bhutanese public, some of the notable benefits of the healthcare delivery system resulted are its high universality, equity, portability and accessibility (Qian, 2018), the effect of which was particularly exemplary during the 2019-2020 COVID-19 crisis where the state continued to pay for all the diagnostic and treatment costs. Had them been an individual burden, the pandemic would surely widen the gap of the rich and poor and render those underprivileged unable or even fearful of obtaining the care needed as shown in many other countries including Nepal.

Bhutan had its own resource limitations when it comes to combating coronavirus, especially surrounding the lack of medical professionals. At the onset of the pandemic, a sheer number of 50, or 13% of the country's entire body of 376 registered doctors were undergoing training in foreign institutes because the country has no medical school. Therefore, the government took a decisive move to recall this precious workforce back home (Dorji & Lucero-Prisno, 2020). Returnees were installed at the forefront of COVID-19 treatment, quarantine and contact tracing, as well as deliveries of other medical procedures and services, besides local doctors and more than 250 nurses. Every healthcare professional had been insured by the state against COVID-19 death to obtain the peace of mind for themselves and their families. In addition, as the number of hospitals in Bhutan took a drastic increase from 31 in 2016 to 48 in 2020 (Dorji, 2021; Qian, 2018), together with 186 basic health units and three regional referral hospitals, they reached a 95% population coverage (Dorji, 2021). This ease of access enabled the subsequent nation-wide distribution of rapid COVID-19 antigen test, to complement the

country's five major hospitals that offered RT-PCR testing in Thimphu, Monggar, Gelegphu, Phuentsholing and Dewathang. By the end of 2020, the country was known to have tested every one of its adult population twice, a feat seen in no other. As a result of this national unified effort, and the tremendous extent the government is shifting the financial burden to itself, it was reported that some citizens even offered to reimburse the state for their quarantine costs (2020) including budget hotels and PCR tests. For this the Ministry of Health published a fee table to give people the transparency should they insist to cover their own healthcare expenditure (Riley et al., 2020). This mentality of the citizens reflects a wish to take personal accountability as a goodwill to their king whom they have a lot of respect for.

Bhutan since the beginning has realized the importance of isolating people who came for general health services from fever patients to prevent cross-contamination during hospital visits, therefore it transformed 54 existing flu clinics into COVID-19 surveillance and testing locations for the susceptible, meanwhile allowing other services to run unimpeded such as mental health, other infectious diseases including HIV and tuberculosis, maternal and neonatal services and treatment of chronic conditions (Gyeltshen & Dorji, 2020). For example, contraceptives were delivered on-schedule to pre-determined places using mobile technology, and mothers of newborn also had their conditions monitored remotely using tele-communication. Such emphasis on primary healthcare delivery and essential drug supply has been sown long ago in 1998 when a health trust fund was established (Sithey et al., 2015), serving the foundation for emergency readiness during the COVID-19 pandemic as identified by the WHO (Kaul, 2021b).

Interview with an ex-tour operator in Bhutan uncovered a drukyul mentality best characterized by "chill" rather than "fear"; he was able to visit the monastery, play archery, have a drink with friends amidst the global panic (Qian & Sonam, 2022), thanks to the little haven his religious country provided, albeit a travel ban taking heavy tolls on Bhutan's tourism sector, similar to that of Nepal. The travel ban occurred as early as March, but the nation-level lockdown didn't happen until August, 2020 (2021; Samarasekera, 2021), giving citizens enough time to make preparation both mentally and logistically. While lockdown is of reactionary nature, Bhutan also actively takes on preventative measures. In January, 2021, upon the receipt of the Oxford-AstraZeneca (Covidshield) vaccine from India (Dorji & Tamang, 2021), Bhutan waited for two month until the propitious moment was determined by religious clairvoyants, and began the national vaccination roll-out on March 27, inoculating nearly 40% of the population in just two days

(Kaul, 2021b), and 66% in the subsequent two weeks (Rocha, 2021), greatly advancing the goal towards 70% herd immunity. While literature findings did not stop there, it is very clear from the publishing academia that the emphasis on the pandemic's adverse effect was ebbing away from general public's attention since the mass vaccination campaign started.

Nepal's Case Study

Nepal adopts a national health insurance (Mishra et al., 2015), but unlike Bhutan, its health system is heavily-underfunded, therefore Nepal takes on resource allocation very differently. Nepal's National Health Insurance Program (NHIP) was made official in 2016 with a goal to advance equity and universal access for its citizens. It is a promising improvement of the earlier community-based health insurance (CBHI) scheme in place since 1970s, thus higher enrolment and less pro-rich bias was expected (Mishra et al., 2015). As the decade-long civil war come to an end in 2006, Nepal's health system experienced a process of decentralization. While key decision-making power shifts from the Ministry of Health and Population in Kathmandu to provincial governments, notwithstanding a wishful thinking to improve efficiency and resource allocation, healthcare policies at the responsibility of provincial leaders, saw mixed results, both positive and negative as occurred in other federalized healthcare societies (Rushton et al., 2021). In actual implementation, budget allocation constraint, precious healthcare resources and a strong private sectors presence meant that the delivery of care is still subject to factors such as socioeconomic differentiations, ethnicity and caste. For instance, a study found that households of higher status were 4 times more likely to enroll in the NHIP (Ghimire et al., 2019). Other studies found that medical expenditure rendered one in ten Nepali experiencing episodes of colossal financial downturn and 1.67% falling below the poverty line (Thapa et al., 2018).

A literature review found more than thirty articles on the impact of COVID-19 poor response on Nepal's healthcare delivery, and the most conspicuous of all, a systemic failure of resource allocation in Nepal, and pointed to a unanimous complaint that by prioritizing COVID-19, Nepal's healthcare delivery is allowing people to die from other diseases (Aacharya & Shah, 2020; Bhatt et al., 2020; D. R. Singh et al., 2021). As the lockdown occurred without prior warnings or in any phased manner in March, 2020, Bhatt et al. (Bhatt et al., 2020) and Singh et al. (D. R. Singh et al., 2021)

identified the poor coordination between three tiers of government, namely, federal, provincial and local as an important factor for inferior healthcare quality and accessibility. While facilities dedicated to COVID-19 were set up, few resources were allocated to none-COVID illnesses (Aacharya & Shah, 2020; D. R. Singh et al., 2021), especially for chronic conditions. And as the pandemic fear intensified, local hospitals closed down and the remaining operating on full capacity were reluctant to admit patients; when people could not get service from the public hospital, the instinct was to go to private providers, a sector comprising a whopping 78% of Nepal's total hospital share (Mishra et al., 2015). However, these patients were often faced with a more pressing financial constraint including a $55 out-of-pocket charge for a negative COVID-19 test as the pre-requisite of admission (D. R. Singh et al., 2021), even when they exhibited no signs of infection. This fear of being denied care at the clinic doorstep for not having the test simply barred many from seeking care in the first place, if not coupled with the public mistrust of the healthcare system and the general perception that hospitals served as transmission hotspots (Karkee & Morgan, 2020; D. R. Singh et al., 2021).

In addition, due to fear and poor planning, clinics of Marie Stopes International, a major provider of family planning products and services in Nepal were shut down during the lockdown (Riley et al., 2020). Together with other factors that debilitated maternal and reproductive health, neonatal death in Nepal during the same period experienced more than 200% surge (from 13 to 40 per 1000 livebirths), and institutional stillbirth 50% (from 14 to 21 per 1000 total birth) (KC et al., 2020). Studies hailed the improved hand hygiene practice as a positive spillover effect of the pandemic, and this would in turn curb sepsis (Karkee & Morgan, 2020; KC et al., 2020). However, it seems that its effect was very limited for neonatal health. Exacerbating the situation was the closing out of transportation facilities and sanctioned freedom of movement for the sick and needy: as public transportation was discontinued and ambulance service put on hold, means to get around were restricted, deterring those seeking immediate care from getting one, including a leg fracture patient and a pregnant woman as identified in a qualitative study (D. R. Singh et al., 2021). This effect is augmented in rural areas where the accessibility was already low prior to the pandemic.

Several studies pointed to a unified concern that the paternalistic official response to the pandemic have also exacerbated the divide between the rich and poor. A Fukushima study (Neupane et al., 2021) identified daily wage workers and impoverished households engaging in informal livelihoods to be among those most severely affected, as was also discovered by Bhatt's team

(Bhatt et al., 2020). Singh et al. shone a spotlight onto the discrimination of marginalized groups based on wealth, political associations and caste when they tried to access services. Unlike in Bhutan where police tend to talk people into wearing a mask or returning home without applying paternalistic measures like issuing arrests (Qian & Sonam, 2022), institutional violence is a recurring issue in Nepal as identified by various studies (Aacharya & Shah, 2020; Bhatt et al., 2020; D. R. Singh et al., 2021). Other incidents of police abuse in enforcing lockdown, maltreatment of migrant returnees and gender-based violence at quarantine centers were also reported (Aacharya & Shah, 2020; Bhatt et al., 2020; Shakya, 2020; D. R. Singh et al., 2021), continuing to raise questions of an endemic power imbalance of the state and individual under a pandemic facade.

In addition to the above direct means, socioeconomic stratification also occurs in subtle forms such as the reduced access of basic necessities like food. As the production and supply chain of grocery items were disrupted (B. Adhikari et al., 2020), by collecting and analyzing various food price data for a pre-and-post-pandemic comparison, Singh el al.(S. Singh et al., 2020), found a substantial increase in prices of all five food categories: pulses/legumes (18%), vegetables & fruits (14%), roots & tubers (10%), cereals (10%) and animal proteins (2%), in all three study districts, Sindhupalchok, Bandiya and Jumla. As a consequence of this inflation and reducing affordability, both school meal food basket and typical household food basket contained less nutrition: vitamins, minerals, proteins, fats and calories. The study also quantified the percentage of nutrient reduction per rupee increase, the result of which, drastic enough to warn policy makers of the negative ramification of lockdown on vulnerable populations, particularly the female and children of poor households whose coping mechanism to inflation was to change their consumption patterns. This is the beginning of a vicious cycle because poor nutrition in utero and at a young age has long-term adverse implications on lost human capital, decreased productivity and lower income upon adulthood (Hoddinott et al., 2013), perpetually fixating the poor at the bottom of a rigid social hierarchy. To serve a comparison, Bhutan rolled out the cash transfer program from April to September, 2020, providing immediate financial relief and free commodities like rice, vegetables and meat to ensure food security for individuals and families at risk (Pelayo et al., 2022; S. Singh et al., 2020).

Nepal's healthcare workers including doctors, nurses, medical assistants, midwifes, hospital wards, security, lab assistants and ambulance drivers (Gupta, Mehra, et al., 2020) on the other hand, experienced high prevalence of depression and anxiety: 38%, twice that of the general population in non-

pandemic years (Gupta, Sahoo, et al., 2020; Risal et al., 2016). This was due to volatile workload, uncertain pay, shortage of protective equipment and social stigma. Being seen as the at-risk individuals, some were even asked to leave their rental apartment and rendered homeless (Aacharya & Shah, 2020; Bhatt et al., 2020; Gupta, Mehra, et al., 2020). While far from being an occupation-specific problem, mental health issues were on the rise even for the general population. Suicide cases increased by 20% before and after the lockdown (R. Singh et al., 2020), and other mental health morbidities became more and more prominent as a consequence of social distancing, house entrapment, domestic violence, stigma against infected or recovered individuals and a lack of consultation services (Bhatt et al., 2020; D. R. Singh et al., 2021; R. Singh et al., 2020). By comparing suicide deaths with that of pandemic casualties it was found that the former overpowers the latter, echoing the concern that a national agenda prioritizing COVID-19 is harming the population in other unforeseen ways.

In contrast to Bhutan's adoption of the Druk Trace app which requires individuals to register every visit to a public place in a database thus greatly facilitating surveillance (Dorji & Tamang, 2021), Nepal had no established e-health system. As scientists called for more aggressive contact tracing procedures in Nepal (Bhatt et al., 2020), it is likely that the country would benefit systemically from similar mobile technologies. Yet grass-root adoptions of telemedicine during the lockdown had been attempted with success by a few specialist doctors of dermatology and blood cancer, in saving a rural girl from a life-threatening toxic epidermal necrolysis (Paudel & Chudal, 2020), as well as ensuring timely follow-up and scheduling of chemotherapy sessions for leukemia patients (Poudyal et al., 2020). Among these civilian efforts, Poudyal et al. obtained positive feedback from leukemia patients who were consulted through a free text/call app Viber, the use of which is encouraged to continue post-pandemic (Poudyal et al., 2020). This smart phone technology enables the timely exchange of critical test results between patients and doctors and during the trial none of the registered user missed his or her chemotherapy. Interesting enough to also note that the Viber text exchange also helped bypass police custody and eliminate hostile interaction as patients were traveling to get their chemo sessions, although no formal agreement was made with the law enforcement (Poudyal et al., 2020). Sanguine outcomes using telemedicine were also found in another study, as WhatsApp (similar to Viber) was used to communicate the impression of toxic epidermal necrolysis (TEN) between a female patient and her dermatologist in order to prevent life-threatening drug-induced TEN due to the use of

antiepileptic carbamazepine (Paudel & Chudal, 2020). Thanks to the prompt visual diagnosis using video calls, the culprit drug could be discontinued, and treatment initiated for the severe skin condition which, had it been left untreated, could cost the rural girl her life during the lockdown. Another focus, as Singh et al. pointed out, should be for chronic disease patients, such as those with obstructive pulmonary disease, hypertension and diabetes (D. R. Singh et al., 2021), to ensure stable communication throughout infrastructure shutdown. These interventions carried out by doctors from the Kathmandu Civil Service Hospital and Nepal Police Hospital reflected the active participation of civilian Nepalese practitioners in trying to better the healthcare delivery for patients during lockdown.

Conclusion

The intense national focus on COVID-19 has undermined the attention deserved by other types of health burdens in Nepal. Among infectious disease burdens, Nepal's COVID-19 mortality remained low at 1.09% (3,061 deaths out of 281,564 infections) as of April 14, 2021(MOHP). In comparison, the 2009 influenza A/H1N1 pandemic had a case fatality ratio of 1.74% (B. R. Adhikari et al., 2011). Moreover, influenza data during the pandemic became skewed, if not a complete absence of flu case reporting between July and September in 2020, posing a stark contrast from previous years when incidence increased steadily in the same period (Pun, 2020). Similarly, another study warned of a large dengue outbreak as a result of the overwhelmed, fully-allocated health system and the fact that COVID-19 high impact areas overlap with dengue-endemic regions (Koirala & Tamrakar, 2020). Regarding non-communicable diseases (NCD), those with chronic conditions like cardiovascular disease or diabetes, those who suffered from physical injuries and mothers of newborns simply could not receive timely care as mentioned. According to WHO, as of 2016, NCD account for two thirds of deaths (66%) in Nepal (WHO, 2016), therefore to forgo this bulk interest areas for the low-fatality COVID-19 is considered unwise, not to mention that there are other more deadly infectious agents worth pursuing as discussed above. One might argue that the low fatality was a result of focusing national effort on coronavirus prevention and treatment, as shown in a survey which found a satisfactory rate as high as 71.4% in Nepal's general public's perception of the government's response to the crisis (Madhu et al.), whereas a more targeted study on frontline healthcare workers reported a grisly 36% satisfaction that

in turn, predicts health workers' unwillingness to fulfill duties during the pandemic (Upadhyaya et al., 2020). People's perception gradually improves as time goes on; as the surprise factor ebbs away and fear diminishes, the system gets better at adapting. This foretells the benefits of a phased measure of lockdown, the kind Bhutan had adopted (Samarasekera, 2021): although Bhutan's travel ban occurred as early as March, the nation-level lockdown didn't fully take place until August, 2020 (2021); even so, the lockdown enforcement in Bhutan was moderate: police would not arrest or manhandle those who violated the curfew or mask rules, and routine primary care services such as maternal health and chronic disease care would be kept uninterrupted (Dorji, 2021).

Therefore, the cause of system chaos might not be of poor healthcare quality as much as of the paternalistic fashion with which the society shuts down itself, taking everyone and every sector by surprise. In late April, 2021, another indefinite lockdown of Kathmandu Valley was proposed by the Coronavirus Control and Management Committee (CCMC), this time, with a three-day notice only (Awale, 2021). This is not to say that response shouldn't occur fast. As noted by many studies, the case of Nepal was particularly challenging because it shares a 1600km porous border with India (B. Adhikari et al., 2020; Bhatt et al., 2020; D. R. Singh et al., 2021), where cases were constantly imported with Indian visitors traveling to or migrant workers of Nepali origin returning to the country (Chalise, 2020; K. Sharma et al., 2021). It is also because of this particular geographical reason that experts believe Nepal would benefit from aggressive contact tracings (Awale, 2021). However, as was mentioned above, Nepal is a country where there is a state power lean and of whom the lockdown had the gravest impact are the poor and meek subject to maltreatment by the law authority. Therefore, it is difficult to ensure that rigorous contact tracing like the one they did in Bhutan won't cause woes in Nepal. Regarding the need for better allocation of resource or funds referred to by various studies, in hindsight of this pandemic (Aacharya & Shah, 2020; D. R. Singh et al., 2021) and as a perpetual theme of studies on Nepal's healthcare system (Mishra et al., 2015; J. Sharma et al., 2018; Thapa et al., 2018), it is due to such complexity of the healthcare structure, the inter-working of different tiers of government and the many stakeholders at play that it is far from a simple task to come up with the perfect action plan for an imminent threat like COVID-19. Nevertheless, useful advice is present in many of these studies and all that the policy maker has to do was to crowdsource insights. By doing so one is surely to be impressed by some of the spontaneous development of the field, for instance of the adoption of

telemedicine in bridging the gap of the urban and the rural, rich and poor, healthy and sick.

The in-depth comparison of Nepal and Bhutan also unraveled a power wrestle between the two South Asia giants: China and India. India's first batch of vaccine export sufficed by the "Neighborhood First" narrative of advancing international goodwill and offering humanitarian aid to countries in need was to Bhutan and Maldives; one day after, another batch containing one million doses was shipped to Nepal (B. Singh et al., 2022). Seen as a strategic move to redefine its relation with Nepal, India's pandemic diplomacy would potentially alleviate some of the Chinese political influence in the region, including early on during the crisis as showcased in the donation of medical supplies such as masks, PCR testing kits, PPEs of a joint effort of the Chinese government as well as private sectors such as Alibaba Jack Ma Foundation (Nirola et al., 2021). This comparison of the two countries' response is nonetheless limited due to the following. First, Nepal's porous border with India made it more vulnerable to cross-country transmission; its population is more than 38 times that of Bhutan, rendering the latter nimble at maneuvering through crisis and adapting to shifting demands, and compared to Bhutan's rather homogenous society made up of Tantric Buddhists, Nepal's ethnic composition is diverse; people follow a number of religions and speak a range of dialects, thus making it difficult to transmit critical information and prevent the spread of fake news. And last but not least, there could be a fundamental selection bias in conducting review of this kind, as the diverse Nepali academia is robust and avid in publishing critiques of its own healthcare system, when a similar introspection could be lacking in Bhutan, potentially due to a self-censoring mentality as a result of the 1992 constitution banning criticisms of the king and the system (DOS; Zam, 2018). With this in mind, this chapter hopes to draw insights among the two, not to be critical towards any one party, but to outline the lessons learned to bring out better change in the future.

