miRNA based pathophysiological control of key signaling pathways involved in Inflammatory Bowel Disease

By

Absar Mazhar

00000117162

Supervised by

Dr. Najam Us Sahar Sadaf Zaidi

Co-Supervised by

Dr. Amjad Ali

DEPARTMENT OF INDUSTRIAL BIOTECHNOLOGY,

ATTA-UR-REHMAN SCHOOL OF APPLIED BIOSCIENCES,

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY,

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A thesis submitted as a MS project in partial fulfillment of the requirement for the degree of MS in Industrial Biotechnology.

Thesis Supervisor:

Dr. Najam Us Sahar Sadaf Zaidi

Thesis Supervisor's Signature: _____

Thesis Co-Supervisor:

Dr. Amjad Ali

Thesis Supervisor's Signature: _____

DEPARTMENT OF INDUSTRIAL BIOTECHNOLOGY

ATTA-UR-REHMAN SCHOOL OF APPLIED BIOSCIENCES

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY

ISLAMABAD

DECLARATION

I certify that this research work titled *"miRNA based pathophysiological control of key signaling pathways involved in Inflammatory Bowel Disease"* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged/referred.

Signature of Student

ABSAR MAZHAR

00000117162

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Signature with stamp: _____

Name of Supervisor: Dr. Najam Us Sahar Sadaf Zaidi__

Date: _____

Signature with stamp: _____

Name of Co-Supervisor: Dr. Amjad Ali__

Date: _____

Signature (Dean/Principal): _____

Date: _____

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This thesis has been checked for Plagiarism. Turnitin report endorsed by Supervisor is attached.

Signature of Student

ABSAR MAZHAR

00000117162

Signature of Supervisor

Signature of Co-Supervisor

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And above all,

To the Almighty ALLAH!

ABSTRACT

Inflammatory Bowel Disease (IBD) is comprises of two different phenotypes Ulcerative Colitis (UC) and Crohn's Disease (CD), both have many conjoint genetic and mechanistic features while having distinctive clinical manifestations. Intestinal epithelial barrier serves as a first line of defense for incoming toxins and plays a crucial role in maintaining the intestinal homeostasis. Any transformation in the integrity of intestinal barrier can lead to multiple maladies as well as IBD. Tight Junctions are crucial part of intestinal barrier, which help in regulating cellular polarity and adhesion of intestinal wall. These tight junction proteins are found to be regulated by certain microRNAs. Identification and analysis of the interaction network of candidate miRNAs and their target gene products would increase our understanding of how to maintain the integrity of intestinal barrier, generating a comprehensive model of cellular signaling pathways associated with IBD will unravel the key components whose imbalance may lead to disease onset. This study attempts to identify the crucial players in the interactome, which regulate important targets and may lead to disease outcomes. In addition, to elucidate how over-expression of certain microRNAs lead to dysfunction of signaling pathways leading to IBD. Antagomir therapy show potential to restore deregulated signaling pathways, which play vital role in homeostasis regulation. Furthermore, we observed how Antagomir application could revert the disease condition by bringing down the up-regulated microRNA. We applied Antagomir therapy to target key microRNAs involved in IBD progression and gauge their impact in recovery.

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LIST OF ABBREVIATIONS

AMOs	Anti-mRNA oligonucleotides
AAV	Adeno-associated virus
β-Catenin	Beta-catenin
BRN	Biological Regulatory Network
CD	Crohn's disease
CD4	Cluster of differentiation 4
CTL	Computation tree logic
CORD15	Caspase recruitment domain-containing protein 15
ECM	Extracellular matrix
FOXO-3	Forkhead box O3
IBD	Inflammatory Bowel Disease
IL	Interleukin
IFN-γ	Interferon gamma
miRNA	Micro RNA
NFκB	Nuclear factor-kappa B
NOD-2	Nucleotide-binding oligomerization domain-containing protein 2
NOS	Nitric oxide synthases
ODE	Ordinary differential equations
PN	Petri nets
RNase III	Ribonuclease III
RNA	Ribonucleic acid
TNF-α	Tumor necrosis factor alpha
TH1	Type 1 T helper
TH2	Type 2 T helper
TGF-β	Transforming growth factor beta
UC	Ulcerative colitis
UTR	Untranslated regions

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CHAPTER 1: INTRODUCTION

The primary integral components of Inflammatory Bowel Disease (IBD) Figure 1 i-e Ulcerative colitis (UC) and Crohn's disease (CD) are instigated by a complicated interaction of genetic, environmental, and immune-regulatory elements. (Gerich & McGovern, 2014). Industrial nations in the north with ethnicity especially Caucasians and Ashkenazic Jews have been reported with increasing rates of IBD (M'Koma, 2013). Since population mix and cross-racial marriage are very common, therefore, racial gaps are thinning and environmental elements may contribute to disease onset. Despite of the underlying genetic predilection, an increasing insight indicates a debilitated mucosal immune reaction to gut microbes in the proliferation of IBD. Major prompted factors comprise a prolonged inflammatory reaction enhanced by contagion caused due to pathogen, virus or a deficient mucosal barrier (Y.-Z. Zhang & Li, 2014). The distinctive inflammatory response initiates with penetration of macrophages and neutrophils, which later liberated chemokines and cytokines. In result, triggers the debilitated immune reaction and stimulate either TH1 or TH2 cells present in the gut lining, accomplice with CD and UC respectively (Matricon, Barnich, & Ardid, 2010a). Classification of factors including both immunological and genetic demonstrate numerous checkpoints at which the inflammatory cascade may be interjected, resulting in generating clear, directed remedies for IBD. The endorsement of IBD is enduring, unsuppressed swelling of the intestinal wall, that can perturb any fragment of the abdominal tract (Hanauer, 2006).

IBD interpretation depends on the existence of architectural biasness (e.g., transmural or frivolous erratic granulomatous intrusion) and acute inflammatory cells (Tontini, Vecchi,

Pastorelli, Neurath, & Neumann, 2015). On the other hand, chronic inflammation diagnosed with zero deformity could also be indicated as characteristic of the healthy gut. The line of distinguish between IBD and inflammatory responses in the healthy abdomens lies in an incompetence to conduct those responses. When a potential pathogen enters a healthy gut, it causes inflammation and then acquire tolerance when that pathogen is extinguished from the abdomen. Despite of this, inflammation is incessant in intestine and the mucosal immune system of patients suffering from IBD (DeRoche, Xiao, & Liu, 2014).