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Chapter 6

Immune Function Is Depressed with Aging While Inflammation Is Heightened: An Enigma

Stephen C. Bondy[1,*] and Meixia Wu[2]

[1]Department of Occupational and Environmental Health and Department of Medicine, University of California, Irvine, Irvine, CA, USA
[2]Evergreen World ADHC, Westminster, CA, USA

Abstract

There is evidence of declining effectiveness of the immune system with aging. This must reflect a diminution of immune surveillance mechanisms, and gradually leads to the emergence of several harmful tendencies. The ability of the aged to mount an effective defense against micro-organisms and to respond well to vaccination are both impaired. These account for the increased susceptibility of the aged to bacterial and viral disease. Such diminished immune function with aging takes place despite increasing levels of inflammatory activity. The ensuing elevation of inflammation with no readily recognizable antigenic trigger, leads to compromised tissue function. The increased presence of irrelevant inflammatory activity can result in elevation of oxidative stress and these events together can proceed to adversely effects including organ damage. Additionally, failure of the immune system in the elderly is also reflected by a growing incidence of autoimmune disease. It seems that the defective immune system of age is not just increasingly quiescent but progresses to a different form of action that, while being ineffective in performing its classical role, is expressed in a new faulty configuration leading to harmful rather than favorable outcomes. This article describes some possible mechanisms underlying such a transition. Finding means

* Corresponding Author's Email: scbondy@uci.edu.

In: Advances in Health and Disease. Volume 57
Editor: Lowell T. Duncan
ISBN: 979-8-88697-098-2

of impeding of this shift is also discussed since this could alleviate many of the diseases associated with senescence.

Introduction

It is well known that the elderly are especially susceptible to infectious disease. This is a reflection of the declining ability of the cells involved in the immune response to mount an operative defense against invasive microorganisms. This shortcoming is evident in both the innate and adaptive immune systems. The innate immune system is intrinsic and is rapidly and non-specifically activated in response to a pathogen or injury. The adaptive immune system involves a slower creation of specific antibodies designed to neutralize an invading antigen. The immune response changes with age so as to diminish both resistance to infection and the ability to respond favorably to vaccines despite heightened levels of inflammation [1]. Chronic activation of monocytes and macrophages can limit their subsequent ability to respond to vaccines and to novel pathogens.

"Inflammaging" is a term used to describe the increase in inflammatory markers found in the elderly, with no apparent utility and not in consequence of reaction to an identifiable exogenous stimulus. The transition from active immune intervention to a muted but ongoing inflammatory state is especially pronounced in the aging CNS where persistent neuroinflammation characterizes several neurodegenerative states and is particularly marked in Alzheimer's disease. Redundant neuroinflammation in COVID-19 may further accelerate the progression of age-related brain inflammation. Elderly individuals are more at risk of developing lethal inflammatory outcomes after SARS-CoV-2 infection [2, 3]. Inflammatory cytokines can certainly be of benefit to the CNS in the presence of invasive organisms, but chronic microglial activation can lead to adverse pathological and behavioral modifications [4]. While neuroinflammation may have a beneficial effects in promoting tissue defense and repair, with age, this positive aspect can be overshadowed by more random destructive inflammatory events. The age associated decline in immune function following a weakened response to vaccination results in to increased enhanced susceptibility to bacteria and viruses [5]. In addition, inaccurate targeting by immune cells with aging can lead to an elevated incidence of autoimmune disorders [6].

Several factors have been suggested to be the cause of these undesirable changes of immune functioning during senescence. These features are

discussed in conjunction with mechanisms that may that lead to reduced effectiveness of immune function despite increased inflammatory activity.

Events That May Initiate Age-Related Excessive Inflammation

Several of the attributes of aging may foster deleterious changes in immune function. These are separately discussed and also summarized in Figure 1.

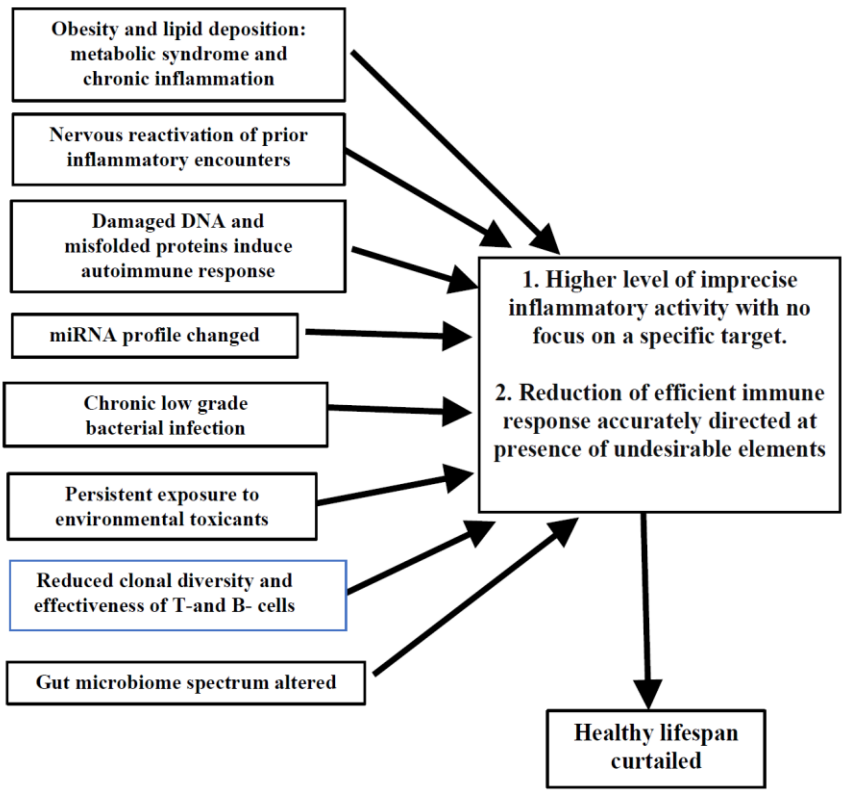

Figure 1. Age-promoted Changes in Immune Functioning. This figure summarizes the metabolic and physiological changes, that take place with aging that adversely influence efficient immune function and elevate non-productive inflammation.

Age-Related Changes in Profile of Immune Cells

The generation of new immune cells by hematopoiesis is needed for the whole lifespan. However, the variety of stem cells effecting this becomes reduced with age. A decrease of clonal diversity is accompanied by an increased expansion of surviving memory clones which often have pro-inflammatory characteristics [7]. This reduced genetic variance leads to both shrinkage of the repertoire of circulating pluripotential naive cells, and to increased emphasis on memory cells [8]. Less new B cells are generated with age, and their responsiveness to antigens is diminished. B-1 cells from the aged have a lower secretory capacity and repertoire diversity and also are reduced in number [9]. This is combined with the recruitment of less effective T cells and dendritic cells [10]. Naive T-lymphocyte production is further reduced in number due to age-related thymic involution. In this manner, older adults have less naive T-cells able to respond to the stimulus of novel antigens, and a higher proportion of experienced T-cells with memories of past challenges. Naive B cells also decline in number due to their reduced proliferation [11].

The elderly would seem to be protected a large library of immune memory relating to past infectious diseases and the ability to respond to their recurrence. However, such remembered responses are not always effectively retained since T cell memory established in youth, can deteriorate in the latter part of life [8]. The antibodies produced by B cells of the aged are generally of lower affinity and are less protective than those produced by young animals [12]. In consequence, the ability to adapt to new infectious stimuli is reduced with age.

The age-related remodeling of the immune system (immunosenescence) involves the adaptive immune system and to a lesser extent, the innate immune system. While there is a higher basal level of NF-kB and TLR-initiated inflammatory activity in the intrinsic as well as the adaptive components of the immune system of the elderly [13] there is also a diminished inflammatory upregulation of inflammatory cytokines after exposure to an exogenous antigenic stimulus [14]. This diminished responsivity in conjunction with elevated basal immune activity is characteristic of inflammaging. This altered status accounts for the increased vulnerability of the aged to many infectious diseases including flu and COVID-19 [7, 11, 15, 16]. Many aspects of COVID resemble an accelerated form of inflammaging in that both are typified by increased numbers of inflammatory monocytes, depletion of effective lymphocytes, and depressed numbers of naive T cells [17]. The continuing elevated basal level of inflammatory activity found with senescence results in

reduced functioning of the innate immune system by desensitizing constituent cells, thus impairing their capacity to react to key immune signals such as such as those derived from infection or vaccines [18]. Loss of effective adaptive immunity combined with increased nonspecific innate immunity, leads to older individuals being less resistant to infection and cancer while being more liable to chronic tissue inflammation.

Although functioning of both B and T lymphocyte components of the adaptive immune system are known to decline with aging, the effect of aging on the performance of the innate immune response is less well understood. Functional changes occurring with age have been reported for neutrophils, macrophages and dendritic cells [19]. In the case of macrophages, aging promotes a shift from the more inflammatory M1 phenotype, toward the more anabolic M2 phenotype which can lead to impaired immunosurveillance [20]. Dendritic cells appear to enhance background inflammation and autoimmune reactions while simultaneously hindering robust responses to infection [21]. These changes are aligned with, and can enhance, parallel changes in the adaptive immune system.

Continued inflammation can produce a counterbalancing immunosuppressive effect by way of expansion of myeloid-derived suppressor cells (MDSC) which activate a general immunosuppressive network [22]. MDSC are also overexpressed in tumors where they protect cancer cells from immune attack and are elevated with age especially in the frail. However despite handicapping the immune response, MDSC can also produce inflammatory cytokines and thus form the basis of indiscriminate inflammation [23]. Decreased cell responsiveness to immune stimuli together with heightened indices of inflammation have been specifically attributed to defective signaling of the JAK-STAT pathway [24].

Changes in the Immune System with Aging May Be Affected by the Bacterial Composition of the Gut Microbiome

The intestinal microbiome may cause priming of T-cells and this may broaden the spectrum of memory cell types. Around 20% of all lymphocytes are found in the gut where they are exposed to a wide range of potential antigens. In consequence, the microbiome plays a significant role in influencing the development of both memory T and B cells [25]. However, the bacterial diversity of immune regulating bacterial species within the gut declines with age while the number of pathobionts potentially provoking an inflammatory

response is increased [25]. These age-associated shifts in the spectrum of gut microbiota may promote inflammation and inhibit effective immune reactions.

Age-related changes in the composition of gut microbiota may impact the chronic inflammatory status often associated with aging [26]. Transfer of the gut microbiota from old mice to young mice initiates innate immune and inflammatory responses mimicking "inflammaging" [27]. Conversely, a mixture of live organisms derived from healthy infants administered to aged mice, prevented fat-induced inflammation and metabolic dysfunction, and retarded physical decline [28]. These reports constitute good evidence for the causal role of gut bacterial composition in the emergence of age-related inflammation.

All adult age groups, appear to possess a common core of the more abundant bacterial taxa in their microbiomes. However, a shift toward more *Ruminococcus, Coprobacillus,* and *Eggerthella* genera becomes marked with increased biological age, and this is independent of chronological age [29]. As biological age increases, overall gut microbiota diversity decreases, while some microbial taxa associated with unhealthy aging emerge. The changes taking place in the gut microbiota with advancing biological age can differ strikingly from those occurring with chronological age. Thus it is important in aging studies to use a biological or functional measure rather than to simply rely on chronological age [30]. Aging is obviously a very heterogeneous event with much variance between the elderly. Very old adults show a high degree of diversity among colonic microbial taxa. *Proteobacteria* and *Firmicultes* appear to have a higher abundance in this successfully aging population. Overall it appears that the presence of certain bacterial species within the gut such as *Akkermansia* may reduce inflammation, and diminish risk for onset of metabolic and cognitive dysfunction among the aged. However the very aged also have higher levels of *Proteobacteria*, in the gut, a phylum generally associated with increased inflammation. Inflammation is lower in the healthy long-lived, than in more typical older adults, and their pro-inflammatory status appears to be compensated for by parallel anti-inflammatory activity [31].

While there are major differences between healthy and hospitalized aged, underlying conclusive generalizations have yet to emerge. The lifestyle of the aged is subject to a very large number of variable factors. Together with very diverse life histories, this complexity accounts for the present unsatisfactory state of knowledge concerning aging and the microbiome. Further understanding of the topic is important as it offers a practical means of modulating the aging-inflammation paradigm.

Age-Related Epigenetic Changes Are Likely to in Part Account for the Reduced Effectiveness of the Senescent Immune System

Epigenetic changes effected by the environment rather than the nature of coded DNA itself are likely to underlie the malfunctioning of the senescent immune system. Different miRNAs involved in regulation of inflammation can act either by altering rates of production of inflammatory mediators or by influencing the extent of DNA acetylation and histone methylation at distinct sites. These have been well-tabulated in a recent review [32]. The up-regulation of specific miRNAs promoting anti-inflammatory activity such as miR-21, miR- 146, miR-223, and miR-29, found in the elderly, may represent attempted compensatory processes to curb the excessive inflammatory activity occurring with age [15].

Analysis of age-related epigenetic changes showed distinct gender based differences. Older females had higher genomic activity for adaptive immune processes while older males had higher activity for innate immune function and inflammation [33]. The precise means by which miRNAs can act to alter gene expression of immune-related proteins remain to be unraveled [34, 35].

Chronic Low Level Infection May Be a Contributor to Many Age Related Diseases

There is evidence that both viral and bacterial infections that are continuing can provoke continuous inflammation. It is now believed that this can contribute to diseases not originally suspected to have an association with exogenous organisms. This has been most unequivocally found in the case of the association with *Heliobacter pyloris* and gastric ulcers, but emerging evidence implicates the potential importance of infectious agents in Alzheimer's disease [36] and with intervertebral disc degeneration [37]. Chronic low grade viral infection may also cause T-cells to develop shortened telomere length leading to replicative senescence. This is associated with loss of memory cell function and diminished immune self-tolerance [38, 39].

Accumulation of Damaged DNA May Underlie Some Age-Related Autoimmune-Based Inflammation

Aging is associated with increased levels of damaged extranuclear DNA which can form an inflammatory stimulus [40]. The accumulation of damaged DNA resulting from inadequate repair can be a source of auto-immune reactivity [41]. An association between impaired DNA repair processes and systemic lupus erythematous (SLE) has been reported [42]. The cytosolic appearance of oxidatively damaged mitochondrial DNA is known to contribute to SLE progression [42]. A parallel intolerance to self-DNA may also contribute to the inflammatory changes found in rheumatoid arthritis [43], another disease of autoimmune origin. A distinctive form of myasthenia gravis involving loss of tolerance to the nicotinic acetyl choline receptor, has a late onset only appearing in those over 50 years age. This is characterized by defective mitophagy and extrusion of oxidized mt-DNA [42]. Mitochondrial DNA can be released into the cytosol following mitophagy and this leads to activation of inflammasomes and production of pro-inflammatory cytokines [44].

Neurons within the Insular Cortex Be Activated Thus Restoring Memory of Earlier Systemic Inflammatory Experiences

A recent report found that inflammatory processes incurred early in life, can be restarted long after the experience, upon re-activation of that cortical region originally responding to an inflammatory event [45]. It appears that immune reactivity can be influenced by mentation alone. Such recollected events are more liable to occur in older individuals who have accumulated a larger number of inflammation- initiating experiences. It has been noted “it was shown almost 150 years ago that presenting patients allergic to pollen with an artificial flower is sufficient to induce an allergic response” [45]. This type of recapitulation which has potentially maladaptive consequences illustrates the close relation between immune and nervous function.

Tissue Barriers to Pathogen Entry Are Weakened in the Elderly

The skin is a primary means of defense against environmentally prevalent pathogens. With age, dermal tissues display reduced effectiveness of immunological vigilance. The failure of barrier immune function leads to both increased susceptibility to infection and to cancer [46]. Diminished immune efficacy is likely to contribute to the increased incidence of cancer that develops with aging [25]. The blood-brain barrier (BBB) is weakened, allowing increased infiltration of peripheral T and B cells from the adaptive immune system into the brain. Age-related dysfunction of these penetrating cells may modify their predisposition from acting in promoting regenerative processes, to a more pathogenic inflammatory style. This takes place during normal aging and even more so with neurodegenerative disease [47]. While both restorative and pathogenic formats encompass activation of immune responses, regenerative episodes are more usefully targeted than more indiscriminating diffuse inflammation [13, 48].

Extended Exposure to Air Pollution and Other Xenobiotics May Elevate Systemic Inflammation and Depress Immune Efficiency

Exposure to urban air high in nanoparticulate content is known to lead to systemic inflammation [49]. The cells of the nervous system seem especially susceptible to inflammation induced by contaminated air. Such exposure has been associated with elevated levels of protein markers of neurodegenerative disease. This has been attributed to abnormal activation of microglia, the major resident intrinsic immune cells of the brain [50]. An acceleration of normal aging processes by poor air quality is likely, and polluted air has been linked to several dementias and to age-related cognitive decline [51]. The relation between particulates and the bound metallic and organic components bound to their surfaces, are significant determinants of their overall immunotoxicity [52, 53].

Misfolded Proteins in an Aggregated Form within the Cell, Can Cause Persistent Inflammation

Since intraneuronal amyloid protein is apparent prior to the appearance of markers of inflammation in Alzheimer’s disease, it is probable that the

presence of such aggregated proteins can cause persistent harmful inflammation. Misfolded proteins containing β-sheets are not readily degraded and accumulate with age. They are likely to catalytically induce similar undesirable structural changes in neighboring normal proteins and thus effect spreading of enduring proteinaceous intracellular inclusions [54]. These can induce a pro-inflammatory response and bring about the chronic, low-grade inflammation that typifies aging [55].

The Incidence of Type 2 Diabetes and Metabolic Syndrome Are Increased with Age

Metabolic syndrome is a combination of disorders including glucose intolerance, hypertension, dyslipidemia and obesity, centered on insulin resistance as the likely underlying cause of pathogenesis. It poses a significant risk for progression to Type 2 diabetes. Many of the changes involved in metabolic syndrome are associated with elevated levels of inflammation [56]. There is evidence that vascular inflammation precedes and initiates the development of metabolic syndrome, and T2D rather than being a result of these disorders [32] Obesity is increased in the elderly population, and adipose tissue itself is a source of macrophage activation, production of pro-inflammatory cytokines, and thus a state of systemic inflammation [57]. Such metabolic stress can expand the decline of immune effectiveness occurring with age [58]. Hyperglycemia resulting from Type 2 diabetes is known to advance senescence [59]. Even a small number of senescent cells can produce widespread effects due to the ability of senescence-associated secreting phenotype(SASP) to produce inflammatory factors that can be systemically distributed [60].