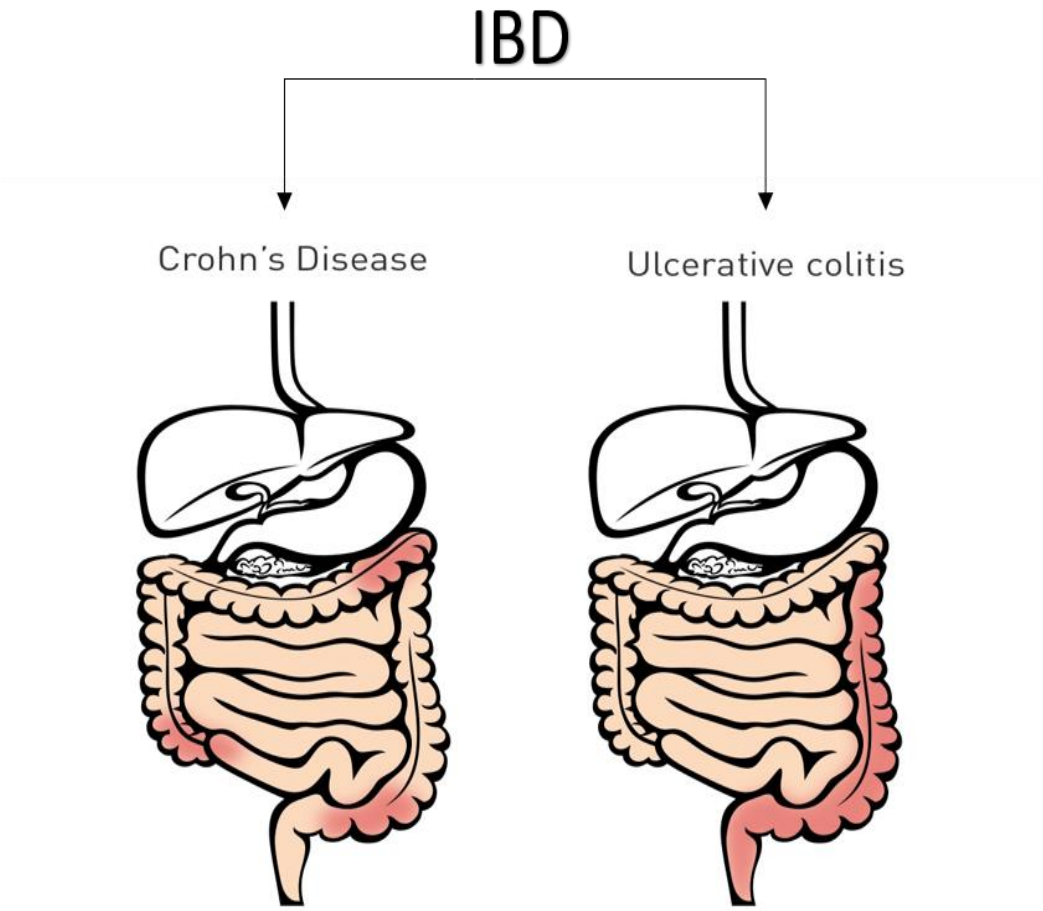

Figure 1: Inflammatory Bowel Disease Phenotypes: 1) Crohn's Disease 2) Ulcerative Colitis.

UC and CD have differentiated clinical, anatomical, and histological findings and they are generally accepted as clinically definite conditions, therefore, a standardize diagnosis remains ambiguous (Vito Annese et al., 2013). Around 10% of patients show undetermined characteristic between UC and CD that is difficult to distinguish. This condition is name as ‘‘indeterminate colitis’’ until the above mentioned symptoms of UC or CD become visible subsequently (Schreiber et al., 1998). Distinct pathogenic mechanisms lies beneath UC and CD is indicated by increased diversity of its symptoms. It is essential that for an ameliorated therapy for IBD, a greater knowledge of the ecological, hereditary, and immunological systems that leads to UC and CD is necessary. (Tontini et al., 2015).

1.1 Computational Biology to Study Dynamic Systems

Biological systems are very dynamic in nature. Over the past decade, computational tools have been vigorously used to analyze biological systems in real time. The core of systems biology consists of computational modeling of systems to build an integrative and coherent picture (LI et al., 2006). It not only helps in investigating the relationships and behavior of elements involved in a biological system but also explains how the system functions as a whole. Moreover, diagrammatic models summarizing biological systems improve mechanistic understanding of the observations (Goel, Chou, & Voit, 2006).

Modulation of gene expression determines overall dynamics of each cell and this is a complex process (de Jong, 2002). Computational and mathematical approaches in systems biology expedite to observe dynamics of a biological system where entities like RNA, DNA, proteins, enzymes and other biological molecules are involved (Glass & Kauffman, 1973). The qualitative modeling framework is amongst the most recognized methods to study the dynamics of gene expression (DEJONG et al., 2004; Thomas & D’Ari, 1990) by

constructing biological regulatory networks (BRNs). Systems in biology are usually modeled using partial or ordinary differential equations, which depend on time derivatives of expression levels, along with kinetic rates of entities. The system is represented in a detailed manner by using quantitative models, which requires data which is either specific or too difficult to find. Hence, qualitative models (BRN, Petri nets) are preferred to understand the complex dynamics of the biological systems. Construction of the BRN requires qualitative thresholds and logical parameters, which can be easily adjusted according to the system dynamics and biological observations.

An established technique for modeling of biological systems is Petri nets (Chaouiya, 2007). Petri nets consider concurrency of a system which is vital to model biological systems. A Petri net contains two sets of vertices called places and transitions. Resources of the system are depicted by places while the events that change the resource's state are represented by transitions. A place hold tokens that might define for example the number of molecules involved in the system. Edges move the tokens causing a change in the system through a transition. Recently, Petri nets have widely been used to study and model metabolic pathways. They have a number of benefits as they are more visual, offer variety in designing the system and also help us analyze systems through a range of tools (Reddy, Mavrovouniotis, & Liebman, 1993).

1.2 Our Contribution

In this study, we develop a model that integrated signaling pathways namely apoptosis, cellular adhesion, extracellular matrix integrity and inflammation in co-relation with their regulation controlled by the expression of miRNA under the influence of signals from intra/intercellular interactions and outside environment.

Different parameters of the model were generated from prior experimental knowledge by using the model checking technique. The parameters are then rendered into a qualitative model to analyze the significant system behaviors, such as, cycles and paths that lead to recovery and pathogenesis. Therefore, extensive analysis of the resulting model is performed to find these paths. This was followed by a prediction of paths that symbolize the condition of homeostasis in the overall system, along with states that reveal the over-expression of the entities, from where the system can't move towards recovery. The results of this study suggest an alternate approach to treat inflammatory bowel disease by regulation the signaling pathways at cellular levels using Antagomirs. Our findings suggest that the Antagomirs can help in regulating significant signaling pathways which were expatriate from homeostasis under the influence of miRNAs expression and aiming to target regulatory proteins modulators may exemplify a novel therapeutic approach to make progress to treat inflammatory bowel disease.

1.3 Problem Statement

The complex interactions in pathophysiological pathways have significant role in inflammatory bowel disease progression. However, such large number of entities involved and their complex regulatory correlations needs to be modeled and explored for unraveling the IBD integrome. Current therapeutics do not sufficiently contribute towards the cure of inflammatory bowel disease. Hence, there is a need to introduce targeted therapies to exploit the miRNA-based regulation in inflammatory bowel disease.

1.4 Research Objectives

The objectives of my research work are as follow:

- Construction of qualitative baseline model of inflammatory bowel disease signaling pathways.
- Investigating the role of miRNA in IBD regulation.
- Analysis of inflammatory bowel disease regulation through simulating baseline and disease models.
- To investigate the antagomeric therapy (miRNA targeted therapy) to treat inflammatory bowel disease.

CHAPTER 2: LITERATURE REVIEW

2.1 EPIDEMIOLOGY OF INFLAMMATORY BOWEL DISEASE

IBD has been witnessed since early times (Kirsner, 1988, 2001) and has arisen as a emergent issue in developing countries (Molodecky et al., 2012). In 1875, Wilks and Moxon coined the term ulcerative colitis into the medicinal dialect. As the occurrence of ulcerative colitis started to increase in the start of twentieth century (Mulder, Noble, Justinich, & Duffin, 2014), 'regional ileitis' was officially acknowledged in 1932 after a publication by Crohn et al (CROHN, GINZBURG, & OPPENHEIMER, 1932).