Potential Therapeutic Strategies

While the deterioration in immune function with aging may to some degree, be inevitable, there are several means by which the speed of this waning may be slowed down. These are discussed and in addition summarized in Figure 2.

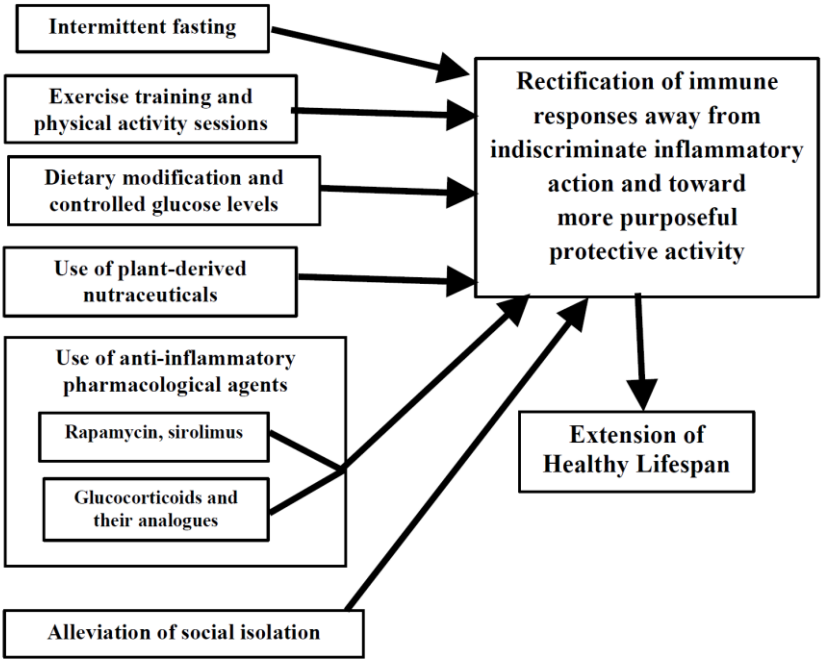

Figure 2. Strategies for Maintenance of Immune Functioning with Age.
This figure encapsulates the range of potential approaches to the problem of maintaining effective immune reactions during senescence. These potentially synergistic lines of attack discussed in the text, are suggested to improve quality of life in the aged.

Use of Materials Derived from Plants

Many plant extracts are known to curb basal ongoing inflammation but do not appear to block desirable immune responses to pathogens or vaccines. These include well known chemically defined materials derived from plants such as resveratrol, curcumin and berberine. Curcumin increases expression of TREM2, a receptor expressed on microglia that stimulates phagocytosis and suppresses inflammation, while decreasing expression of CD33, which inhibits microglial phagocytosis [61]. These immunomodulatory changes may be mediated by the ability of curcumin to downregulate miRNA-155 which is involved in inflammatory processes [62]. Other agents in this group are omega-3 polyunsaturated fatty acids and melatonin [63]. There are few reports of adverse effects following extended use of such agents. Their mechanisms

of action are diverse and generally not focused on a single intracellular target. Sites at which they may be active include modification of the miRNA expression profile, induction of sirtuins, support of pancreatic pancreatic beta cell functioning [64]. In addition to suppression of background inflammation, these phytochemicals can inhibit cell division, protect against pro-oxidant events and modulate gene expression. As a result they have been posited to act as tumor suppressors and general age-retardants [65]. The breadth of targets impacted by these chemicals renders understanding and documentation of their chief beneficial mechanisms difficult, but such intricacy could also account for their lower toxicity relative to more selective pharmacological agents. It may be that the intervention at multiple sites provided by nutraceuticals is optimal for combatting the many-faceted inflammaging process.

Diet, Fasting and Physical Activity

The qualitative and quantitative nature of the diet are critical determinants of the inception of metabolic syndrome. Where diet is appropriate in both constitution and amount, the likelihood of immunosenescent change is diminished [66]. Periodic fasting and dietary restriction-mimicking drugs have anti-inflammatory effects by blocking the secretion of inflammatory cytokines. These treatments may allow cellular reprogramming and suppress delay the onset of senescence [67]. The dietary approach may slow the onset of several age-related neurodegenerative conditions by mitigation of inflammation [68].

There is good evidence that physical activity can have a positive effect on immune aging and decrease the extent of maladaptive responses [69]. Regular exercise reduces age-related chronic inflammation, increases autophagy, improves mitochondrial function, and insulin sensitivity [70]. Some benefits of physical activity may ensue from the ability of exercise to modify the composition of the gut microbiota [71].

Chronic Use of Anti-inflammatory Drugs

Steroids

Dexamethasone has a potent anti-inflammatory capacity and, when administered at an early stage, its regular application has to been reported to

retard the rate of progression of scrapie, a prion disease of sheep [72]. Treatment with this glucocorticoid analog, reduced the cytokine profile away from inflammatory forms and toward more anabolic and immunosuppressive peptide species. Despite the finding that dexamethasone administered in the preclinical stage of scrapie resulted in reduction of vacuolation and scrapie pathological protein deposition, there was also evidence of treatment leading to increased astroglial and microglial activation and GFAP levels were raised [72]. It is likely that glial immune responses can play protective or destructive roles depending on the nature of their activation. This may explain why, in a human trial, minocycline which has immunosuppressant properties, worsened the condition of ALS patients rather than effecting any improvement [73]. While steroids can be very useful in mitigating effects of acute inflammation such as that found in COVID-19 where the inflammatory response is especially hazardous for the aged [74], extended treatment with endocrine anti-inflammatory agents may not be helpful in treating age-related inflammation.

Rapamycin and Metformin

The mechanistic target of the rapamycin (mTOR) signaling pathway is a highly conserved network involved in the maintenance of immune homeostasis. Downregulation of this system leads to immunosuppression, extends lifespan and delays the onset of several age-associated diseases [75]. Malfunctioning of this pathway can result in autoimmune disease. Therefore, targeting mTOR may be a strategy to attenuate overall aberrant inflammaging [76].

The biguanide metformin is an anti-diabetic drug which can mimic calorie restriction and effect SASP inhibition, is able to reduce age-related inflammation and promote longevity in experimental animals [77, 78]. A clinical trial of the ability of metformin (1500 mg per day), to retard age-related processes is currently in progress.

Sociological Considerations

The elderly are more likely to become socially isolated. A meta-analysis of 14 papers indicated that some indices of inflammation such as increased C-reactive protein (CRP), and cytokine IL-6 are associated with the self-reported experience of loneliness [79]. Feelings of isolation can negatively affect the health of older people and immune malfunction may be a major factor in this. Addressing this problem can be accomplished with relatively ease and could lead to widespread benefits.

There is a good evidence that inadequate or disrupted sleep can initiate excessive inflammation and that some of its adverse health effects are due to this [80]. Another remediable factor is excessive exposure to a high level of noise. This can be considered an environmental pollutant and can both directly and secondarily produce heightened levels of general inflammation [81].

Conclusion

While sporadic immune activation is important in contending with infection and injury, unrelenting inflammation is the major cause of death in the world. Over 50% of all deaths have been ascribed to diseases encompassing inflammation-linked deficits, including cardiovascular disease, neurodegenerative disease, cancer, Type 2 diabetes, kidney disease, fatty liver disease and autoimmune disorders [82].

The diminished signal/noise ratio of the aging immune system triggers an impaired ability to react to antigenic stimuli, combined with excessive but not selective inflammation. While some of these changes may be inescapable, the rate of establishment of this state can be influenced by several factors which relate to the overall aging process. Specific disorders such as neurodegenerative diseases, diabetes, and excessive weight can all accelerate inflammaging. Such adverse changes characteristic of aging cannot be totally avoided, but addressing these issues by medical or dietary interventions, is to be expected to reduce the tempo of the progressive buildup of the inflammatory burden. Nonetheless, the maintenance of acute inflammatory responses is essential for normal physiological regulation and for survival [57]. Successful aging is likely to involve a good balance between pro-inflammatory and anti-inflammatory influences. It has been proposed that immune changes observed during aging may in part only represent an adaptation to a challenging environment [83]. The challenge is to channel the dynamics of immune function away from indiscriminate responses and toward more targeted optimum performance.

The Roman dramatist Terentius believed *"senectus ipsa est morbus"* (old age itself is a disease). Such a perspective should encourage early intervention in the aging process in order to mitigate or delay some of its consequences.

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Chapter 7

Pyogenic Spondylodiscitis

Balaji Zacharia[1,*], MD and
Afsal Valiyaparambil[2], MD

[1]Department of Orthopedics, Government Medical College, Kozhikode, Kerala, India
[2]Department of Orthopedics, Government Medical College, Kozhikode, Kerala, India

Abstract

Infection of the spine can involve the vertebral body, intervertebral disc, spinal canal, or adjacent soft tissues. The most common mode of infection is hematogenous. Contiguous spread from the nearby focus of infection and direct inoculation are other routes of infection. It constitutes approximately 2 to 7% of infections of the musculoskeletal system. Pyogenic spondylodiscitis is common in young children and the elderly. Diabetes mellitus, immunodeficiency, HIV infection, and intravenous drug abuse are some of the predisposing features. Staphylococcus aureus is the most common pathogen, and Staphylococcus epidermidis, E. coli, and Pseudomonas are other organisms causing pyogenic spondylodiscitis. The infection can be acute, subacute, or chronic. Pyogenic spondylodiscitis has an indolent course. Most of the cases present with low back pain, which is not related to the activity. Rest pain and night pain are common. Constitutional symptoms are less common. Abscess formation and neurological symptoms are rare. The most common investigations include routine blood examination, ESR, and CRP. Blood cultures are positive in 25% of cases. Radiographs, CT scans, MRI scans, and PET scans are useful imaging modalities. The majority of cases can be treated nonoperatively using antibiotic therapy. However, surgery is indicated in cases with instability and neurological

* Corresponding Author's Email: balaji.zacharia@gmail.com.

In: Advances in Health and Disease. Volume 57
Editor: Lowell T. Duncan
ISBN: 979-8-88697-098-2

deficits and in cases that do not respond to antibiotic therapy. In this chapter, we discuss the epidemiology, clinical features, investigations, and management of pyogenic spondylodiscitis.

Keywords: vertebral osteomyelitis, pyogenic spondylodiscitis, pyogenic discitis; infections of the spine

Highlights

- Infection of the vertebra and intervertebral disc is called spondylodiscitis.
- It is common in children and the elderly
- Pyogenic spondylodiscitis commonly affects a single level.
- Hematogenous spread is the most common mode of spread
- Staphylococcus aureus and E. coli are the most common
- MRI is the most sensitive investigation
- Nonoperative treatment is effective in most cases.

Introduction

Spondylodiscitis is an inflammation of the vertebral bodies and the intervertebral disk space. It is a complex disease of multifactorial etiology. The majority of pyogenic spondylodiscitis (PSD) is due to monobacterial infection. Pyogenic spondylodiscitis represents approximately three to five percent of cases of osteomyelitis. An annual incidence of 0.4 to 2.4 per lakh population is reported from Europe [1, 2]. There is high mortality in PSD. The overall mortality is 5 to 10% [3]. There are two peaks of occurrence, one in patients younger than 20 years and another in elderly between 50 to 70 years. There is a male predominance with a male to female ratio of 1.5 to 2:1 [4]. Malnutrition, chronic steroid intake, immunosuppression, diabetes mellitus, HIV infection, spinal surgeries, rheumatoid arthritis, and obesity are common risk factors [5]. The common modes of bacteria reaching the spine are hematogenous, direct invasion by trauma or surgery or contagious spread from an adjacent source of infection.

Pyogenic vertebral osteomyelitis can affect vertebral bodies, intervertebral disc space, spinal column, and adjacent soft tissues. The vertebral body is the most common site. The intervertebral disc can be affected

by the hematogenous route or direct inoculation during spinal procedures. Spinal column infection is an infection developing around the dural sheath. It can be within the epidural space, a spinal epidural abscess, or within the spinal cord, an intramedullary abscess. Adjacent soft-tissue involvement is common in children. They present as a paraspinal abscess, retropharyngeal, or psoas abscess. The lumbar spine is the most common area, followed by the thoracic and cervical spine [6].

Bacteriology

Spondylodiscitis can be divided into pyogenic and nonpyogenic. Nonpyogenic infections are due to tuberculosis, fungi, and parasites. Staphylococcus aureus is the most common organism causing PSD. Its incidence is 30 to 80%. Escherichia coli is responsible for 25% of infections. E. coli infection is associated with genitourinary infections. Pseudomonas aeruginosa infection can occur in intravenous drug abusers. Staphylococcus epidermidis is another organism causing PSD. Salmonella infections are common in people with sickle cell anemia. Group B streptococci can cause PSD in patients with IE (infective endocarditis). Skin commensals can rarely cause vertebral infections, so we must be careful before labelling such organisms as contaminants when we obtain them in cultures. Anaerobic organisms are rare, as are multiple organisms causing PSD. Anaerobes such as Propionibacterium acne can cause indolent postoperative discitis. Another anaerobe, Bacteroides fragilis, can cause a contiguous spread of infection from the pelvis and abdomen [7].

Pathogenesis and Pathology

The infecting organism can reach the spine by three routes. Hematogenous, direct inoculation or contiguous spread from a nearby source. Hematogenous spread can occur either via the venous route or via the arteriolar route. The hematogenous spread from a distant focus in descending order is the genitourinary tract (17%), the skin and soft tissues (11%), intravascular devices (5%), the gastrointestinal tract (5%), the respiratory tract (2%), and the oral cavity (2%) [8]. The Batson plexus of the vertebral venous plexus has direct communication with the extra vertebral pelvic and retropharyngeal venous plexuses. There are valveless veins connecting the perivertebral

venous plexus with the meningorrachidian veins. Infection from other sites can spread through the Batson plexus of veins to the spine [9, 10]. According to Trueta, the bacteria lodge in the endarteriolar capillary loops in the vertebral body. The arterial supply to the vertebrae comes through the metaphyseal vessels. These vessels bifurcate and provide blood supply to adjacent endplates. This is due to the peculiar development of the spine, where the upper half of the lower sclerotome and lower half of the upper sclerotome fuse to form the vertebral body. Hence, infections are common in the paradiscal regions of the adjacent vertebrae [11, 12]. Percutaneous or open surgical procedures can inoculate infecting organisms into the intervertebral discs. For postoperative infection to occur, large quantities of bacteria ($>10^5$ organisms) must be present at the surgical site, soiling of the site of surgery in the immediate postoperative period, or there must be bacteremia to bring the organisms through the hematogenous route [13]. Penetrating injuries of the spine can also lead to direct inoculation of the organisms and cause PSD [14]. Infections from the surrounding areas, such as retroperitoneal, intraabdominal, or retropharyngeal areas, can cause contiguous spread into the vertebral column [15].

In adults, the intraosseous arteries within the vertebral bodies are endateries. Septic emboli can be trapped in the vertebral body and can cause extensive destruction of the vertebral body. The destruction produces wedging of the body and kyphotic deformity. Kyphosis leads to instability and neural compression. Later in the infection, paravertebral abscesses are formed. Tracking of this abscess into the epidural space can cause neurological deficits due to compression. Thrombosis of vessels can cause ischemia of the cord and neurological deficits. A rare cause of neurological deficit without vertebral body or disc involvement is a spinal epidural abscess. It is characterized by a triad of fever, back pain, and signs of meningeal irritation [16].

The adult intervertebral disc is a relatively avascular structure. The destruction of the disc in adult PSD is not due to direct involvement. This is due to a lack of diffusion of nutrients from endplates. The endplates are destroyed by proteolytic enzymes. Therefore, intervertebral disc destruction occurs late in adults. In children, there is extensive anastomosis of vessels in the vertebral body, and there is direct communication between intradiscal vessels and metaphyseal vessels. Therefore, there can be early infection and destruction of discs. The rich vascular anastomosis in the vertebral body prevents its early destruction. Therefore, pyogenic discitis is a common presentation in children younger than 8 years [17].

Once the infected emboli reach the vertebra through the end arteries. It causes local inflammation. Fluid extravasation from the vessels causes a rise in intratrabecular space pressure. This causes a further compromise in vascularity. This leads to necrosis of the vertebral body. This ischemic cascade leads to vertebral body destruction. The enzymatic destruction of the endplates leads to the involvement of the intervertebral disc. The pus formed can track posteriorly and form a dorsal abscess in the paraspinal region. It can track beneath the anterior longitudinal ligament, but it is rare in PSD. Anterior tracking in the cervical spine can cause a retropharyngeal abscess. In the lumbar spine, it can lead to psoas abscess. If the destruction is above the levator ani muscle, it can track to the inguinal region, and if it is below the levator ani, it can produce an abscess in the ischiorectal fossa. The abscess can also track along the vessels and produce abscesses in the thigh, popliteal region, or beyond that. The neurological deficits are due to direct compression by epidural abscess, necrotic material, granulation tissue, sequestrum, and disc fragment. Vertebral destruction and the resulting instability can lead to deformities. This deformity can cause compression of the spinal cord. Rarely ischemic injury to the cord can occur due to thrombosis of the spinal arteries. The prognosis of such injuries is poor. Hematogenous pyogenic spondylodiscitis can affect any region of the spine. The most common site is the lumbar spine (58%), the second thoracic spine (30%), and the least common in the cervical spine (11%) [18].

Clinical Features

Pyogenic infection of the spine usually has an indolent course. The clinical features depend on the virulence of the organism and host defense mechanism. The extent of destruction of the vertebral body or disc can cause an abscess, neurological deficits, or deformity secondary to instability.

Most patients have a predisposing factor for infection. Many of them may remember recent infections in the genitourinary tract, respiratory tract, gastrointestinal tract, or skin. The initial symptom may be nonspecific low back pain. There can be a delay of one to two months for the diagnosis. The most common symptom is unremitting low back pain. The pain is usually constant and exacerbated by movements. The pain worsens during the night and is often not relieved with rest. It can radiate to the abdomen, perineum, hip, buttock, or legs. A low-grade fever is present in the majority of patients.

The patients notice an evening rise in temperature. Some may complain of loss of weight and appetite [19].

The abscess formation can present as severe stiffness, dysphagia, or torticollis. In the dorsal spine, it can cause neurological deficits in the lower limb or girdle type of pain in the chest. As described above, the lumbar abscess can track many regions. An epidural abscess can cause neurological deficits. An untreated abscess can cause persistent discharging sinuses [20].

Vertebral body destruction can lead to loss of height or wedging of the vertebral body and localized segmental kyphosis. A lateral listhesis can lead to scoliosis; both of these can cause central canal or neural foraminal stenosis [2].

Neurological symptoms can occur suddenly or in a gradual manner. The involvement of vertebrae above D11 can cause paraplegia. Lumbar spine involvement can cause cauda equina syndrome. Neurological symptoms can occur in one-third of cases. The severity can range from mild dysesthesia or weakness to severe paralysis with sphincter loss or radiculopathy. Motor symptoms are common because the compression is from the ventral aspect. Sensory symptoms are usually delayed [21].

Compared to adults, children with pyogenic infections of the spine usually have discitis. Vertebral body involvement is rare in children younger than 8 years. Similar to adults, it has an indolent course. The diagnosis is usually delayed by 2 to 4 weeks. The infection is severe in neonates and young children. In this age group, it is usually associated with sepsis and multiple infections. There can be the destruction of the vertebra with kyphosis. The symptoms and signs are mild in toddlers and preschool children. They often have a fever, and neurological deficits are rare in this age group [22].

The spinal epidural abscess has a dramatic course. It classically presents with back pain, back tenderness, and fever. Later, signs of meningeal irritations appear. There can be radicular symptoms that are followed by motor weakness, bowel or bladder involvement, and sensory deficits evolving very rapidly (within 24 hours). It is an orthopedic emergency [23].

Investigations

Pyogenic osteomyelitis of the spine may be misdiagnosed initially, or the diagnosis is usually delayed. A high index of suspicion must be present on the part of the treating physician. They must suspect the possibility of PSD in

patients complaining of back pain, fever, and/or neurological deficit following a recent infection.

A hemogram shows an elevated leukocyte count with neutrophilia. In some cases, a hemogram may be normal. Acute serum inflammatory markers, such as erythrocyte sedimentation rate (ESR) and C reactive protein (CRP), will be elevated in most cases of PSD. However, both of these parameters lack specificity. CRP is a more sensitive measure than ESR. A reduction in CRP levels can be used to monitor the response to treatment [24].

Conventional radiographic changes of spondylodiscitis may appear later. At least 30% of bone destruction may be there to be detected on conventional radiographs. It is difficult to diagnose infection using radiographs in areas such as the posterior elements such as the facet joint and sacrum or junctional areas such as the cervico-dorsal, dorsolumbar, or lumbosacral areas. The earliest changes in the vertebral body are demineralization or osteoporosis. Thereafter, there is blurring, resorption, or erosion of the vertebral endplates. Later destruction and collapse of the vertebral body result in deformity. Finally, there is a loss of intervertebral disc space due to its destruction. However, in children, there is the early destruction of the intervertebral disc. In short, plain X-ray shows osteoporosis with endplate destruction and loss of intervertebral disc space [25].

A computed tomography (CT) scan has the advantage that it can detect the infection early. It is also helpful in detecting infections in the junctional areas and posterior elements of the spine. It can also detect the extent of bone destruction, fragmentation, epidural compression by bone fragments, and soft tissue calcification in PSD. The deformity can be assessed better with a CT scan. The air pockets detected in the vertebrae due to gas formation detected by the CT scan. These are known as emphysematous osteomyelitis of the spine [26].

Magnetic resonance imaging is the most sensitive and useful investigation in PSD. It is non-invasive and has no risk of radiation. It can detect infection very early. The bone marrow edema was detected as hypointense on T1WI, isointense on T2WI, and hyperintense on STIR images. It can detect the extent of involvement in the vertebral body, posterior elements, endplates, and discs. It can also show the extent of epidural compression by the disease and deformity. Another advantage of an MRI scan is that it can detect satellite and skip lesions [27].

Fluorodeoxyglucose positron emission tomography (FDG-PET) is a sensitive and specific test to detect infections of the spine. However, both infections and malignancies will show increased uptake, and hence, differentiation between the two may be difficult. This is a very useful investigation when MRI is contraindicated for various reasons. When both MRI and FDG-PET scans are contraindicated, a bone scan is an alternative for detecting spondylodiscitis [28].

Isolation and identification of the organism from tissues is the gold standard for establishing the diagnosis of infections. Blood culture will be positive in the phase of bacteremia. It has been found that in spondylodiscitis cases, positive blood culture results will be obtained in 30 to 78% of cases. In the majority of cases, the patients might have taken empirical antibiotics or antibiotics for a pre-existing infection, which may decrease the chance of obtaining a positive blood culture. We have to do both aerobic and anaerobic cultures. It has been shown that there is an increased chance of obtaining a positive blood culture immediately following an open biopsy.

Regarding vertebral biopsy, one can perform an open biopsy or a CT-guided percutaneous biopsy. A CT-guided biopsy is the preferred initial method. Due to the low amount of sample obtained, CT-guided biopsy can be negative in many cases. In such cases, an open biopsy is indicated. Specimens should be sent for culture for atypical organisms and an extended bacterial culture that can yield organisms with low virulence. The use of molecular methods such as polymerase chain reaction (PCR) allows a more accurate diagnosis [29, 30].

Classification

According to the duration of the disease, spinal infections are classified as acute, subacute, and chronic. Acute spondylodiscitis is one with symptoms of less than 3 weeks duration. When the symptoms persist for more than 3 weeks up to 3 months, it is subacute PSD, and when symptoms persist for more than 3 months, it is chronic. In 2017, Pola et al., proposed a classification based on clinical and radiological features. This is an MRI-based classification with major criteria, such as spinal instability, epidural abscess, and neurological compromise, and minor criteria, such as an intramuscular abscess. They also proposed a treatment algorithm based on this classification [31] (Table 1).

Table 1.

Types	Features	Treatment
A	All cases without biomechanical instability, epidural abscesses, or neurological involvement	
A1	Simple discitis without the involvement of vertebral bodies	Rigid orthosis immobilization
A2	Spondylodiscitis involves the intervertebral disc and adjacent vertebral bodies	Rigid orthosis immobilization or percutaneous stabilization
A3	Spondylodiscitis with limited involvement of paravertebral soft tissues	do
A4	Spondylodiscitis with unilateral (A.4.1) or bilateral (A.4.2) intramuscular abscesses	do
B	Includes cases with radiological instability of significant bone destruction without epidural abscesses or neurological involvement	
B1	Destructive spondylodiscitis without segmental instability	Rigid orthosis immobilization or percutaneous stabilization
B2	Destructive spondylodiscitis extended to paravertebral soft tissues without segmental instability	do
B3	Destructive spondylodiscitis with biomechanical instability and segmental kyphosis	Destructive spondylodiscitis with biomechanical instability and segmental kyphosis
C	All cases with neurological compromise or epidural abscesses	
C1	Epidural abscess without neurological symptoms neither segmental instability	Rigid orthosis immobilization or percutaneous stabilization with closer clinical-radiological monitoring
C2	Epidural abscess and segmental instability without neurological impairment	Open debridement and stabilization
C3	Epidural abscess and acute neurological impairment without segmental instability	Open debridement and decompression
C4	Epidural abscess and acute neurological impairment with segmental instability	Open debridement, decompression, and stabilization

Differential Diagnosis

The most common differential diagnosis for pyogenic spondylodiscitis is nonpyogenic spondylodiscitis. Tuberculosis of the spine must be considered and ruled out in all cases of PSD. This is particularly true in TB endemic areas. It is difficult to differentiate between the two by clinical and imaging features. Biopsy and culture are used to differentiate between the two. The course of pyogenic spondylodiscitis is more acute than that of tuberculosis. Skip lesions are common in tuberculosis. MRI scans can differentiate between the two; a

large thin-walled abscess with subligamentous spread and skip lesions on MRI are usually suggestive of tuberculous spondylodiscitis [32].

Brucellosis is another infection of the spine that can mimic pyogenic spondylodiscitis. It is a systemic disease involving the heart and nervous system. In younger patients, it can present with arthritis or sacroiliitis, and in elderly patients, it can cause spondylodiscitis. L4/L5 or L5/S1 is the most common site. It can cause a local infection in the anterior vertebral body or diffuse involvement of the whole vertebra. It can cause isolated discitis or spondylodiscitis. On imaging, the vertebral architecture is maintained despite panvertebral involvement. There can be sclerosis in the vertebra. Small paraspinal abscesses or gibbus deformities may be present. Large parrot beak osteophytes may be seen in some cases in X-rays. The serrated appearance of the anterior vertebral margin is also seen in the X-ray. The combination of anterosuperior corner destruction (Pedro Pons' Sign) in combination with vertebral sclerosis and osteophyte formation is pathognomonic of brucellosis [33]. Fungal infections such as aspergillosis, blastomycosis, and candida infections are common in immunocompromised patients. Parasitic infections such as hydatid disease can also involve the spine.

The other differential diagnosis includes inflammatory and degenerative disorders or malignancies affecting the spine. Inflammatory conditions such as acute pyelonephritis, appendicitis, intraabdominal abscess, or bowel ischemia can mimic acute PSD. The Anderson`s lesion in ankylosing spondylitis is a disco vertebral lesion due to inflammation that can mimic spondylodiscitis. Both primary and secondary tumors of the spine are the differential diagnosis for PSD [6].

Treatment

The aims of the treatment of pyogenic spondylodiscitis are 1. To eliminate the focus of infection. 2. To relieve pain. 3. To regain function 4. To prevent and correct complications such as deformities or neurological deficits. The majority of cases of spondylodiscitis can be treated by nonoperative methods. Some cases require surgical treatment.

Nonoperative treatment is indicated for mild uncomplicated spondylodiscitis. However, there are reports of complete recovery of neurological deficits with antibiotic treatment alone. The nonoperative method consists of antibiotic therapy for the elimination of infecting organisms. The antibiotics are chosen according to the culture report and their bioavailability

in the spine. In cases where culture is negative for any organism, an antibiotic sensitive to Staphylococcus aureus or E. coli must be given. Flucloxacillin, cefazolin, and ceftriaxone are common drugs used for MSSA. Vancomycin, daptomycin, linezolid, and levofloxacin are used for MRSA. For enterococcal infections, penicillin G or ampicillin is commonly used. In penicillin-resistant cases, vancomycin or daptomycin can be used. The present consensus on the duration of antibiotics is six weeks. During the initial two weeks, intravenous antibiotics are given followed by four weeks of oral antibiotics. There is a recommendation for longer treatment with antibiotics in immunocompromised patients, but literature support is lacking for this recommendation. Percutaneous aspiration of an abscess such as a psoas abscess can be performed as part of the conservative treatment. There is a high risk for failure of nonoperative treatment in epidural abscess, recurrent osteomyelitis, or diabetes mellitus. If no clinical or radiological improvement is seen after four weeks of conservative treatment, we have to consider it a failure. A repeat MRI may be required if a plain X-ray shows features of deterioration in a patient failing to improve with nonoperative treatment. We have to monitor the treatment by weekly checking inflammatory markers to assess the effectiveness of treatment [34].

Antibiotic treatment is combined with analgesics for pain relief. There is no literature evidence for bed rest as a treatment for PSD. However, a limitation of activity has been shown to reduce pain. The orthosis cannot reduce pain or prevent deformity in spinal osteomyelitis. However, orthoses are commonly prescribed for pain relief in PSD.

Surgical Treatment

The most common indication for surgery in PSD is taking a biopsy when CT guided biopsy fails to yield a positive culture. Currently, endoscopic biopsy has replaced the need for open biopsy in most cases. It can also be used for debridement and drainage of an abscess.

The indications for surgery are as follows:

- Failure of conservative treatment
- Spinal epidural abscess
- Neurological deficit

- Presence of a ventral paravertebral abscess > 2.5 cm
- Sepsis
- Kyphosis >15 degrees
- Vertebral body collapse >50%
- Translation >5 mm.

The surgery must be performed as early as the earliest indication of a nerve root or cord compression. There are reports of complete recovery even after paralysis. The prognosis is not good in cases where surgery is performed after the onset of paralysis. Surgery aims to decompress the neural tissue, debridement of the infected vertebrae and discs, and prevent or treat instability and deformity by instrumentation. The approach for surgery in PSD can be anterior, posterior, or combined. Anterior debridement and stabilization were the standard procedures used for spondylodiscitis in the past. With modern advances in surgery, we can perform decompression, debridement, and stabilization through the posterior approach alone. In cases where an anterior void is created by extensive debridement, additional PEEK or titanium cages can be used for reconstruction of the anterior column. The posterior approach is suitable if multiple-level surgery is needed. Decompression without stabilization should be avoided. Minimally invasive techniques, such as percutaneous pedicle screw stabilization and transpedicular drainage of abscesses, can be performed. Other approaches, such as transpsoas XLIF, can also be used. There is no difference in the outcomes with various approaches if the aims of the procedure are met [34, 35, 36].

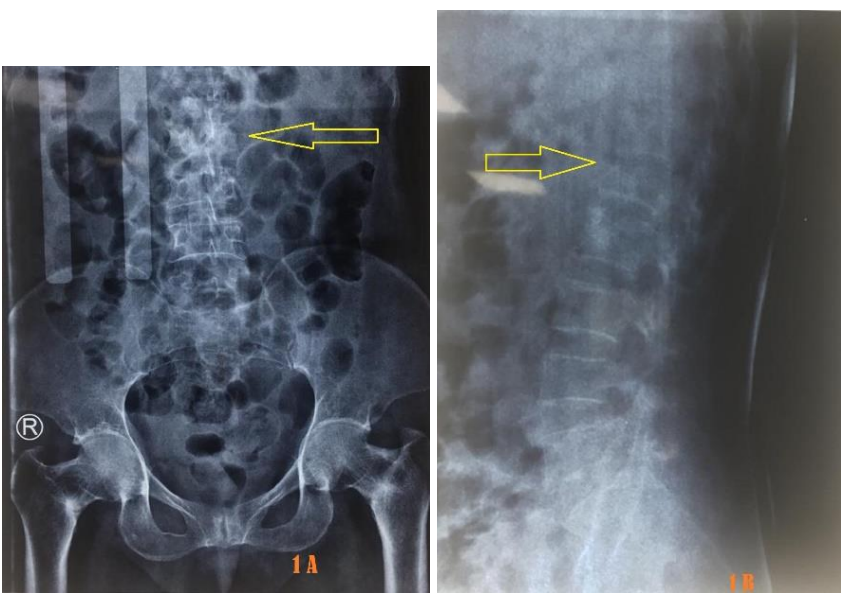
Conclusion

The diagnosis of pyogenic spondylodiscitis is often delayed. We have to suspect PSD in any patient with low back pain and constitutional symptoms. Staphylococcus aureus and Escherichia coli are the most common pathogens causing it. MRI is the most sensitive diagnostic modality. Nonoperative treatment is effective in most cases. Instability, deformity, and neurological deficits are the most common indications for surgery.

Case Examples

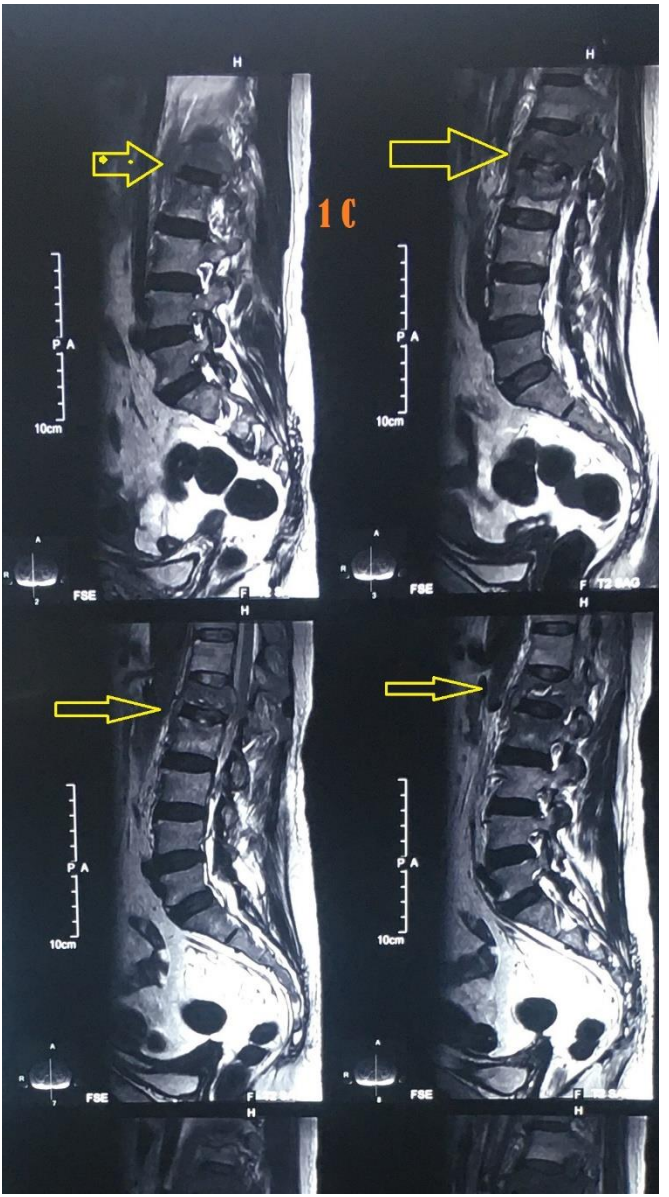
Case 1

Figure 1 (A-E): A 58-year-old woman presented with a 3-month history of progressive low back pain. She had an evening rise in temperature. Conservative treatment with analgesics and physiotherapy showed no improvement in her symptoms. An anteroposterior and lateral radiograph (1 A&B) showed destruction of the L2 vertebral body and superior endplate. There was more than 50% height reduction of the vertebral height. An MRI scan (1 C) showed STIR hyperintensities in the L1 and L2 vertebral bodies with the destruction of the body and superior endplate of the L2 vertebral body. There was an intraosseous abscess within the L2 body. There was an epidural abscess with mild compression of the thecal sac. Additionally, bilateral psoas abscess. CT-guided aspiration failed to give a diagnosis. An open biopsy (1 D) was performed through a posterior approach. Posterior instrumentation and fusion were performed (1E).