A standardized evaluation of over 200 population-based studies assessing the occurrence or pervasiveness of IBD has drawn the worldwide epidemiological structure of IBD. Several countries in the west have no record on the rate or incidence of IBD, a number of geographic arrangement can be concluded with the on hand published data (Figure 2). The incidence of IBD higher in western countries, affecting almost 0.5% of the population (Molodecky et al., 2012). A number of studies have proven that IBD has cemented foundations in the newly developing nations (Burisch et al., 2014; Ng et al., 2013; Niu et al., 2016; Park, Kim, & Cheon, 2014; Sood, Midha, Sood, Bhatia, & Avasthi, 2003; Tozun et al., 2009; Victoria, Sassak, & Nunes, n.d.). The rise of IBD in these nations matches outlines witnessed in the west in the start of twentieth century (Kirsner, 2001). The worldwide stress of IBD in 2025 in shape of direct and indirect health-care burden management of IBD in the Western world is significant (Kappelman et al., 2008; Rocchi et al., 2012). Since the start of the twenty-first century, the management of IBD in the

western countries has radically progressed. The induction of biologic mediators (namely, anti-TNF therapies) decreased the number of surgeries for IBD (Frolkis et al., 2013; Ma et al., 2017). In the last ten years, the application of biologic mediators has gradually improved (Hazlewood et al., 2015). The advent of vedolizumab and many other upcoming immunological agents (for example, ***ustekinumab***) (Rocchi et al., 2012; Sandborn et al., 2012), therapists will have better decisions to distinguish biologic treatments for IBD (Gerich & McGovern, 2014). Developing states must get ready for the swift upsurge in occurrence and prevalence of IBD. With more people to get IBD, the absolute number of patients in developing countries in 2025 is likely to approach that in the western countries (Figure 3). Justifying the weight of IBD will call for a multifaceted tactics including inventing the distribution of healthcare, increasing the usage of biologic agents, and funding preventative medicine research. This communal methodology may lead us to restriction of global rise of IBD (Kaplan, 2015).

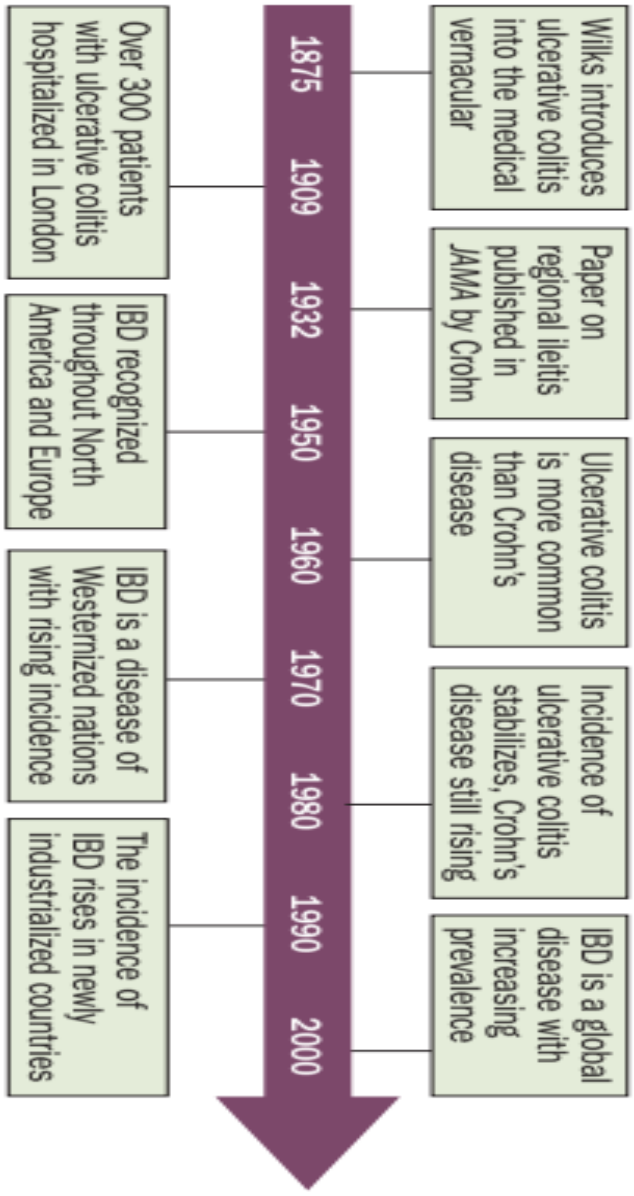

Figure 2 Historical timelines of Crohn's disease and ulcerative colitis throughout the world adopted from Kaplan et al. 2015

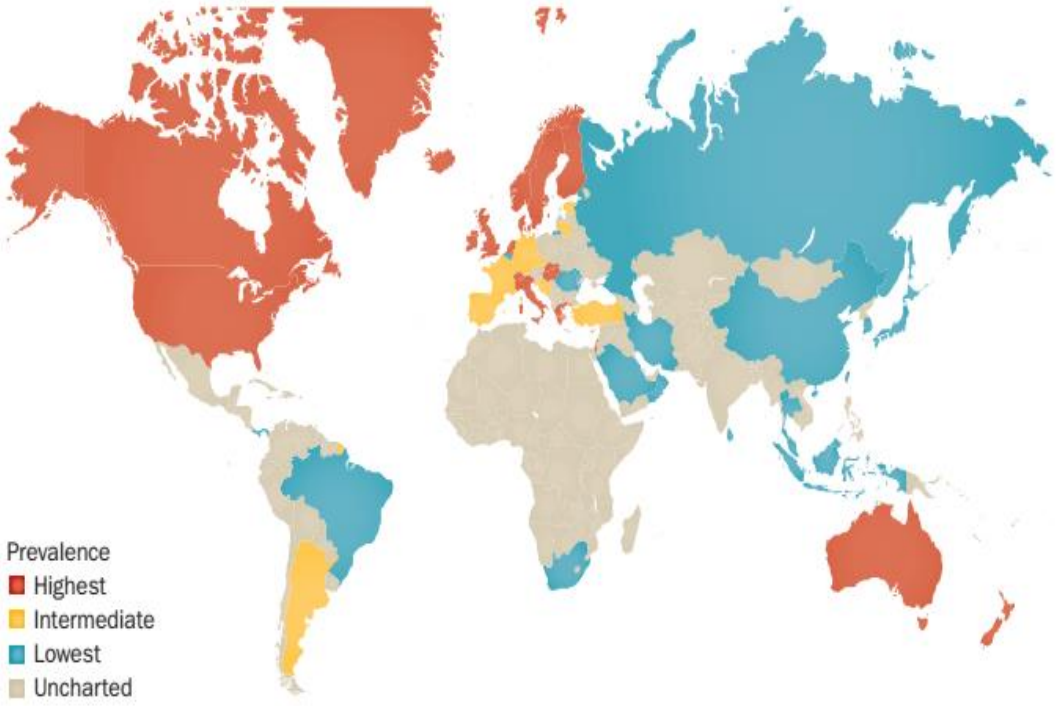

Figure 3. The global prevalence of IBD in 2015. Data from Molodecky et al.4 Adapted from an image provided by PresenterMedia.

2.2 Etiology of Inflammatory Bowel Disease

Multiple aspects force the construction of mucosal irritation. In addition, variations in impress may elucidate the clinical distinction seen in UC and CD. For precedent, a particular family could have more than one infected members, signifying amplified genetic susceptibility. In weigh, sporadic disorder, and that accounts for almost all of IBD cases, is prone to be triggered by a distinctive environmental cause or due to subtler irregularity in the gastrocolic immune structure. Current etiologic theories pertaining to IBD center around environmental set offs, genetic factors, and immunoregulatory deficiencies and microbial exposure (Hou, El-Serag, & Thirumurthi, 2009).

2.3 Hereditary Dynamics

Epidemiological studies exhibit, hereditary features play role in the proneness of IBD (Chen & Bierut, 2013). The disease is hereditarily convoluted and difficult to be elucidated by a particular gene-model only. It is assumed that UC and CD may be polygenic conditions partaking some vulnerable loci. Intuitively, the disease phenotype is assessed by a number of reasons, inclusive of the intercommunication between allelic variants at various loci, in addition genetic and environmental stimuluses. Subsequently, the existence of a altered gene does not pledge the onset of IBD, nor it tells who will be affected by it, underlining the significance of cofactors in triggering the disease (Cătană, Neagoe, Cozma, Magdaș, & Tăbăran, 2015; Khor, Gardet, & Xavier, 2011; Kirsner, 2001).

2.4 Correlation between NOD2 Gene and IBD-1 Loci

Genome-wide scrutinizing using microsatellite DNA markers has recognized numerous hereditary positions possibly related with UC or CD (Chapman & Pekow, 2015). Substantial connections have been described on chromosomes 1, 3, 6, 7, 12, 14, 16, and 19. One of the strongest links is for IBD-1, a susceptibility locus in the pericentromeric section of chromosome 16. Comprehensive study yields out nucleotide-binding oligomerization domain 2 (NOD2) gene and protein which is also called as caspase activation and recruitment domain 15 (CARD15). NOD2 is the foremost gene to be palpably related with IBD, (V Annese et al., 2006) and 3 reported mutations of NOD2 have been linked to the progression of CD (Chapman & Pekow, 2015; M'Koma, 2013).

The mechanistic behind the alterations in the NOD2 gene, which cause the progress of IBD, is still vague. NOD2 gene express primarily in cell lines, where it has a part in host-signaling pathways. NOD2 is involved in stimulation of nuclear factor NFκB, which leads to assembly of various non-specific mediators of infection. These consist of cytokines, growth factors, and metabolites of arachidonic acid and reactive oxygen, these all causes inflammation and results in tissue damage. Another theory centers on interruption of epithelial homeostasis consequential from the deficiency of NOD2-mediated acclimatization of antigen-presenting cells to prompt regulatory and effector T cell responses (Chapman & Pekow, 2015).

2.5 Microbial Acquaintance Causing Immunoregulatory Blemish

In fit gut epithelium, the manifestation of potentially proinflammatory gut microbes is endured deprived of neutrophil enrollment within the epithelial wall. It could be triggered in a stepwise manner by the distinctive phenotype of the native macrophage populace in

the intestine. Initiating the receptor expressed on myeloid cells (TREM-1) is a cell surface molecule present on neutrophils, monocytes, and macrophages that augments the inflammatory response by enhancing degranulation and secretion of proinflammatory cytokines (Mueller & Macpherson, 2006). A number of concepts are put forward regarding the pathogenesis of this very process: impaired immune host reaction to normal gut constituents, infection with a explicit pathogen, and/or defective epithelial barrier to gut antigens (Matricon et al., 2010a).

2.6 Impaired Immune Host Response to Intestinal Components

The typical relationship amongst epithelial microbes and the host is mutually beneficial (Latella & Papi, 2012). It is postulated that gut microbiota lowers the expression of genes for the inflammatory response and slabs stimulation of the NFκB pathway, thus preventing the inflammatory immune reaction of the gut to the innumerable microorganisms and food antigens which it fronts persistently (Xu, Liu, Feng, & Liu, 2014). In IBD, this restraint is lost. The contact with gut microflora now initiates an inflammatory reaction by the mucosa, leading to a prolonged, damaging immune response. Mouse models exhibit no progression of colitis in altered strains kept under controlled conditions (Knight-Sepulveda, Kais, Santaolalla, & Abreu, 2015; Matricon et al., 2010a; Persson, Ahlbom, & Hellers, 1992). Research suggests that genetic modifications cause wide-ranging immunoregulatory reactions to the matching bacteria. Hence, in patients with IBD, it is probable that divergent bacteria are accountable for the inflammatory effect in different individuals (Z. Liu et al., 2012).

2.7 Faulty Barrier Lining

IBD is related with augmented porousness of the intestinal wall causing uninterrupted spur of the abdominal immune system. It is hypothesized that this might be the major deficiency in individuals with IBD. Studies conducted on animal models shows a trend for the development of stark inflammation near leaky epithelial walls. Gut bacteria appear to increase the barrier dysfunctionality, forming a repetitive cycle of mucosal inflammation that permits for entry and translocation of bacteria (Odenwald & Turner, 2013; Tripathi et al., 2009).

In humans, the microbiota usually contains 1014 organisms from 30 known genera and 500 species. Healthy epithelium, with its greatly developed tight junctions, typically offers an operational fence against gut microbes and antigens. Also, the intestinal epithelial cells have regulator mechanisms that controls unfitting activation of immune responses. If, however, bacterial yields are able to bypass the epithelial wall, this will result in direct interaction of microbes and their products with immune cells leading to adaptive immune response. Different inflammatory cytokines will be produced, enlisting further cells into the intestine barrier. These comprise cytokines that reduce the tight junctions between the endothelial cells in the gut vasculature, which consecutively aids hiring of neutrophils to the inside of epithelial from the peripheral blood (Chapman & Pekow, 2015; Mogensen, 2009; Y.-Z. Zhang & Li, 2014).

2.8 Role of miRNA in inflammatory diseases

MicroRNAs (miRNAs) are small nucleotide sequence of 25 nucleotides which does not code for a protein but regulate expression of gene by epigenetic changes. miRNAs binds to 3′ UTRs of complementary mRNA stand subsequently diminishing constancy and hinder

the process of translation. Research revealed the function of miRNAs shows an emerging mutual theme of become accustomed to physiologic and pathophysiologic set of conditions strains and restoring or modifying gene expression in completely differentiated tissues (Leung & Sharp, 2010; Mendell & Olson, 2012).

Latest research have confirmed the dysregulated expression of miRNAs in the blood of IBD infected people. Studies to confirm vital miRNA controlled cellular and animal systems with experimentally inflicted IBD. This enabled us with better understanding of importance of miRNA in the progression of IBD. With confirmations and understanding of miRNA role and their dysregulated levels in IBD, the prospect for unique miRNA biomarkers and treatments is around the corner (Chapman & Pekow, 2015).