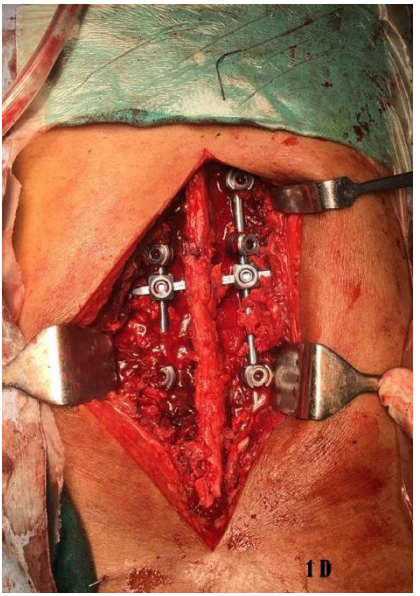
(A) (B)

Figure 1. (Continued)



(C)

Figure 1. (Continued)



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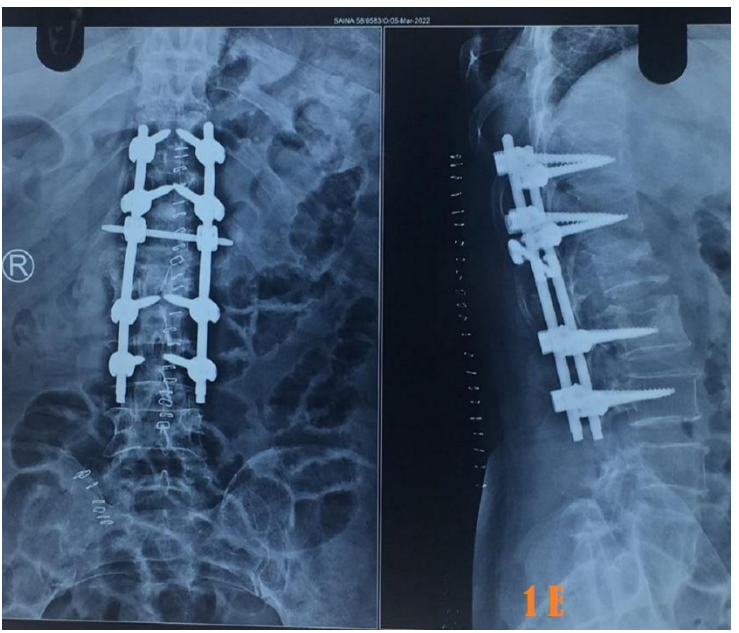
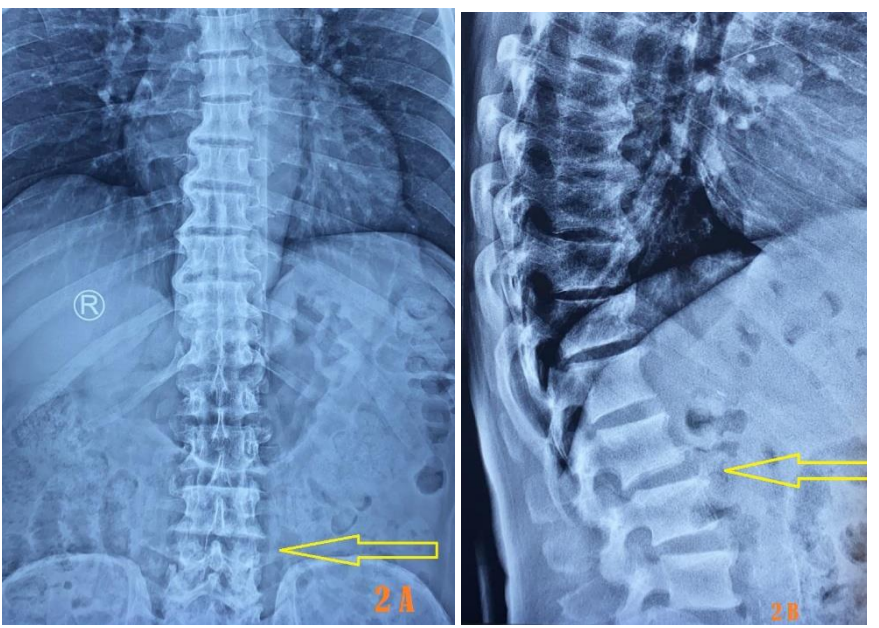


(E)

Figure 1. A-E.

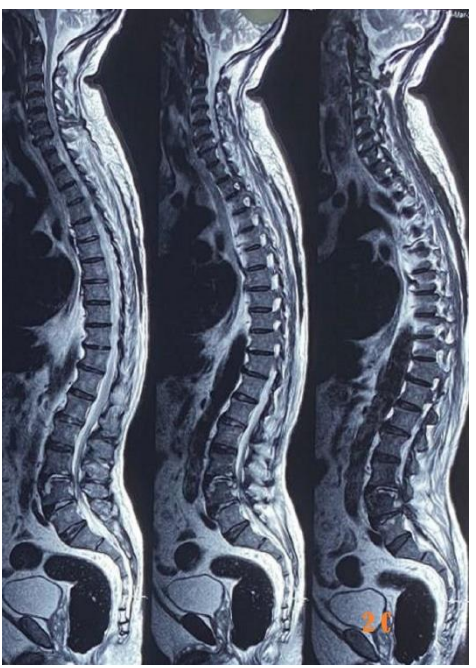
Case 2

Figure 2 (A-E): A 43-year-old man presented with a history of recent onset low back pain. He had rest pain and pain during the night. At the time of examination, there was a severe limitation of spine movements. Tests for root compressions were negative, and there were no neurological deficits. A course of analgesics for 3 weeks showed no improvements in his symptoms. Anteroposterior and lateral X-rays (2 A&B) showed irregularity in the L3/L4 disc space with syndesmophytes. MRI scan (2 C) hyperintensity in T2WI and hypointensity in T1WI with minimal anterior vertebral collection in the L3/L4 area suggestive of infection. A bone scan (2 D) showed increased tracer uptake in the same region. A CT-guided biopsy was suggestive of pyogenic spondylodiscitis with MSSA. He was given parenteral antibiotics for 2 weeks followed by 4 weeks of oral antibiotics. Posttreatment radiogram showed complete healing of the lesion (2 E).



(A) (B)

Figure 2. (Continued)



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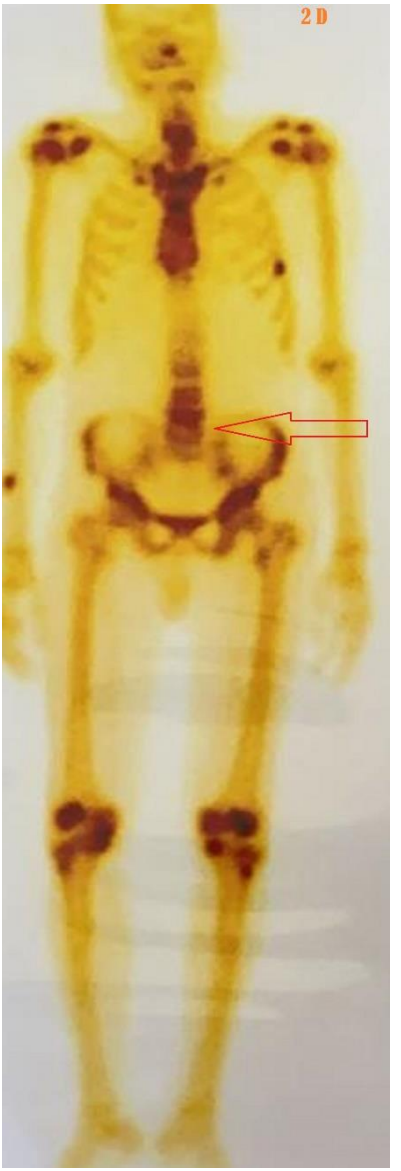
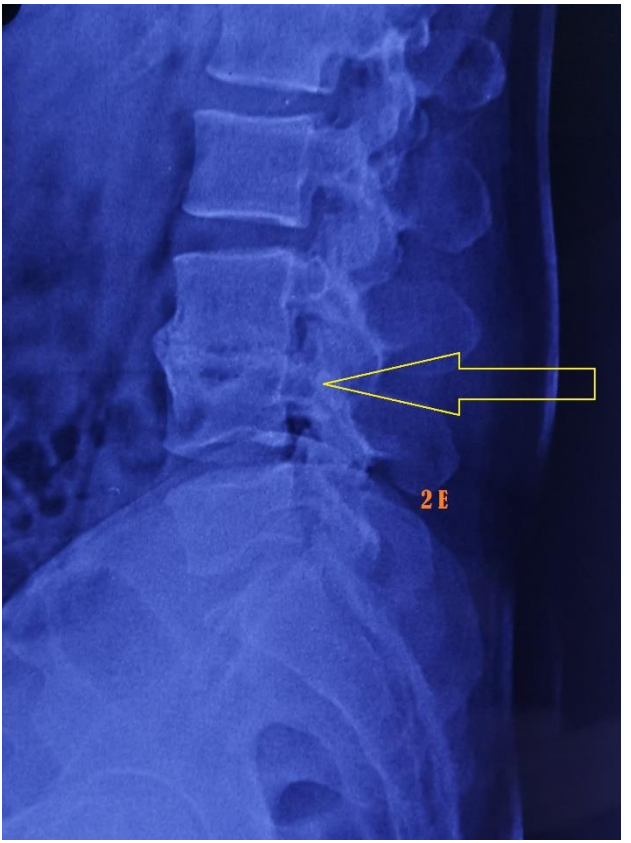


(D)

Figure 2. (Continued)



(E)

Figure 2. A-E.

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Chapter 8

Characterization of Vitamin B_{12} Compounds in Foods

K. Koseki, T. Bito, PhD and F. Watanabe*, PhD
The United Graduate School of Agricultural Sciences, Tottori University, Tottori, Japan

Abstract

Vitamin B_{12} cannot be synthesized by animals or plants as it is only produced by some archaea and bacteria, some of which can also synthesize various vitamin B_{12}-associated compounds bearing distinct base moieties in their lower ligand. Animal-derived foods, such as meat, milk, and fish are major dietary sources of vitamin B_{12}. Various inactive vitamin B_{12} compounds have been identified in foods using liquid chromatography/electrospray ionization-tandem mass spectrometry analysis approaches. In this chapter, we describe the latest information on the characterization of vitamin B_{12} compounds discovered in dietary sources.

Keywords: inactive vitamin B_{12} compounds, pseudovitamin B_{12}, vitamin B_{12}

Introduction

Vitamin B_{12} (B_{12}) can only be synthesized by some archaea and bacteria; it cannot be synthesized by animals and plants (Scheider and Stroiñski 1987). Thus, B_{12} is the only the vitamin that is absent in plant-based foods. Our investigation of B_{12} compounds in various foods revealed that some foods

* Corresponding Author's Email: watanabe@tottori-u.ac.jp.

In: Advances in Health and Disease. Volume 57
Editor: Lowell T. Duncan
ISBN: 979-8-88697-098-2

contain several corrinoid compounds with the different base moieties, such as Factor IIIm, pseudovitamin B_{12} (PseudoB_{12}), Factor A, Factor S, and B_{12} [*c*-lactone], and several modified B_{12} compounds such as B_{12} carboxylic acids (Figure 1). These B_{12}-associated compounds are inactive in humans (Stupperich et al., 1990, Hoffmann et al., 2000, Miyamoto et al., 2006, Santos et al., 2007, Watanabe et al., 2007a, Yabuta and Watanabe 2009, Hashimoto et al., 2012, Teng et al., 2014a, Okamoto et al., 2020a).

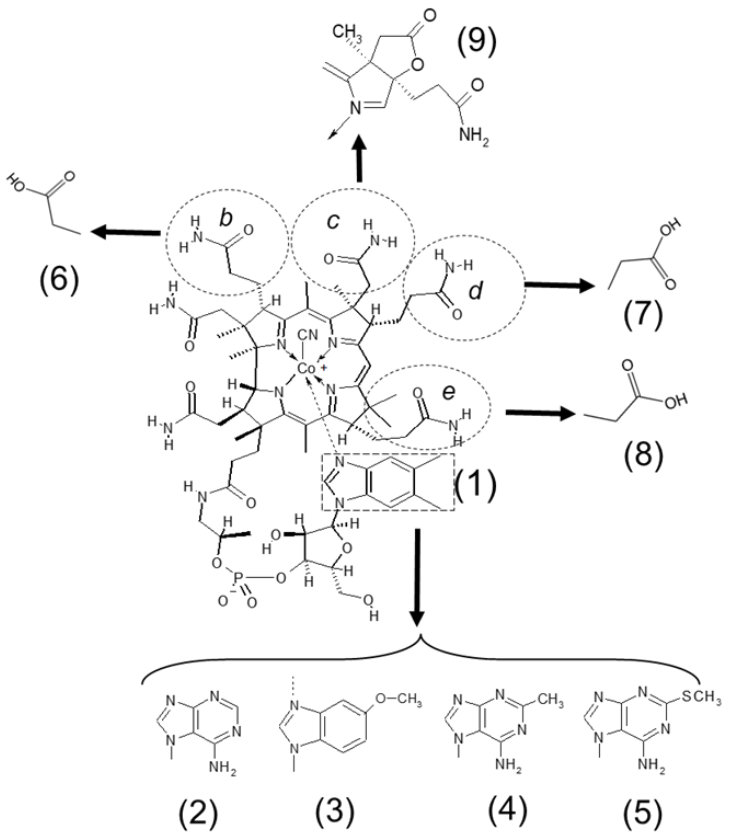

Figure 1. Structural formula of B_{12} and partial structures of inactive B_{12} compounds detected in foods: (1) B_{12}, (2) PseudoB_{12}, (3) Factor IIIm, (4) Factor A, (5) Factor S, (6) B_{12}-*b*-monocarboxylic acid, (7) B_{12}-*d*-monocarboxylic acid, (8) B_{12}-*e*-monocarboxylic acid, and (9) B_{12}[*c*-lactone].

The recommended dietary allowance (RDA) for B_{12} is determined as 2.4 µg/day in Canada, Japan, and the USA (Institute of Medicine 1998, Shibata et

al., 2013). This value is the lowest among all vitamins' RDA. To prevent B_{12} deficiency, information on the precise content of biologically active B_{12} compounds is essential. The current chapter summarizes the latest information on B_{12} compounds detected in foods.

I. Determination of B_{12} in Foods

Previously, B_{12} was present as protein-B_{12} complex forms in foods. For the investigation, B_{12} needs to be released from food proteins and converted into a specific stable form of B_{12} (cyanocobalamin) (Watanabe and Bito 2018a). The most common method for extracting B_{12} is boiling with potassium cyanide at acidic pH (pH 4.0–4.5) for 30 min (Watanabe and Bito 2018b). Furthermore, B_{12} can be directly determined in the extract using a microbiological assay strategy. *Lactobacillus delbrueckii* subsp. *lactis* ATCC7830 is widely used in the bioassay (Watanabe and Bito 2018b). However, PseudoB_{12} has the B_{12} activity in this bacterium (Watanabe et al., 1998), but it does not present any physiological activity of B_{12} in humans. Aiming to overcome the potential deficits of this bioassay, high-performance liquid chromatography (HPLC) and liquid chromatography/electrospray ionization-tandem mass spectrometry (LC-MS/MS) are commonly utilized. Before the determination of B_{12} using these integrated systems, pretreatment procedures including solid-phase extraction and/or immunoaffinity columns are widely used as efficient purification methods (Watanabe et al., 2022). Detailed methods are presented in a recent review from our group.

II. Animal-Derived Food Products

Liver and Meat

During the discovery of B_{12}, it was extracted in its red crystalline form from the liver since a diet containing a large amount of liver dramatically improved pernicious anemia, a B_{12}-deficient symptom (Scott and Molly 2012). Livestock livers containing high B_{12} are therefore a decent source of B_{12} in humans. Raw livers of cow, pig, and chicken indeed contained 52.8, 25.2, and 44.4 μg of B_{12} per 100 g of wet food weight, respectively (Ministry of Education, Culture, Sports, Science and Technology 2010). These livestock

livers contained major B_{12} and minor inactive corrinoids (approximately 10% of the total detected B_{12}), such as Factor III, Factor A, Factor S, PseudoB_{12}, and four types of B_{12} monocarboxylic acids, which were hypothetically identified as B_{12}-*d*- monocarboxylic acid and B_{12}-*e*-monocarboxylic acid, as well as two unidentified B_{12} monocarboxylic acids (our unpublished data).

Livestock meats contains approximately 2 µg of B_{12} per 100 g of wet food weight (Ministry of Education, Culture, Sports, Science and Technology 2010, Gille and Schmid 2015) while there is no information on the occurrence of the inactive corrinoid compounds.

Milk

B_{12} levels in bovine and sheep milk (0.4 µg and 0.7 µg/100 g, respectively) were considerably higher than that in human milk (0.04 µg/100 g) (Raynal-Ljutovac et al., 2008). Bovine milk has been previously reported as an important source of B_{12} in humans (Matte et al., 2014). B_{12} concentration in bovine milk varies based on the breeding state, milking time, and cow type. B_{12} levels in milk from Jersey cows are commonly lower than that in milk from Holstein cows (Miller et al., 1966, Duplessis et al., 2016). Currently, no information is available regarding the incidence of inactive corrinoid compounds from the liver in cow's milk.

Egg

Although boiled and raw whole chicken eggs contain approximately 0.9 µg B_{12}/100 g of wet food weight (Ministry of Education, Culture, Sports, Science and Technology 2010), the majority of B_{12} can be detected in the egg yolk (Doscherholmen et al., 1975) (Figure 2). Low bioavailability of B_{12} (approximately 10%) is observed in egg dishes because of the poor egg B_{12} absorption (Doscherholmen et al., 1976). In the case of Chinese traditional food product, the century egg, all the B_{12} detected in the egg yolk is associated with macromolecules (Teng et al., 2016). However, approximately half of the B_{12} egg yolk is released from the macromolecules during *in vitro* digestion. It was previously reported that chicken egg intake does not contribute significantly to an increase in B_{12} serum levels in humans (Bunchasak and Kachana 2009).

III. Fish and Shellfish Product

Fish Meet

Although B_{12} content (per 100 g of each portion) of the whole fish body is significantly higher (several times greater) in the viscera than in the meat, approximately 70% of total B_{12} detected in whole fish body (except for head and bones) were derived from the meat (Nishioka et al., 2011). Sardine and bonito dark meat contain approximately 31% and 14% of B_{12}, respectively, against the total B_{12} detected in total (dark and light) edible meats (Tanioka et al., 2012; unpublished data) (Figure 3). In particular, B_{12} content per 100 g of wet food weight was significantly higher in tuna dark meats (approximately 53 µg) when compared to the light meats (approximately 6 µg) (Nishioka et al., 2011). B_{12} content of fish meat is generally higher in big piscivorous fish compared to small fish (Watanabe and Bito 2018a). B_{12} serum levels of subjects consuming herring diets were significantly increased when compared to the livestock meat diet (Scheers et al., 2014). Thus, fish is an important contributor to high B_{12} levels in humans.

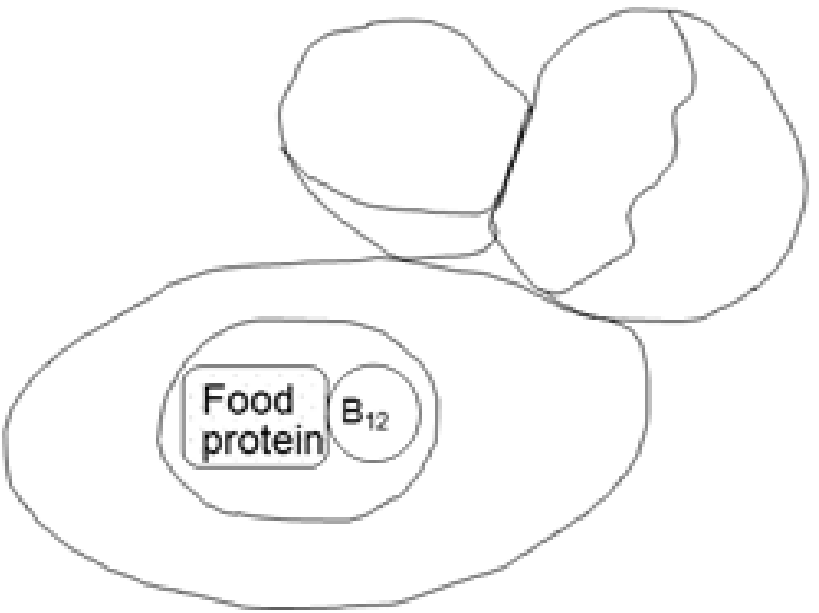

Figure 2. Distribution of B_{12} in chicken egg. B_{12} is detected only in the egg yolk and is bound to proteins.