2.9 Current treatments for IBD

Diseases have no more ways of treatment as well as if we talk about the surgical treatment it also has too many restrictions against non-responders. Primarily we used the therapeutics achieve the goal in improvement of patient quality of life by different ways i.e. inducing and maintaining flux, restoring the health against disease complication. This approach must be effective against body weakness, psychological fluctuations and control the fatal diseases without showing bad or major side effects. IBD treatment in concern of pharmaceutical way we have five major categories i.e. anti-inflammatory drugs, biological agents, drugs for symptomatic cure and immunosuppressant. Despite this, many pharmaceutical drugs and other things have been manufactured and applied in recent years, their implementation results increased our knowledge in systematic study and pathophysiological mechanisms. Now there is concord that IBD may be product of combined effects of different factors: genetic variations, environmental influences,

fluctuation in the innate and adaptive immune responses and changes in intestinal microbiota. Combination of different factors are indeed for disease to confirm itself clinically. There is no thumb rule for each patient having same combination of factors cause of disease it could vary patient to patient in response to therapy (SCHIRBEL & FIOCCHI, 2010). In the body, the largest lymphoid organ is intestine because on regular basis it faces the large amount of antigens. There are many defense mechanisms present inside the intestine like mucosa-associated lymphoid tissue present in the bowel effective against inflammations and oral defense line stability, and the lymphocytes also plays the important role in activation of alternate pathways. A strong immune response always reflect the presence of specific antigen presenting cells.

Immune responses against the IBD can be innate and adaptive responses produces different elements against its pathogenicity. Innate immune response always play a role in its recognition and provide first cell line defense mechanisms, which stimulates cell signaling.

The abnormal functioning may lead to abrupt inflammatory responses, in this response the adaptive immune channels activated which may lead to production of cytokines those are derived from CD4+ T cells, now this innate and adaptive immune response against inflammation normally provides immunoregulation and tolerance (Danese & Angelucci, 2009). Intestinal epithelial cell barrier function providing main defense mechanism in body, it controls or regulate antigens in mucosal immune system, this barrier is main strength against antigens, dysfunction of this barrier may cause large antigen exposure which also in an important part of IBD pathophysiology. Genetic variations also affects the regulation of immune responses like production of cytokines, immune cells differentiation and regulations, different stimulating factors secretion and structures maintenance and

regulate the pathways of leukocytes. Changes in endothelium may changes the route of leukocytes that could be effect the therapeutic targets. In immunological disrupt functioning CD and UC has some differences like in CD APC and macrophages produce by IL-12 and IL-18 in result of TH1 response and secretion of inflammatory cytokines like TNF-α, IFN-γ, and IL-2. In addition all these cytokine stimulate APCs to produce other cytokines i.e. IL-1, IL-6, IL-8, IL-12, and IL-18,and cycle continues (Plevy, 2002). In case of UC we have the TH2 type response which is characterized by increment in production of IL-4, IL-5, and IL-13 and reduction in IFN-γ. Th17 cells also found which belongs to T helper cells but not relate to Th1 and Th2 cells, these cells are differentiated under the of IL-1β, IL-6, IL-21, and IL-23 (Z.-J. Liu, Yadav, Su, Wang, & Fei, 2009). Th17 cells differentiation are also regulated by TGF- β, cytokines also regulate the differentiation of it (Brand, 2009).

Adhesion molecules directly involved in the trafficking of leukocyte cells, chemokines and tissue repair molecules also plays important role in pathogenicity of disease. In disease condition the pro-inflammatory cytokines continuously secreted and increased in expression of adhesion molecule which is not normally expressed by specific lymphocyte population (Rivera-Nieves, Gorfu, & Ley, 2008). From all these knowledge of all complexes and processes, there are number of different pharmaceutical agents deigns for its treatment which actually only minimize inflammatory pathways through different targets inhibition (Triantafillidis, Merikas, & Georgopoulos, 2011).

2.10 Antagomirs and their role in treatment

Anti-microRNA oligonucleotides are manufactures with tailored specification having reverse complementary to their targeted miRNA for greater binding ability and enhanced stability. As unstable strand of RNA is always deteriorate in the bloodstream because of nuclease enzyme presence (Czauderna et al., 2003). These oligonucleotides are chemical modified may also include nucleotide modifications (may be phosphate or sugar backbone modification). Mostly sugar modified at antisense 2′-*O*-methyl (2′-OMe) and 2′-*O*-methoxyethyl (2′-MOE) oligoribonucleotides, and 2′,4′-methylene bridge (LNA), it increases attaching power with target miRNA and reduction in deterioration. Phosphorothioate (PS) modified *via* the replacement of a sulfur in phosphate backbone to prevents enzymatic degradation but on the other hand reduction in binding affinity (Lennox & Behlke, 2011). LNA modified Anti-microRNA oligonucleotides have very maximum affinity to bind, which gives the benefit of providing for smaller sequence lengths, storing of specificity, maintenance of core sequence and enhance effectiveness. Pure LNA modification may cause self-dimerization of oligonucleotides reducing effectiveness (Janssen et al., 2013).

2.11 Delivery of miRNA agents

2.11.1 AMOs with chemical modifications

The major hurdles in treatments based on nucleic acid is to determine mechanism of delivery because of its anionic nature, higher molecular mass and possible decay from enzyme reactions. However, scientists have recognized the importance of controlling gene expression at post transcription level and are trying to overcome such obstacles. Chemically modified AMOs such as AMOs conjugated with cholesterol have been

developed to overcome delivery limitations. Targeted tissue delivery has been successfully demonstrated by using high amounts of "antagomirs", which include modifications in 2′-OMe and phosphate backbone of AMOs. Such modifications has made possible to deliver AMOs without using any other materials like nanoparticles, microvesicle and exosome. (Chapman & Pekow, 2015; Krützfeldt et al., 2005).

2.11.2 Viral vectors

Various Viral vectors can be used as a vehicle for the delivery of miRNA particles. Examples of such vectors include adenoviruses, retroviruses, lentiviruses and AAV (Adeno- associated viruses). Among such vehicles, AAV has shown greater efficiency because they are usually not able to integrate into the genetic material of host. In addition, AAV viruses are nonpathogenic, have high transduction ability and have specificity. AAV viruses through replacement of miR-26a have been used to obtain antitumor activity in Hepatocellular carcinoma of human models(Kota et al., 2009). They are also been used for treatment of hemophilia B (Nathwani et al., 2011). Some miRNAs expressed by AAV2 are being used to inhibit miRNAs present in retina of mice (miR-96, miR183 and miR-182) in vivo via direct injection of AAV2-expressed miRNA sponges into the eyes. (Krol et al., 2010). Human clinical trials for the usage of AAV vectors have been conducted describes such vectors as well tolerated. However, toxicity due to AAV capsid is one of the big concern for using such vectors

(Mingozzi et al., 2007).

In Inflammatory Bowel Disease, therapies with help of viral vectors have been used in experimental models affected with colitis. Rectal and intravenous infusion of adenoviral vector containing interleukins-10 (1L-10) in 2,4,6-trinitrobenzene sulfonic acid have been

reported by Lindsay and colleagues (J. Lindsay et al., 2002) . Knockout of IL-10 has been reported to reduce colitis in infected mice (J. O. Lindsay, Ciesielski, Scheinin, Brennan, & Hodgson, 2003). Pseudotype 10 AAV-mediated IL-10 delivery via superior mesenteric artery injection can heal colitis in IL-10 knockout mice. Transduction efficiency and tropism of AAV serotypes 1-10 have been tested by Polyak and colleagues via small bowl lavage, SMA injection and enema. (Polyak et al., 2012). Efficient intestinal transduction have been observed inly in case of SMA injection by 4,7,8,9 and 10 serotypes of AAV. However, transduction via AAV 7, 8 and 9 serotypes have been observed in non-intestinal tissues such as liver and stomach. In monkeys an interesting fact has been observed that AAV10 has been associated to contain higher levels in the lymphoid tissues, ileum, lymoh nodes and spleen (Mori et al., 2008).

2.11.3 Nanoparticles

Conjugation of AMOs with cholesterol has increased effect on cellular entry of AMOs. However, this mode of delivery is dependent on presence of a transmembrane protein S1D1 (Wolfrum et al., 2007). Leukocyte have low levels of S1D1 therefore AMO delivery with cholesterol conjugation is considered less effective for IBD therapy (H. Zhang, 2009). Different nanoparticles based delivery system are being investigated. These include lipid-based mechanism, dendrimers, polylactide-coglycolide and polyethylenimine systems. Natural polymers(chitosan, atelocollagen and natural protamine) as well as inorganic materials and exosomes are also being investigated (Yu Zhang, Wang, & Gemeinhart, 2013). Usage of such nanoparticles offer huge advantages that includes cost effectiveness reduced toxicity and reduced immunogenicity. Though nanoparticles are less effective than

viral vectors, still they can be considered as more efficient because they offer the alteration of surface ligands enabling targeting of specific receptors of cell surface followed by cellular uptake. β7 integrin-targeted nanoparticle system have been used to deliver cyclin D1(a siRNA) to target certain sets of leukocytes responsible for intestinal inflammation. (Peer, Park, Morishita, Carman, & Shimaoka, 2008).

These nanoparticles were attached covalently with monoclonal antibody targeting integrin β7 and have capacity for attachment with outer surface liposomes. Deliver of CyD1 siRNA with help of this nanoparticle has been found to repress TNFα and IL-12. In addition, naked siRNA were unable to penetrate into mucosal tissues. When liposomes were used as carrier vehicle for Anti-TNFα and delivered through rectal anema, a significant reduction in TFNAα mRNA was observed which results in reduction in histological inflammation (Yingjie Zhang et al., 2006).

2.11.4 Cell-to-cell delivery

A nonpathogenic E. coli strain has been engineered in order to produce short hairpin RNA (shRNA) which can target a mammalian gene in vivo and in vitro. This phenomenon has been referred as transkingdom RNA interference. This E. coli, after successful intravenous and oral administration, caused silencing in human colon and intestinal epithelium. (Xiang, Fruehauf, & Li, 2006).

Another delivery model based on cells system has been developed which uses the fact that primary B-lymphocytes have Ranse III enzymes. These enzymes are important for miRNA synthesis. B-lymphocytes have been successfully programmed with help of non-viral plasmid and is being used for delivery of AMOs. (Almanza et al., 2013).

2.12 Study of Biological signaling pathways using computational approaches

With overflow of experimental data, there is a need to develop computational tools in order to interpret the expression data. Vast amount of data discloses a large number of issues for interpreting the biological systems instead of giving the strong solution and explanation. This problem is catered by increasing the modeling and simulation methods to analyze data for biological systems in light of computational system biology. This field allows to develop distinct procedures and methods with regard to biology and allows us to understand biological system structures (Wolkenhauer, 2001). Developing dynamics of such systems can help to put a foundation for the control, modification and designing of system to confirm properties predicted by biological scientists (Kitano, 2002a).

2.12.1 The formal modeling approach

Various modeling approaches are applied for more than a decade in order to verify several simultaneous systems. Various tools and procedures are utilized from this field in order to observe the behavior of biological systems. They are also used to model and verify such complex systems.

2.12.2 Continuous Petri nets (PN) formalism

Petri net was named after a scientist named as Carl Adam petri, who proposed a graphical and mathematical formalism in 1960s responsible for simulation and modeling of Petri net (PN) has been named after Carl Adam Petri who, in the mid-sixties, proposed a mathematical and graphical formalism reasonable for the modeling of scattered and extensive frameworks. (René David & Alla, 1992; Murata, 1989; Reisig, 1985). PNs used

for quantitative and qualitative. In last 40 years, PNs have connected to vast varieties of uses and an extensive work has conducted on hypothetical advancements.

'Common' frameworks have explained by PN modeling especially for molecular networks. Reddy et al. has plied PN structure to demonstrate biochemical response (Reddy, Liebman, & Mavrovouniotis, 1996). PNs are useful mathematical formalisms allowing a natural portrayal of biochemical networks. This is for the most part because of their graph-based structure (Nagasaki, Doi, Matsuno, & Miyano, 2004). Over the years, a number of boosts and extensions of the Petri Nets formalism have been developed by a few studies (Jensen & Rozenberg, 1991; Koch, Reisig, & Schreiber, 2011). Out of these, continuous Petri Nets formalism has arisen as a potent tool to model the substantial entities involved in the several signaling pathways that function intracellularly (Westenskow et al., 2013). In more broad-spectrum terms, a continuous PN is arranged representation of a system's ODEs, but it is not as much of prone to error and it is easier to understand as compared to the differential equations

CHAPTER 3: MATERIAL AND METHODS

A biological system is comprised of numerous elements that are tightly regulated through signaling networks in which the expression level of an entity may either trigger or inhibit the synthesis rate of additional entities or itself. Generally, conventional models (such as ODE and PDE models) are used to represent the entities in a biological system through differential equations that implicate time derivatives for the estimation of various quantities like levels of concentrations, the rate of reactions and temperatures. These models are continuous in nature and rely on specific kinetic values, which are typically not available for all of the entities in the network and have been to be assumed. Hence, the modeling approaches based on graph theory make use of linear methods to observe the behaviors exhibited by biological systems. The methods employed in the current study are divided into consequent sub-sections presented in Figure 4.