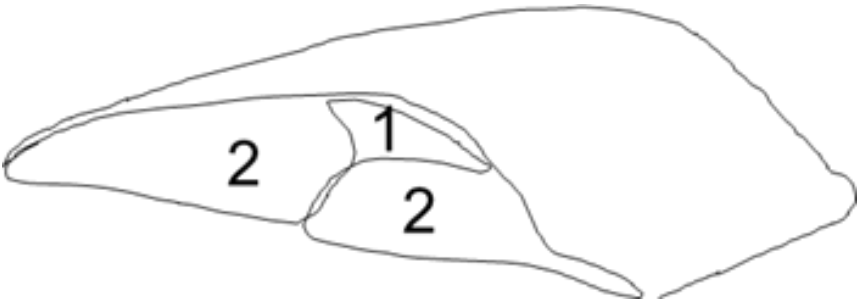

Figure 3. Cross-sectional views of fish meats. (1) dark meats and (2) light meats.

Shellfish

Although edible bivalves, such as mussels, oysters, and clams contain high levels of B_{12}, PseudoB_{12} is rarely detected in shellfish. B_{12} levels were significantly higher in edible bivalves (approximately 60 µg/100 g wet food weight) compared to edible snails (approximately 20 µg/100 g wet food weight) (Tanioka et al., 2013) (Figure 4). The variations B_{12} content and constituent B_{12} compounds among these edible snails are associated with their dietary habitats (Watanabe and Bito 2018a). B_{12} contents of shellfish meats are reported to be approximately 27 and 3 µg/100 g of wet food weight in the carnivorous ivory shells (*Babylonia japonica*) and herbivorous turban shells (*Turdo Batillus cornutus*), respectively (Teng et al., 2015a) (Figure 4). Inactive corrinoids, such as Factor IIIm and Factor S are lead compounds in escargot products, although their B_{12} and inactive corrinoid contents are relatively low (Teng et al., 2015b).

Shrimp and Crab

Approximately 2−4 µg B_{12} per 100 g of wet food weight was detected in shrimp muscles but the viscera contained significantly higher levels of B_{12} (12−33 µg/100 g of wet food weight, Figure 4) (Okamoto et al., 2020a). Commercially available shrimp viscera products were found to contain approximately 30 µg B_{12}/100 g of wet food weight. B_{12} content in lobster, crayfish, crab, and large shrimp viscera contained elevated B_{12} (7.2–118.6 µg/100 g of wet food weight) (Koseki et al., 2021). The viscera tested contained high levels of B_{12}-*b*-, -*d*-, and -*e*-monocarboxylic acids and B_{12}-*be*-

dicarboxylic acid (Okamoto et al., 2020a, Koseki et al., 2021). These B_{12} carboxylic acids are specifically located in their viscera edible portions and are inactive in humans.

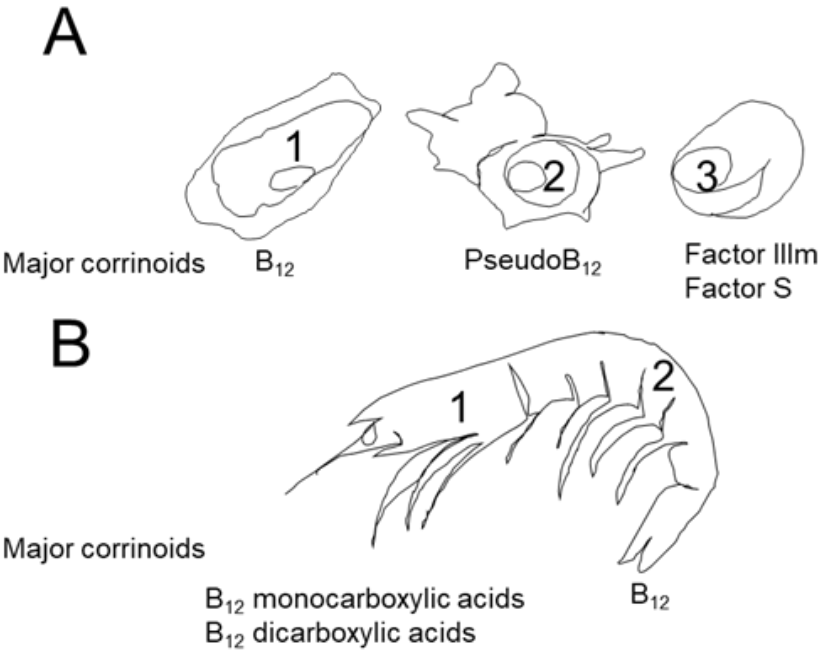

Figure 4. Major corrinoid compounds in shellfish and shrimp. (A) shellfish, (1) oyster (*Crassostrea nippona*), (2) turban shell (*Turdo Batillus cornutus*), and (3) escargot products. (B) Argentine red shrimp [*Pleoticus muelleri* (Bate, 1888)], (1) viscera and (2) muscles.

Others

Food protein-bound B_{12} malabsorption is common in elderly people (Baik and Russell 1999). Various studies have revealed the efficacy of oral administration of free B_{12} in elderly people with the B_{12} malabsorption (Vidal-Alaball et al., 2005). Foods fortified with free B_{12} are considered thus an important B_{12} contributor in elderly people (Figure 5).

In Japan, different types of fish soup stocks and extracts that are prepared with dried bonito and sardine are commonly used for flavoring or seasoning. Although the B_{12} levels in many commercially available soup stocks are very

low (trace levels), some liquid-type soup stocks contain B_{12} (approximately 5.0 μg/liter) (Nishioka et al., 2008). Most B_{12} detected in specific fish extracts are derived from free B_{12} (Nishioka et al., 2008). Short-necked clam extracts contain significant levels of free B_{12} (approximately 132 μg/100 g of food weight), although no or trace B_{12} were detected in scallop and freshwater clam extracts (Ueta et al., 2010). These results suggest that certain fish or shellfish extracts are decent sources of free B_{12} for elderly people with food-bound B_{12} malabsorption.

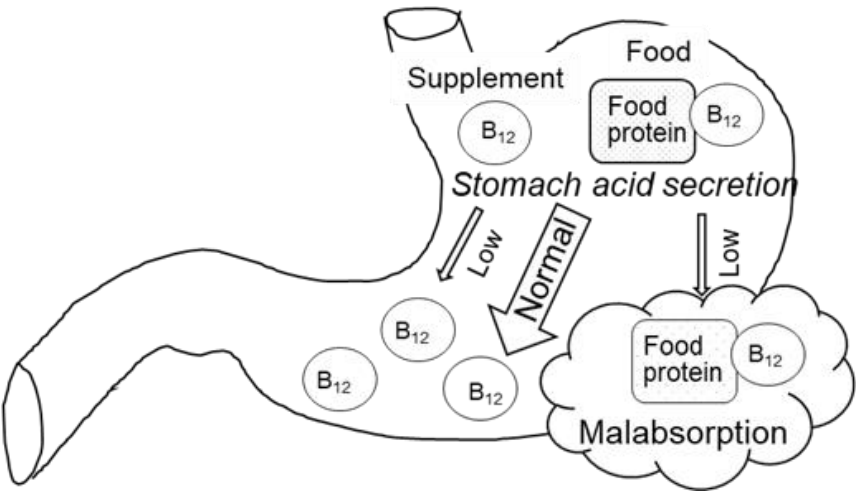

Figure 5. Outline of food protein-bound B_{12} malabsorption.

IV. Edible Insect Products

Edible insect products such as giant water bug, bee larva, grasshopper, and weaver ant contain low B_{12} levels using a bioassay. However, diving beetle and cricket products contain high levels of B_{12} (approximately 90 and 66 μg/100 g of dry food weight, respectively) (Okamoto et al., 2021). In the cricket products with high B_{12}, PseudoB_{12} (approximately 74%), and Factor S (approximately 21%) are the predominant corrinoid compounds, with B_{12} constituting only 5% of the total corrinoids (Okamoto et al., 2021). These inactive corrinoid compounds might have been synthesized by the intestinal bacteria of crickets.

V. Plant-Derived Foods

Our evaluation of plant-derived food sources with high B_{12} levels indicated that a red algae *Porphyra* products are the most optimal source for naturally occurring plant B_{12} sources (Watanabe and Bito 2018a). The red algae *Porphyra* sp. are commonly known as sea vegetables and consumed worldwide as dried nori sheet products. Various species of *Porphyra* contain substantial amounts of B_{12}, dried Chinese nori products (Zicai, approximately 60 μg/100 g), dried New Zealand nori products (Karengo, approximately 29 μg/100 g), and dried Korean nori products (Kim, approximately 67 μg/100 g) (Miyamoto et al., 2009, Watanabe and Bito 2018a). Although B_{12} detected in dried nori products was found to be absorbed in the intestine and to function as the coenzymes in B_{12}-depleted rats (van den Berg et al., 1991, Takenaka et al., 2001), the bioavailability of nori B_{12} is still unclear in humans.

VI. Microorganism-Derived Food Products

Edible Microalgae

Chlorella sp., a group of green alga, is commonly used as human food supplements (Kittaka-Katsura et al., 2002, Yang et al., 2006, Chen et al., 2008). B_{12} compounds were determined in many types of Chlorella products using bioassays. B_{12} contents of the products varied from trace to approximately 400 μg/100 g of total weight (Bito et al., 2016a). The trace B_{12}-containing Chlorella cells are aseptically grown (closed culture conditions), while the high B_{12}-containing Chlorella cells are openly grown in large vessels (open culture conditions) (Bito et al., 2016a). Among Chlorella species, B_{12} contents are significantly higher in C. pyrenoidosa compared to C. vulgaris under open culture conditions. However, Factor IIIm and a cobalt-free corrinoid were detected in certain high B_{12}-containing products (Bito et al., 2016a).

Mushrooms

Even though dried fruiting bodies of various wild mushrooms contain zero or trace levels (approximately 0.09 μg/100 g of dry food weight), B_{12} levels

(approximately 2 μg/100 g of dry weight) of black trumpet (*Craterellus cornucopioides*) and golden chanterelle (*Cantharellus cibarius*) fruiting bodies were slightly higher (Watanabe et al., 2012). However, in cultivated mushrooms, B_{12} levels of shiitake mushroom (*Lentinula edodes*) and white button mushroom (*Agaricus bisporus*) fruiting bodies were approximately 6 and 0.2 μg/100 g of dry food weight, respectively (Bito et al., 2014, Koyyalamudi et al., 2009). As shiitake mushroom fruiting bodies cannot synthesize B_{12} *de novo*, the B_{12} levels detected in the fruiting bodies could derive from concomitant B_{12}-synthesizing bacteria (Bito et al., 2014). These results indicate that these mushroom fruiting bodies should not be considered B_{12} sources in humans due to lower B_{12} content and occurrence of harmful B_{12}[*c*-lactone] even in rare cases (Bito et al., 2014, Teng et al., 2014a).

Edible Cyanobacteria

Spirulina, Aphanizomenon, and Nostoc, are established edible cyanobacteria. Even though substantial levels of B_{12} (approximately 240 μg of B_{12}/100 g of weight) were determined in commercially available Spirulina products using a bioassay (Watanabe et al., 1999, Teng et al., 2014c). PseudoB_{12} was the predominant corrinoid compound in all edible cyanobacteria products including Spirulina products. Thus, edible cyanobacteria products should not be considered decent B_{12} sources for vegetarians and elderly people who are high-risk populations for B_{12} deficiency.

VII. Traditional Fermented Food Products

Milk Products

B_{12} levels were slightly lower in fresh and soft cheeses (approximately 1–2 μg/100 g of dry food weight) compared to hard and semi-hard cheeses and washed rind cheeses (approximately 3–4 μg/100 g of dry food weight) (Bito et al., 2016b). Although the evaluated natural cheeses primarily contain B_{12}, traces of unidentified corrinoid compounds were detected in some cheeses.

Soybean, Vegetable, and Tea Leaf

Although the majority of traditional fermented plant foods such as natto, miso, tempeh, sauerkraut (pickled cabbage), otukemono (pickled vegetable), and Chinese tea leaf were found to contain < 0.5μg of B_{12}/100 g of wet food weight (Okada et al., 1983, Bito et al., 2018, Teng et al., 2014b), there are some types of stinky tofu and pickled vegetable products that contain >10 μg of B_{12}/100 g of wet food weight (Li et al., 2004). To clarify whether these products can cause an increase in the serum B_{12} levels in humans, further clinical studies should be performed.

VIII. Loss of B_{12} in Food Processing and Cooking

Cooking

Considerable decrease in B_{12} in milk products occurs during fermentation, thermal processing, and storage. A remarkable decrease in B_{12} was reported after cooking of chicken, pork, and beef meats (Watanabe 2007b). However, vacuum-packed pouch-cooking significantly reduced the loss of B_{12} in livestock meats (no loss for veal, lamb, and pork; approximately 13% loss for beef) (Nishioka et al., 2011). For fish meats, B_{12} levels of skipjack tuna and round herring meats were reduced up to approximately 85% and 62%, respectively, by grilling, boiling, frying, steaming, or microwaving, respectively (Kojima et al., 2017, Scheers et al., 2014, Nishioka et al., 2006). Accordingly, in livestock meats, B_{12} levels in fish meats were significantly decreased by vacuum-packed pouch-cooking (approximately 8% loss for salmon and approximately 28% loss for cod fish) (Creed 1995). These results indicate that the temperature and cooking time considerably affected B_{12} decrease caused by conventional cooking methods; moreover, other food ingredients were greatly correlated with B_{12} decrease.

Storage

Light exposure to milk during storage stimulated the degradation of milk vitamin B_2, which was thus degraded to form superoxide anion radicals and singlet oxygen (Allen and Parks 1979, Toba et al., 1980). These radicals

induced degradation of B_{12} corrin ring in an aqueous solution (Kräuter and Stepanek 1985). The B_{12} levels of milk products were decreased by approximately 27% after exposure to light at 4 °C for 24 h (Watanabe et al., 2000). These results indicate that a prolonged storage period of milk products under light can induce significant loss of B_{12}. In addition, the, the rate of B_{12} decrease in chocolate-flavored milk was considerably correlated with cocoa polyphenols that can promote the formation of peroxide compounds.

Food Additives

Fruiting bodies from dried shiitake mushroom and Lion's mane mushroom (*Hericium erinaceus*) do not commonly contain B_{12}[*c*-lactone] as an inactive corrinoid (Bito et al., 2014, Teng et al., 2014a). B_{12} [*c*-lactone] can be formed from B_{12} after treatment with the organochlorine antibacterial agent chloramine T. B_{12}[*c*-lactone] binds relatively weakly to an intrinsic factor involved in the gastrointestinal absorption of B_{12}, and it effectively inhibits B_{12}-dependent enzymes, methylmalonyl-CoA mutase, and B_{12}-dependent methionine synthase (Stabler et al., 1991).

Food additives such as hypochlorous acid water significantly influence the chemical and biological properties of B_{12} in aqueous solutions (Okamoto et al., 2020b). When B_{12} was treated with hypochlorous acid water, hypochlorous acid water rapidly reacted with B_{12}. The highest absorption of B_{12} was entirely eradicated while B_{12} activity was eliminated after 1 h. When the effect of the food additive on shrimp and beef meat's B_{12} content was determined, there was no significant difference in the B_{12} content of shrimp and beef meats with or without the treatment, suggesting that the food additive could not react with food B_{12} in food, as most of this vitamin detected in its protein-bound form rather than the free form (Okamoto et al., 2020b).

Author Contributions

K. K., T. B., F. W. wrote the manuscript. All authors reviewed and commented on the manuscript and approved the final version.

Funding

This work was supported by JSPS KAKENHI Grant Numbers 21K05488 (F.W.).

Notes

The authors declare no competing financial interests.

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Chapter 9

Tryptophan: An Amino Acid to Manage Biofilm Threat Efficiently

Payel Paul[1], Sridebesh Ghorui[2], Poulomi Chakraborty[1], Sharmistha Das[1], Prosun Tribedi[1] and Debabrata Ghosh Dastidar[2,*]

[1]Department of Biotechnology, The Neotia University, West Bengal, India
[2]Guru Nanak Institute of Pharmaceutical Science and Technology, West Bengal, India

Abstract

Tryptophan, a naturally occurring aromatic amino acid, is a well-reported versatile molecule in literature. Its importance as an antibiofilm agent against several biofilm-forming organisms has been studied comprehensively. In this study, the antibiofilm effect of tryptophan has been presented against strong biofilm-forming organisms such as *Staphylococcus aureus* (a Gram-positive bacterium) and *Pseudomonas aeruginosa* (a Gram-negative bacterium) as the mentioned organisms have been reported to form a plethora of infections including urinary tract infections, gastrointestinal infections, skin infections, etc. on a human host by exploiting biofilm. Towards this direction, tryptophan has shown promising characteristics in inhibiting the microbial biofilm formation of these two organisms. On exploring the underlying mechanisms, it was found that the compound, tryptophan, inhibited biofilm formation by targeting the quorum-sensing property by downregulating their respective quorum-sensing linked genes. Thus, it can be stated that the biofilm-forming ability of both these organisms can be compromised by targeting their quorum-sensing property. Apart from this, tryptophan

* Corresponding Author's Email: debabrata.ghoshdastidar@gnipst.ac.in.

In: Advances in Health and Disease. Volume 57
Editor: Lowell T. Duncan
ISBN: 979-8-88697-098-2

could also reduce the cell surface hydrophobicity resulting in the inhibition of biofilm development. It was also reported that tryptophan could be used in combination with other antibiofilm molecules for the sustainable management of biofilm threats. Hence, tryptophan could be recommended as a potential antibiofilm agent to manage the biofilm-associated infections caused by *S. aureus* as well as *P. aeruginosa*.

Keywords: biofilm, drug resistance, quorum sensing, tryptophan, quorum sensing

Introduction

According to the Centers for Disease Control and Prevention, 65% of human diseases due to bacterial infections are linked to the formation of biofilm. Biofilms are communities of sessile microorganisms (bacteria or fungi) living in a self-produced matrix. Since antibiotic therapy and host immune defense can not penetrate the biofilm defenses, the biofilm-forming bacteria cause chronic infections. They have complex survival strategies that enable them to pose constant pathogenicity by releasing virulence factors and planktonic forms.

Amino acids are organic molecules that consist of a basic amino group (—NH_2), an acidic carboxyl group (—COOH), and a unique R group (or side chain) attached to a carbon atom (known as alpha carbon atom). The α-carbon is the center of the chirality of amino acids. Therefore, all amino acids other than glycine do exist in D and L form (stereoisomers).

In the environment, bacteria shares their habitat with other diverse organisms. They secret a diverse group of effector molecules to interfere with the growth and viability of nearby organisms. One such class of molecules is D amino acids that regulate diverse cellular processes such as cell wall biogenesis, biofilm integrity, and spore germination. D-amino acid can inhibit cell wall synthesis of bacteria by altering the structure of peptidoglycan in bacterial walls. It has been proved that D-leucine, D-methionine, D-tyrosine, and D-tryptophan could replace the D-alanine in bacterial cell walls. D-amino acid could also inhibit the initial adhesion of bacteria by reducing hydrogen bonding, changing surface potential, and hydrophilicity. Thus D-amino acids, especially D-leucine, D-methionine, D-tyrosine, and D-tryptophan have well-established antibiofilm activity.

Among the amino acids, tryptophan has been extensively studied for its antibiofilm activities. The objective of this article is to elaborate on the current status of research work done on the management of biofilms with tryptophan.

Biofilm and Drug Resistance

The microbial biofilm is a complex structure of surface-adhered microbial cells that are enclosed in a layer of self-secreted extracellular polymeric substances (EPS). Approximately 50-90% of the biofilm mass is made up of the EPS layer which is mainly composed of exopolysaccharides, secreted proteins, lipids, and extracellular DNA. EPS plays a key role in the development of resistance of biofilm cells to host immune defence, as well as several conventional antibiotics, pH stress, chemical exposure, and other environmental hardships (Fernandes et al., 2021; Sharma et al., 2019). The development of microbial biofilm requires five succeeding steps: I) Initial reversible attachment of planktonic cells to any biotic or abiotic surfaces where non-covalent interactions such as van der Waals forces, electrostatic bonds and bacterial appendages plays vital roles; II) Irreversible adherence of microbial cells to that surface by exploiting hydrophobic bonds to break the repulsive forces between the cells and surfaces; III) Formation of microcolony- after reaching certain threshold concentrations, adhered cells start secreting autoinducers following expression of biofilm specific genes thereby secretion of extracellular matrix by sessile cells. Here multilayer formation of biofilm structure starts taking place; IV) Maturation of biofilm- increasing thickness of microcolony eventually directed towards the maturation of biofilm structure; V) Dispersal of mature biofilm cells- the biofilm cells start getting detached to free dispersed cells to get colonized in new surfaces (Gupta et al., 2016). Although free disperse cells seem like their planktonic counterpart, they exhibit little characteristic differences (Chua et al., 2014).

An extensive exploration of microbial existence in the natural world has shown that microorganisms are not only alive but also can flourish in extremely harsh environments which were previously assumed to be lifeless. The environment that is considered as the barrier to microbial survival preferably induces the microbial biofilm formation to provide a suitable shelter for microbial growth and reproduction. Moreover, individual cells in the bacterial community experience increased resistance to antibiotics and predation (Gupta et al., 2016). Furthermore, several works of literature

reported that bacteria in the biofilm show changeable behaviour and are almost 10-1,000 times more resistant to antibiotics than planktonic form (Chakraborty, Daware, et al., 2018; Chatterjee et al., 2021; Paul, Chakraborty, Sarker, et al., 2021). Besides, in clinical disease, the significance of bacterial biofilms is illustrated by an estimation that states that more than 80% of chronic and severe infections are biofilm linked (Chakraborty et al., 2021; Gupta et al., 2016; Paul, Chakraborty, Chatterjee, et al., 2021). Despite this, biofilm formation encourages the microbial cells the production of virulence factors to promote pathogenicity in the human host (Chakraborty et al., 2021; Gupta et al., 2016). Among the biofilm-forming bacterial species, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are well-known biofilm-forming ubiquitous, opportunistic pathogens thar are the leading cause of nosocomial as well as community acquired chronic diseases throughout the world (Sirijan Santajit & Nitaya Indrawattana, 2016). The biofilm mode of such bacterial growth not only offers resistance against conventional therapeutics, but also helps in succession of chronic diseases by dispersing the developed biofilm into new sights to spread infections (Lister & Horswill, 2014). Therefore, impediment of such biofilm linked threats are imposing a significant challenge to biomedical sciences.

Biofilms exhibit diverse adaptations and survival strategy in hostile environments. Biofilms may comprise of homogenous or heterogenous microbial communities. The heterogenous biofilms exhibit higher resistance than the homogenous one. Several strategies have been postulated that could be adapted by bacterial biofilm to develop such resistance. Obstruction to the penetration of antibiotic molecules through the extracellular polymeric matrix of biofilm structure is well established. Since the EPS layer contains several positively as well as negatively charged molecules that can bind to the oppositely charged drug molecules, thereby enhancing the impedance of biofilm. Besides, the extracellular matrix can adsorb antibiotics to decrease their penetration within. It was also found that biofilm adhered cells can release and accumulate various antibiotic degrading enzymes such as β-lactamase which led to hydrolysis of such enzyme sensitive antibiotics (Dincer et al., 2019). Moreover, the divergent pH, temperature, availability of nutrients and oxygen within the microenvironment of biofilm structure brings about inactivation of antibiotics. Secondly, presence of eDNA in biofilm matrix enhances resistance against therapeutics. Being anionic in nature, eDNA can chelate several ions such as magnesium ions, leading activation of quorum sensing specific genes by lowering Mg^{2+} concentrations in outer membrane of biofilm cells thereby imposing antimicrobial resistivity (Dincer et al., 2019).

Table 1. The physicochemical properties of Tryptophan

Serial no.	Characteristics	Description
1.	Molecular Formula	$C_{11}H_{12}N_2O_2$
2.	Molecular weight	204.22 g/mol
3.	Physical state	White- slight yellow crystal powder
4.	Melting point	290.5°C
5.	Solubility (Water)	11.4 g/L at 25°C
6.	Chemical classification	Indole amino acid
7.	logP	-1.1
8.	IUPAC name	(2*S*)-2-amino-3-(1*H*-indol-3-yl) propanoic acid
9.	Taste	Slightly bitter
10.	Odor	Odorless

Tryptophan: A Promising Aromatic Amino Acid

Amino acids can be uniquely categorised based on their physicochemical and biological significance. The physicochemical properties of tryptophan have been shown in Table 1 (https://pubchem.ncbi.nlm.nih.gov/compound/Tryptophan). Tryptophan is an aromatic amino acid. It exists in D and L-form. It can be considered as the most unique and the largest coded amino acid among all others. Interestingly, it is the sole amino acid that consists of an indole ring. This indole ring contributes majorly in hydrophobic characteristics to the tryptophan molecule. Apart from the hydrophobic interactions, tryptophan can form strong hydrophilic noncovalent interactions such as formation of hydrogen bond. The nitrogen atom of indole ring structure is involved in hydrogen bond interaction that in turn facilitates the aqueous solubility. These properties are of crucial importance for its antimicrobial, antibiofilm, and anticancer properties.

Tryptophan and Biofilm Management

Tryptophan has well established anti-biofilm activity. Both the D and L-isoform of tryptophan have been found to inhibit the biofilm development of *Pseudomonas aeruginosa* by interfering with the swimming motility of the bacteria (Brandenburg et al., 2013). The mechanisms behind antibiofilm are

listed in Table 2. Both the D, L-tryptophan inhibit pseudomonal biofilm, but L form of tryptophan is more effective than its D form. It shows antibiofilm activity by increasing bacterial cell motility (swimming/gliding) by increasing flagellar activity (Figure 1) (Brandenburg et al., 2013).

Moreover, L-tryptophan was found to impede biofilm forming capacity of *Staphylococcus aureus* and *Pseudomonas aeruginosa* by interfering with the cell surface hydrophobicity and quorum sensing system (Chakraborty, Daware, et al., 2018; Paul, Chakraborty, Sarker, et al., 2021). As per literature, the D-tryptophan hampers the formation of biofilm by disassembling the pre-matured biofilm of *Pseudomonas mendocina* and *Staphylococcus aureus* pursuing hindrance in adhesion and cohesion of cells (Figure 2) (Ghosh et al., 2019).

The functional amyloid fibres present on bacterial cells have important role in cell-cell and cell-surface interactions. This amyloid fibrillation is accelerated in presence biofilm extracellular components and eDNA. These components also form complexes with amyloid fibres promoting the formation of biofilm. D-tryptophan prevents the formation as well as disintegrate microbial biofilm of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (Warraich et al., 2020) by inhibiting the amyloid fibrillation. Thus, using tryptophan could be considered as an effective strategy in handling microbial biofilm threats.

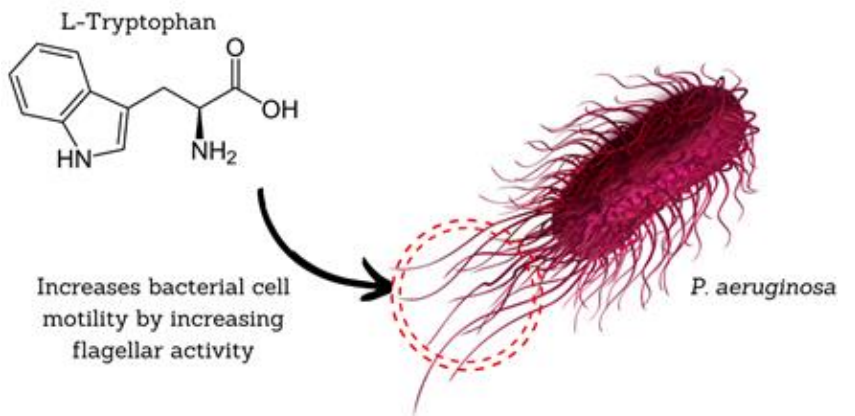

Figure 1. Mechanism of action of L-Tryptophan against Pseudomonas aeruginosa by increasing bacterial cell motility.

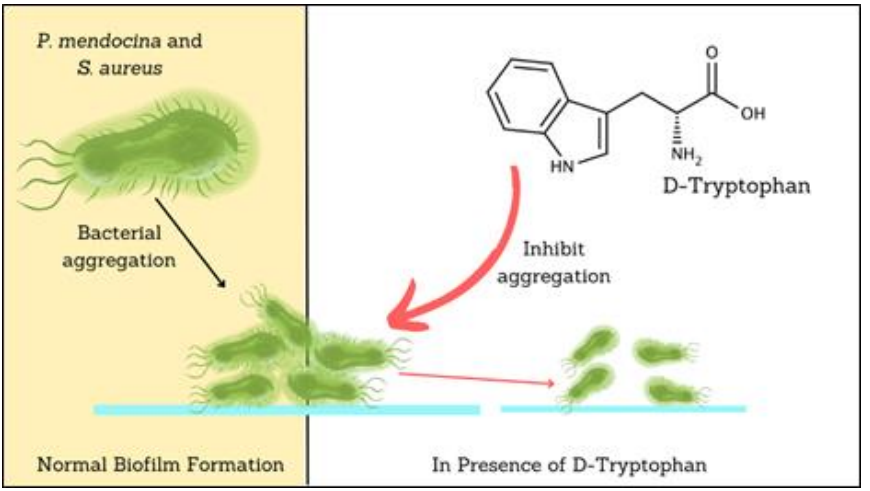

Figure 2. Mechanism of action of D-Tryptophan against biofilm development of *Staphylococcus aureus* and *Pseudomonas mendocina.* D-Tryptophan inhibits the aggregation and intracellular adhesion of bacterial cell by forming tannic acid which is an important step in case of biofilm formation (Ghosh et al., 2019).

Molecular Mechanism of Biofilm Control under the Influence of Tryptophan

Inside the biofilm structure, microbial cells undergo various morphological as well as physiological changes which get assisted by multiple genes and signalling pathways. Toward this direction, a better eradication of biofilm associated threats can be initiated by exploring molecular mechanism of biofilm structure. Since, QS system is very important for microbial biofilm development, many researches come up with targeting QS system as a vital strategy to prevent biofilm. Quorum sensing is basically cell-cell communication process of bacteria during the process of biofilm formation. In this respect, agrA/agrC and lasI/lasR systems were found to be pivoted for quorum signalling pathway of *S. aureus* and *P. aeruginosa* respectively.

Table 2. Mechanism of action of Tryptophan against Bacterial biofilm

Serial no.	Bacteria	Form of tryptophan	Effective Dose	Mechanisms of action against Biofilm	Reference
1.	*Staphylococcus aureus*	D-Tryptophan	0.1-1 mM	Biofilm's Intercellular adhesion and aggregation are affected by the formation of tannic acid	(Ghosh et al., 2019)
2.	*Pseudomonas aeruginosa*	D, L-Tryptophan	1 to 10 mM	Modulating bacterial cell motility.	(Brandenburg et al., 2013)
3.	*Pseudomonas mendocina*	D-Tryptophan	0.1-1 mM	Biofilm's Intercellular adhesion and aggregation are affected by the formation of tannic acid	(Ghosh et al., 2019)

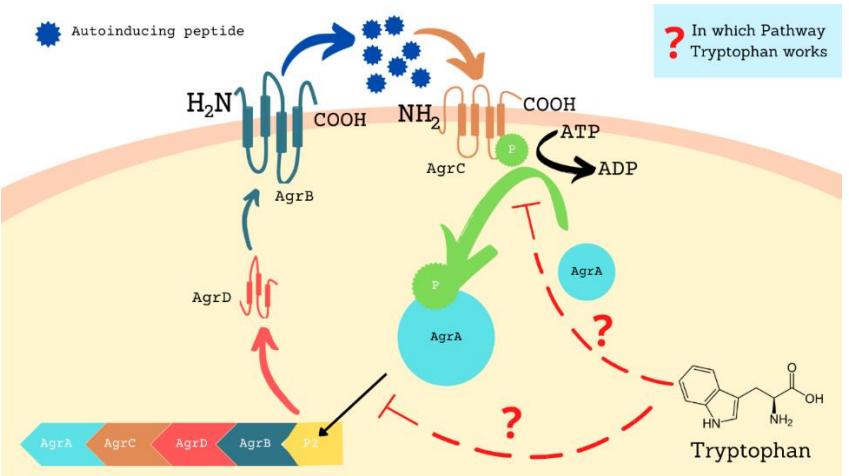

Figure 3. Tryptophan inhibits the *Agr* quoram sensing gene of *Staphylococcus aureus*.

The *Agr* operon constitutes a global regulatory system that controls cell density-dependent virulence factor expression (Figure 3). The P2 promoter of *Agr* operon control the expression of four components of quorum-sensing system*: AgrB, D, C, A*. *AgrB* is a transmembrane protein required for the processing of the pro-peptide *AgrD* to produce the autoinducing peptide (AIP), that is a quorum signal molecule. Binding of AIP to *AgrC* triggers the phosphorylation of *AgrA* that binds subsequently to the promoter region P2 and sustains the cycle (Painter et al., 2014). Tryptophan binds with *AgrA*, as reported by Paul et al. (Paul, Chakraborty, Sarker, et al., 2021). The binding affinity is good enough to form a stable *AgrA-tryptophan* complex. This has a significant effect in inhibiting the formation of biofilm. But, it is not clearly proven that binding of tryptophan with *AgrA* inhibit the phosphorylation of *AgrA* or attachment of phosphorylated *AgrA* to the P2 site.

Exploring further, it was found that the microbial cells required two-types of attachment during biofilm formation, one is adhesion- attachment between cells and the surfaces and other one is cohesion- attachment among cells. For several biofilm forming human pathogens, both this attachment is directly proportionate with surface hydrophobicity of cells. In case of *S. aureus*, *dlt*A was found to enhance Staphylococcal cell surface hydrophobicity by executing d-alanination of teichoic acid, present on bacterial cell surface thereby promoting the microbial biofilm formation. Here it is noteworthy that

DltA could act as d-alanine:d-alanyl carrier protein ligase that results in ligation of d-alanine to teichoic acid. In this regard, tryptophan was found to decrease the expression of *dlt*A gene significantly in order to inhibit the biofilm formation. Besides, another gene- *ica*A is responsible for structural development of Staphylococcal biofilm by producing extracellular matrix (PIA). In absence of *ica*A expression, multilayer formation of biofilm is inhibited. The real-time PCR assay revealed that the expression level of *ica*A gene of *S. aureus* is reduced significantly under the influence of tryptophan. Thus, compromising intra as well as intercellular communication process under the influence of tryptophan might explore new window to manage microbial biofilm threats.

Synergistic Effects of Tryptophan in Combination with Other Antibiotics

As treatment with an conventional antibiotics is inefficient in microbial biofilm inhibition, several combinatorial strategies are followed (Das et al., 2016). It was explored that D and L forms of tryptophan individually can inhibit Pseudomonal biofilm development almost about 80% at 48 h of incubation (Brandenburg et al., 2013). But interestingly, almost about 90% microbial biofilm inhibition was noticed when *Pseudomonas aeruginosa* was exposed to the D form of tryptophan in combination with the L form of tryptophan (Brandenburg et al., 2013). Moreover, it was also explored that tryptophan enhanced the antibiofilm activity of several antimicrobial molecules (Sanchez et al., 2014). In this regard, D-tryptophan was found to enhance the anti-biofilm property of ciprofloxacin and colistin against *Pseudomonas aeruginosa* (Sanchez et al., 2014). On the other hand, the anti-biofilm activity of rifampicin was found to be enhanced when it was introduced to *Staphylococcus aureus* in combination with the D form of tryptophan (Sanchez et al., 2014). This combination mainly works on the existing biofilm which indicates an effective strategy for the subsequent increase in the anti-biofilm property of the antibiotics in a dispersion of microbial cells encompassed with biofilm structure (Sanchez et al., 2014). Furthermore, tetracycline, a conventional antibiotic, alone never shows significant action on Pseudomonal biofilm. But it was addressed by (Jayalekshmi et al., 2016) that its anti-biofilm property gets increased by

almost 90% when it was used in combination with tryptophan (Jayalekshmi et al., 2016).

Tryptophan also increased the anti-biofilm activity of natural and synthetic molecule other than antibiotics. Chakraborty et al. (Chakraborty et al., 2021) reported the synergistic effect of tryptophan in reducing the Pseudomonal biofilm after co-administration with thymoquinone, the major constituent of black cumin seed. Similarly the effect of tetrazine capped silver nanoparticles (TzAgNPs), against *Pseudomonas aeruginosa* (Chakraborty et al., 2021) was attenuated. It was also explored that the combination of tryptophan, thymoquinone, and TzAgNPs reduced the microbial biofilm by accumulating reactive oxygen species (ROS) in the biofilm cells (Chakraborty, Joardar, et al., 2018).

Conclusion and Future Perspective

It can be concluded that the D and L forms of tryptophan alone or in combination with several natural as well as synthetic molecules and nanoparticles can efficiently control the biofilm pathogenicity caused by *Pseudomonas aeruginosa* and *Staphylococcus aureus*. There are multiple mechanisms behind the anti-biofilm activity of tryptophan. Starting from the modulation of bacterial cell motility, prevention of intracellular adhesion and attachment to different surfaces, inhibition of amyloid fibrillation, and suppression of quorum sensing genes are the fundamental mechanisms behind the antibiofilm activity of tryptophan. Being a natural amino acid, tryptophan is biocompatible and biodegradable. Hence, it can appear a potent ingredient in wound healing agent in future.

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Chapter 10

Nrf2 as One of the Potential Targets in Multiple Sclerosis

Samira Shirooie*, PhD
Pharmaceutical Sciences Research Center, Health Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran

Abstract

It has been proposed that the endogenous antioxidant defense mechanisms in MS are deficient and therefore do not prevent the progression of these lesions. In this sense, some of the current therapeutic approaches (for example, fumarate treatment) aim to upregulate the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) as an essential regulator of antioxidant protection. Nrf2 is a critical regulatory factor for many cytoprotective molecules to counter oxidative stress and detoxification gene expression. Therefore, targeting the Nrf2 factor may be a suitable strategy for studying of diseases in whose pathogenesis oxidative stress is implicated. In pre-clinical studies, compounds such as melatonin, curcumin, resveratrol, and sulforaphane have been shown to reduce the symptoms of MS by activating the Nrf2 signaling pathway. Some of the chemical drugs and medicinal plants have been indicated to have beneficial effects in pre-clinical researches and clinical trials. In this chapter, we will discuss targeting Nrf2 by synthetic and natural agents and their impact on MS.

Keywords: Nrf2, demyelination, multiple sclerosis, neuron

* Corresponding Author's Email: shirooie@gmail.com

In: Advances in Health and Disease. Volume 57
Editor: Lowell T. Duncan
ISBN: 979-8-88697-098-2

Introduction

One of the most prevalent autoimmune diseases of the central nervous system (CNS) is multiple sclerosis (MS), causing multiple demyelinating lesions in the brain and spinal cord (Fletcher, Lalor, et al., 2010). The symptoms of MS are various and depend on the affecting nerve (Dobson and Giovannoni 2019). Some of the symptoms of MS are loss of coordination, spasticity, tremor, weakness, blurry vision, and tingling in limbs. Based on the type of MS, the symptoms can progress or not during the time. There are four main types of MS, including Recurrent-remittent (RRMS), Secondary progressive (SPMS), Primary progressive (PPMS), and Progressive-remittent (PRMS) (García-Lorenzo 2010).

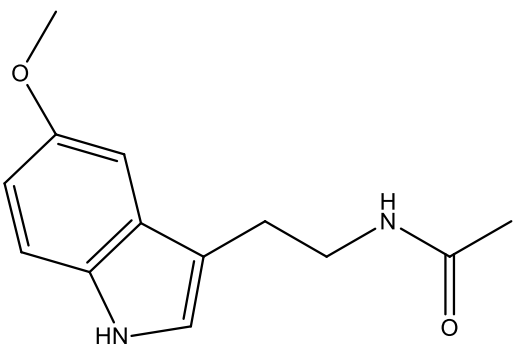
Inflammation and oxidative stress play an important role in the pathogenesis of MS (Pegoretti, Swanson, et al., 2020). Many immune cells, such as $CD4^+$ and $CD8^+$ T cells, B cells, and macrophages involved in the pathogenesis of MS via the production of cytokine, antibodies and, chemokines, which increase inflammation (Pegoretti, Swanson, et al., 2020). Microglia, a type of glial cell that protects the neurons, is another type of immune cell found in the CNS. Activated microglia release pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and iNOS which can activate, and recruit the immune cells (Hanisch and Kettenmann 2007). In addition to inflammation, oxidative stress of macromolecules, including lipids, proteins and DNA, has a significant role in MS lesions and demyelination (Fischer, Wimmer et al. 2013). Activated microglia express NADPH oxidase, myeloperoxidase and increase the production of free radicals and mitochondrial injury (Geng, Galano, et al., 2022).

A transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), encoded by the NFE2L2 gene, is one of the primary defense mechanisms countering oxidative stress via elevation of the expression of cytoprotective enzymes such as NADPH: quinone oxidoreductase (NQO1) glutathione-S transferases, Gpx, SOD1 and heme-oxygenase-1 (HO1) (Swindell, Bojanowski, et al., 2022). In addition to suppression of oxidative stress, Nrf2 shows an anti-inflammatory effect through an inhibitory effect on the NF-kB signaling pathway so, reducing pro-inflammatory cytokine production (Wardyn, Ponsford, et al., 2015). NF-kB involves in apoptosis and upregulation of the inflammatory and oxidative stress-related genes such as NADPH oxidase, nitric oxide synthase, and cyclooxygenase (VanderJagt, Hunsaker, et al., 2022). Nrf2/HO-1 signaling dysregulation in animal leads to microglial activation, over-expression of cytokines, demyelination,

neuroinflammation, and clinical symptoms like MS occur (Upadhayay and Mehan 2021). Downregulation of Nrf2 is involved in many neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD) (Esteras, Dinkova-Kostova, et al., 2016).

One of the oral medicines prescribed for MS is dimethyl fumarate (DMF). Evidence indicates that DMF and its active metabolite monomethyl fumarate activate the Nrf2 signaling pathway and show cytoprotective and anti-inflammatory effects in RRMS (Gopal, Mikulskis, et al., 2017). So, Nrf2 activators have a protective effect in reducing the symptoms of the neurological disorders, including MS. In this chapter, we discussed the targeting of Nrf2 by natural and synthetics compounds in the treatment of MS.

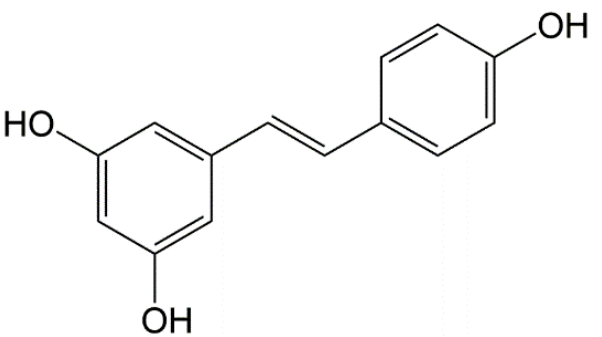
Melatonin



N-acetyl-5-methoxytryptamine (melatonin), which is secreted from the epiphysis gland, shows several pharmacological effects, including circadian rhythms, antiinflammation, immunomodulatory, anti-oxidant, cardioprotective, and antidiabetic (Shukla, Chinchalongporn, et al., 2019). Melatonin physiological activities are mediated by the membrane and nuclear receptors. Melatonin prevented the neurotoxicity effect of aluminum chloride (AlCl3) via up-regulation of the Nrf2 signaling pathway, which increased the brain levels of GPx, SOD, CAT and reduced the levels of NO, and lipid peroxidation in the brain of rats (AlOlayan, ElKhadragy, et al., 2015). It has been shown that melatonin inhibits the pathological effects of manganese (Mn) on neurons, and reduces motor disorders, neurodegeneration, ROS and MDA production by Mn via activation of the Nrf2/ARE pathway (Deng, Zhu,

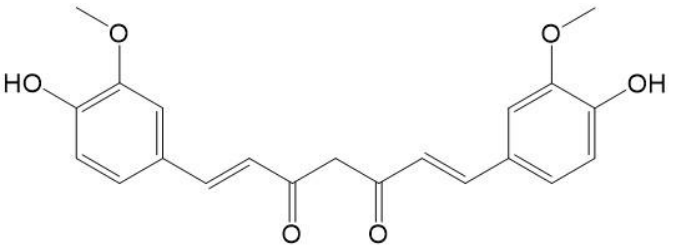
et al., 2015). Kang et al. have indicated melatonin administration reduced infiltration of ED1 macrophages and CD4 T cells into spinal cords and ICAM-1 in experimental autoimmune encephalomyelitis (EAE) (Kang, Ahn et al. 2001). In another study on the cuprizone-induced demyelination model of MS, melatonin induced remyelination, MFN1 expression, and increasing GSH level in mice (Kashani, Rajabi, et al., 2014). The beneficial anti-oxidative effects of melatonin in different animal models of MS are mediated by activation of the Nrf2/ARE signaling pathway, which elevates the anti-oxidative enzymes such as SOD and CAT as well as reduces TBARS and IFN-γ secretion (Long, Yang, et al., 2018).

Resveratrol



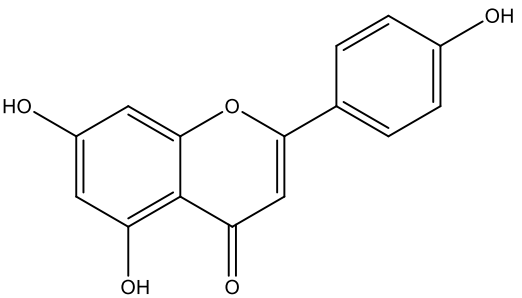
3,5,4′-trihydroxy-trans-stilbene (resveratrol), a natural polyphenol enriched in the skin of fruits, such as grapes and blueberries shows anti-inflammatory effects via targeting Nrf2 and NF-kB (Catalgol, Batirel, et al., 2012). Several animal studies on various models of MS such as Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) and EAE, have demonstrated resveratrol reduced clinical severity of symptoms, neuronal apoptosis and the levels of TNF-α, IFN-γ, IL-2, IL-9, IL-12, IL-17, MIP1A, MCP-1 through targeting Nrf2 (Sato, Martinez, et al., 2013, Wang, Li, et al., 2016). Similarly, Ghaiad et al. have indicated that oral resveratrol administration reversed the motor imbalance and pathological changes induced by cuprizone in mice via down-regulation of the NF-kB signaling pathway (Ghaiad, Nooh, et al., 2017).

Curcumin



1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin) origins from turmeric (Curcuma longa). In an in vivo study on the EAE model of MS, curcumin showed neuroprotective effects, including remyelination, reducing BBB breaking down, IL-17, NF-kB, and TNF-α as well as elevating the levels of IL-4 and IL-10 through the Nrf2/HO-1 signaling pathway (Mohajeri, Sadeghizadeh, et al., 2015). In several MS studies, the anti-inflammation and anti-oxidative stress of curcumin has been demonstrated by improvement in coordination and severity of symptoms, immunomodulatory effect via reducing pro-inflammatory cytokines and increasing the anti-inflammatory cytokines as well as reducing mitochondrial damages (Kanakasabai, Casalini, et al., 2012, Feng, Tao, et al., 2014).

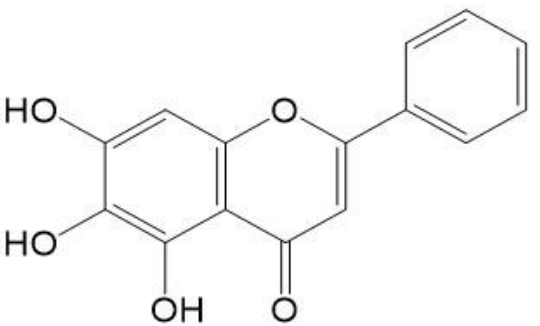
Apigenin



4′,5,7-trihydroxyflavone (apigenin), a natural product is belonging to the flavone, is found in many plants. Apigenin has neuroprotective effects against neurodegenerative diseases such as AD, PD, ALS and MS (Anusha, Sumathi, et al., 2017, Nikbakht, Khadem, et al., 2019, Dourado, Souza, et al. 2020).

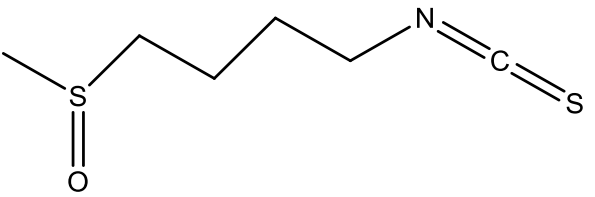
Apigenin, a natural flavonoid, attenuates the EAE severity through the modulation of dendritic cell and other immune cell functions. In the EAE model of MS, apigenin administration reduced the expression of α-4 integrin and CLEC12A on splenic DCs, infiltration and demyelination of neurons in the CNS (Ginwala, McTish et al. 2016). Xu et al. have shown apigenin has a neuroprotective effect against t-BHP in ARPE-19 cells through activation of Nrf2 signaling, thereby elevating the levels of CAT, SOD, and GPx as well as reducing the ROS and MDA levels (Xu, Li, et al., 2016). In an *in vitro* study on PC12 cells, apigenin reduced the apoptotic effect of oxygen and glucose deprivation/reperfusion and neuronal injury by increasing Nrf2 expression, detoxifying enzymes, and reducing the levels of ROS and P53 protein expression (Guo, Kong, et al., 2014).

Baicalin



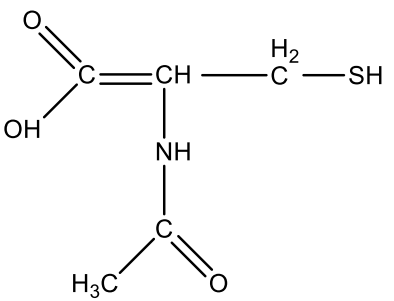
5,6-Dihydroxy-4-oxygen-2-phenyl-4*H*-1-benzopyran-7-β-D-glucopyranose acid (baicalin) is a flavonoid found in the root of *Scutellaria baicalensis*. Baicalin showed neuroprotective effects in EAE MS-model and improved the imbalance, reducing pro-inflammatory cytokines, Th1 and Th17 cell differentiation by suppressing STAT/NFκB signaling pathway and SOCS3 induction (Zhang, Li et al. 2015). Several studies on baicalin have shown that some of the beneficial pharmacological effects are due to Nrf2/Keap1 activation and increasing the expression of antioxidant enzymes (HO-1, NQO-1) (Shi, Hao, et al. 2018, Ma, Wu, et al. 2021).

Sulforaphane



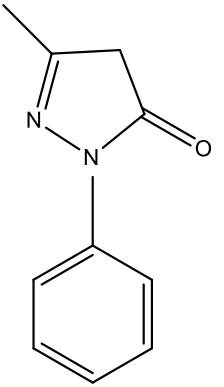
Sulforaphane (SFN), a sulfur-rich natural compound found in cruciferous vegetables, has antioxidant and anti-inflammatory effects (Shi, Hao, et al. 2018). In an *in vivo* study on the EAE model, SFN administration improved the motor coordination, reducing infiltration and demyelination via targeting Nrf2/ARE signaling pathway, increasing the expression of HO-1 and NQO-1 (Li, Cui et al. 2013). Similarly, Galea et al. have studied the neuroprotective effect of SFN on the EAE model and showed the neuroprotective effect of SFN by reducing disability, apoptotic cells, and demyelination (Galea, Copple, et al., 2019).

ASSNAC



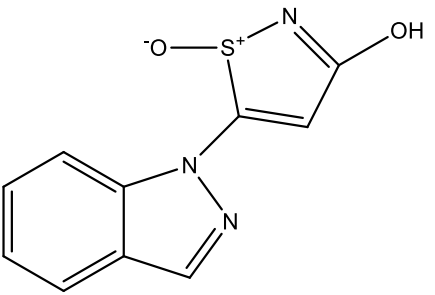
S-Allylmercapto-N-acetylcysteine (ASSNAC), a synthetic compound formed by conjugation between N-acetylcysteine (NAC) and S-allyl mercaptan to improve the cell permeability of NAC (Izigov, Farzam, et al., 2011). Savion et al. have shown that ASSNAC increased the cellular level of Nrf2 in the brain of the EAE animal model, which elevated anti-oxidative biomarkers such as glutathione (Savion, Izigov, et al., 2014).

Edaravone



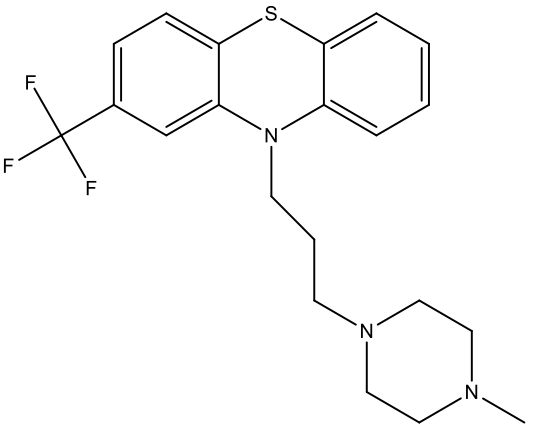
Edaravone, a synthetic neuroprotective compound, is used for the improvement of recovery after stroke and in the treatment of ALS (Bailly, Hecquet, et al., 2020). Several studies have indicated the therapeutic effects of edaravone are mediated by Nrf2 activation which are blocked by Nrf2 gene knockdown (Zhang, Xiao, et al., 2018, Shou, Bei, et al., 2019). In the EAE model of MS, treatment with edaravone reduced clinical severity, iNOS expression and infiltration of lymphocytes to the CNS (Moriya, Nakatsuji, et al., 2008).

TFM-735



TFM-735 is a new Nrf2 inducer that has neuroprotective effects. TFM-735 reduced T-cell proliferation, IL-6, and IL-17, elevating the expression of NQO1 in the EAE model of MS (Higashi, Kawaji, et al., 2017).

Trifluoperazine



Trifluoperazine, an antipsychotic medicine, used for schizophrenia and other psychotic conditions, shows anti-inflammatory and anti-oxidative stress effects (Góngora, Máñez, et al., 2000). In an *in vivo* study on the cuprizone-inducing demyelination mice, trifluoperazine improved motor coordination, reduced nitric oxide, elevated SOD level and remyelination through Nrf2 activation and NF-kB inhibition (Khaledi, Noori et al., 2021).

Conclusion

This chapter resulted Nrf2 and NF-kB signaling pathways having a potential role in the pathogenesis of MS. Targeting Nrf2 with synthetic or natural products showed neuroprotective effects in MS. According to the results, it can be claimed that Nrf2 could be a good target for remyelinating in myelin disorders like MS.

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Biographical Sketch

Samira Shirooie

Affiliation: Pharmaceutical Sciences Research Center, Health Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran

Education: PhD of Pharmacology

Business Address: Kermanshah University of Medical Sciences

Research and Professional Experience:

- Neuropharmacology
- Neurodegenerative diseases
- Inflammation
- Gastrointestinal Pharmacology
- Hepatotoxicity
- Cell culture

Professional Appointments:

Assistant Professor of Pharmacology; Head of Student Research Committee of the School of Pharmacy

Honors:

2nd Best Student in the Entrance Exam of PhD of Pharmacology (2015).
1st Best Ph. D. Student in the Board Exam of PhD course (2017).

Publications from the Last 3 Years:

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Lowell T. Duncan
Editor

Advances in Health and Disease

Volume 57

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ISBN-13: 979-8-88697-098-2
9 798886 970982