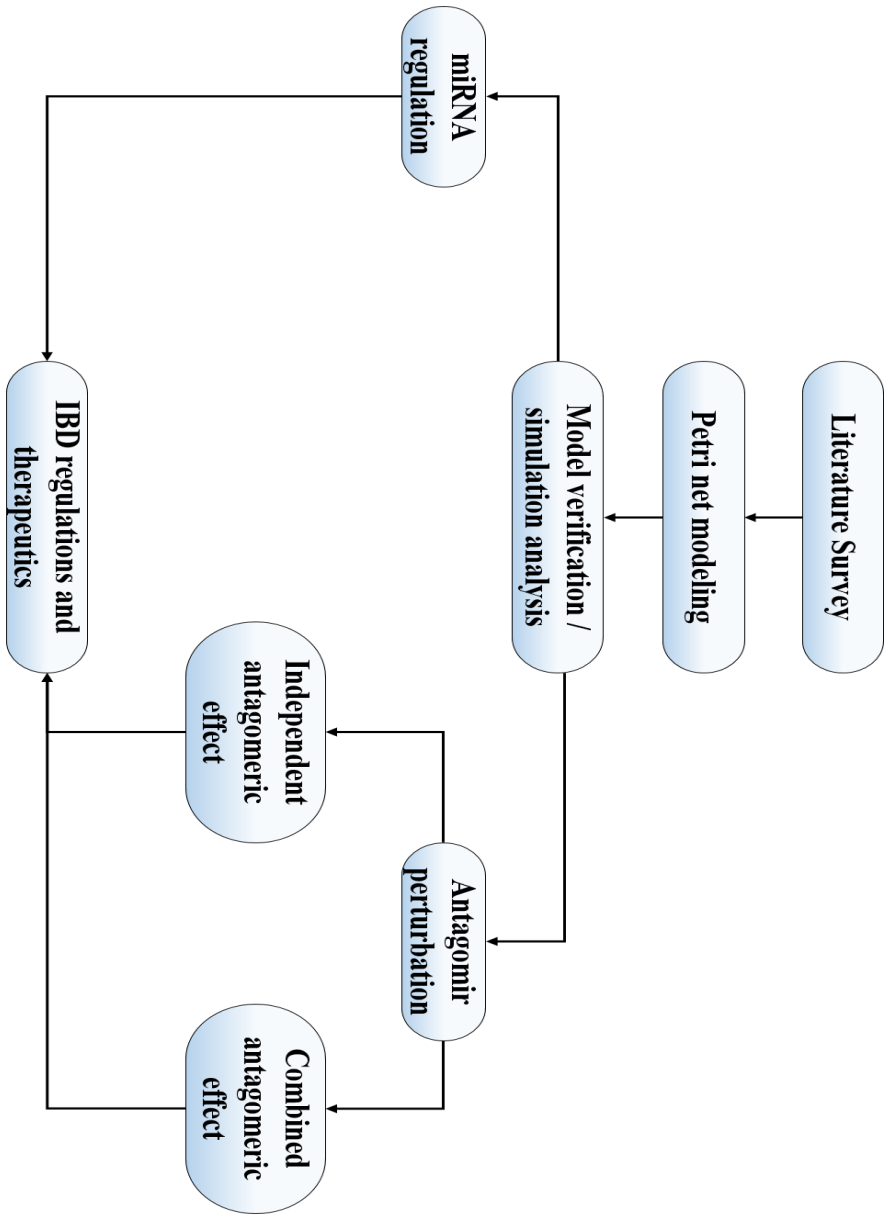

Figure 4 Methodology flow diagram of the study

3.1 PETRI NET MODELING

PNs) were developed by Carl Adam Petri in 1962 for the analysis of technical systems, in particular, the concurrent processes occurring in such systems, and were introduced as a part of his dissertation in 1962 (Ahmad, Niazi, Mansoor, Siddique, & Bibby, 2012). The framework itself is simple and flexible enough, though, that it has been successfully applied in other domains and studies as well, such as biochemical processes, industrial mechanisms, software analysis etc. (Blätke, Dittrich, Heiner, Schaper, & Marwan, 2012). Numerous regulatory pathways and networks have been modeled using Petri Nets to analyze and predict system behaviors. The modularity and flexibility are a major advantage of Petri Nets that allow the user to model a single or a combination of systems to analyze a variety of networks including epigenetic, metabolic, and transcriptional and protein-protein interaction etc.

3.1 Standard Petri Nets

A Petri net is a bipartite graph consisting of two sets of vertices, places and transformations. Places are illustrated with circles whereas boxes or bars indicate transformations. Usually, a place describes a resource or an entity (for example proteins, DNA, RNA etc.) and its state (number of entity present, relative level, cellular concentration etc.). In comparison, a transition describes any process occurring in the system. In a Petri net, the edges or arcs always connect vertices from two distinct sets only, i.e. places connect to transitions. The weight of an arc is equal to 1 by default and it represents the arc multiplicity. An arc never connects two transitions or two places. An arc with an indented dot at its head representing an ‘inhibitory arc’. The function of an inhibitory arc is suppression of token flow as it cease

the firing of a conversion. Places present before transitions are called "input places" while those after transitions are "output places" specific for that particular event. 'Tokens' are denoted as numbers or dots within a place in a Petri net. They are variable and represent states of entities (Blätke et al., 2012). Tokens, in particular, signify relative concentration levels of entities like RNA, proteins, ions, organic and inorganic molecules in a biological system (Chaouiya, 2007). 'Marking' represents the state of the system based on the presence of tokens in a particular place at that instance. In a dynamic system, marking evolves with time as the tokens flow in the model. All the input places must have tokens to fire a transition. In accordance with respective arc multiplicities, the total amount of tokens are withdrawn from input places and deposited to output place after a transition has been fired (René David & Alla, 2010).

Formally,

"*A standard Petri net is a quadruple N = (P, T, f, m0), where:*

P, T are finite, non-empty, disjoint sets. $P = \{P_1, P_2, P_3, \ldots\ldots P_n\}$ *is the set of places and* $T = \{T_1, T_2, T_3, \ldots\ldots, T_m\}$ *is the set of transitions.*

$f: \big((P \times T) \cup (T \times P)\big) \rightarrow \mathbb{Z}_{\geq 0}$ *defines the set of directed arcs, weighted by non-negative integer values.*

$m_0: P \rightarrow \mathbb{Z}_{\geq 0}$ *gives the initial marking".*

[89] (Section 2.2, Page number 6)

Definition (Continuous Petri net):

"A continuous Petri net is a quintuple CPN = (P, T, f, v, m0), where:

P, T are finite, non-empty, disjoint sets. P is the set of continuous places. T is the set of continuous transitions.

$f: \big((P \times T) \cup (T \times P)\big) \rightarrow \mathbb{R}_0^+$ *defines the set of directed arcs, weighted by non-negative integer values*

$v : T \rightarrow H$ *is a function, which assigns a firing rate function* h_t *to each transition t, whereby*

$H := \cup_{t \in T} \{ h_t | h_t : \mathbb{R}^{|\cdot t|} \rightarrow \mathbb{R}^{+} \}$ is the set of all firing rate functions, and $v(t) = h_t$ for all transitions $t \in T$

$m_0 : P \rightarrow \mathbb{R}_0^+$ gives the initial marking"

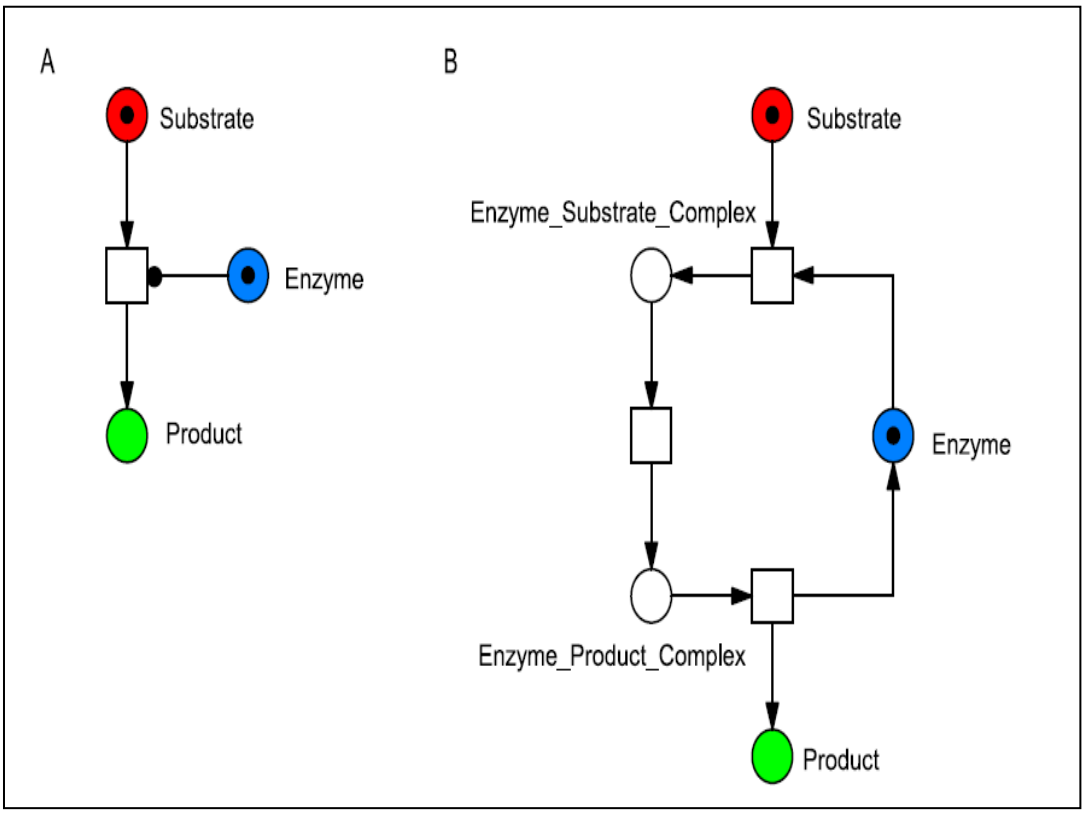

Figure 5: Representation of Enzymatic Reaction:

Figure (A) shows a Petri net of a simple enzymatic reaction. The enzyme is connected with a read edge depicting that the enzyme remains unconsumed. Figure (B) shows a Petri net describing steps of enzymatic reaction. The enzyme is consumed temporarily and released by the end of the reaction. (Blätke et al., 2012)

3.2 Semantics and Properties of Petri Nets

Firing enabled transitions define how the behavior of a system undergoes evolution with time. The behavior and evolution of a Petri Net is described by the firing of permitted transformations. A transition is an enabled, permitted or live transition when arc weights amidst pre places and transition are satisfied with the marking of its pre-places. As the transition is fired, the weighted markings of pre-places are subtracted from it and deposited in the post- places. This property is called firing rule.

In the present study, Petri net model generation and simulations were run using Snoopy version **snoopy-stable-windows-2017-12-13.msi** available at http://www-dssz.informatik.tu-cottbus.de/DSSZ/Software/Snoopy#downloads for Windows (64bit). Snoopy offers a variety of Petri net modeling options. Normally modeling tools do not offer much variety in net classes that can be used to design a model. However, Snoopy includes a number of net classes, for example, continuous, hybrid, stochastic, time-free Petri nets and their colored extensions. It also includes a number of analysis tools such as simulation, built-in animations, and export to other tools for analysis (Heiner, Herajy, Liu, Rohr, & Schwarick, 2012). Snoopy offers a unified environment for modeling of Petri nets. Snoopy provides the following modeling functions:

- Addition of graphical elements (Places, transitions, edges)
- Edit or modify the properties of nodes (e.g. initial marking, name) and edges (multiplicity).
- Define declarations (in colored Petri nets)

3.3 NON-PARAMETRIC STRATEGY FOR PETRI NET MODELING

Numerous studies have reported researches engaging Petri net strategies to model cellular signaling cascades and gene regulatory networks (LI et al., 2006). This formalism has been extended by various groups (Chaouiya, 2007; Hardy & Robillard, 2004; Sackmann, Heiner, & Koch, 2006) to comprehend the intricacy of the cellular environment. Present study is based on the non-parametric approach formulated by (Ruths, Muller, Tseng, Nakhleh, & Ram, 2008) to observe the ambulation of cell-specific signaling pathways using Petri net strategies. The inference on which a PN model base is that the signaling network connectivity is the most compelling element of signal proliferation (LI et al., 2006). Therefore, modifications in the activity levels of the proteins are illustrated in the PN model through token number (Ruths et al., 2008).

3.4 Construction of the Petri net

The chemical reaction regulations may not always be enforced to a signaling pathway. A cell signaling pathway is activated as a foreign particle interacts with receptors present on cell surface. In turn, it activates downstream proteins through modifications like phosphorylation, de-phosphorylation or interaction with proteins. Modeling such a complex and dynamic pathway a modeling strategy was formulated that well suited the network topology of the disease model (Inflammatory Bowel Disease). In the models constructed in the Petri net, places indicate the proteins/genes and miRNA (for instance, insulin receptor, ligands, enzymes, transport proteins, genes etc.). These elements are involved in the pathways in IBD while the transitions represent the procedures like interactions happening among the places (e.g. formation of complex, chemical reactions, posttranslational modification, transport processes, up-regulation or down-regulation of

gene/protein expression etc.). The markings of continuous places are real numbers and the firing of transitions is an extended procedure. Every arcs consist of weight equal to one except for the one indicated otherwise. Moreover, inhibitory arcs are used to display impedimentary effects of miRNAs on cellular procedures or Antagomirs on miRNAs. Our model depicts source transformations as the synthesis of proteins in the pathway while sink transitions represent the decay or dissociation of entities exiting the system.

In our study, Hybrid Petri Net was designed to understand change in the relative activity (up-regulation/down-regulation) and not the precise assessment of the protein concentration/parameters within the signaling pathways of IBD.

A Hybrid Petri net does not require an exhaustive state space and can define a finite system with an infinite state space. It offers features that allow representation of modules in the designed model and analysis of network can be done through linear algebraic methods. Various mathematical models have been proposed in the literature, but there is so far no Petri Net model accepted for IBD that simultaneously incorporates multiple signaling pathways involved in disease.

When studying biological systems, the concentration level or a discrete number of an entity (protein, DNA, RNA etc.) is represented by tokens. In order to perform simulations, it is important to indicate the availability of entities in a biological system. Therefore, initial values were assigned as tokens in the signaling pathways that corresponded to an initial state of entities in the cell

The steps employed to generate the PN model are literature survey to excerpt the critical factors involved in apoptosis, inflammation, cellular adhesion and extracellular matrix

integrity with and without miRNA deregulations; pathway abstraction; generation of Petri net model and its analysis.

3.5 MODEL VERIFICATION THROUGH SIMULATIONS

A model can display all sorts of behavior which established the state space of the model (Ahmad et al., 2012). Analysis of distinct behaviors can be studied using discrete model. Contrastingly, an intense and a recurrent infinite state space is studied through continuous model. It is typically approximated to hybrid or discrete equivalents (Ahmad, Roux, Bernot, Comet, & Richard, 2008).

The state space information is used by model checking methods to check the presence of various behaviors and properties in the system (Clarke, Grumberg, & Peled, 1999). A Petri net carries out simulations of place/ transition network with token flow. It can help us predict the dynamics of model with time. Analysis of a state space subset or all the possible state spaces can be done through simulation runs. Increasing the simulation runs contributes to increased precision of average time. With the passage of time, all simulation runs fluctuate and depict system behavior. Therefore, a model that has either infinite or infeasible state space can be verified through model checking. To validate the designed pathway, our methodology utilized the simulative property of Petri net.

For model verification, we studies on some of the critical properties (impact on signaling pathways in case miRNA over-express and repression of higher miRNAs with anti-miRNA agents) which have been reported in published literature.

When the simulation results were compared to already proven experimental data regarding signaling pathways (apoptosis, ECM integrity, cellular adhesion, and inflammation), the prior findings found in literature were verified.

In crux, our method computed the tokenized activity levels as abstract measures in which the changes over the passage of time depicted changes in active protein concentrations. Therefore, achieving similar system behavior relative to experimental data our model was validated. Henceforth, various biological insights can be driven through extending this model and this signaling pathway can be better understood. The details have been given in Table: 1.

Table 1 List of miRNAs and their target proteins involved in the signaling pathways such as apoptosis, inflammation, cellular adhesion and extracellular matrix integrity in inflammatory bowel disease

Target protein	**miRNA**	**Reference**
NOD-2	**miR-320,miR495,miR-512, miR-671**	Pierdomenico M, Cesi V, Cucchiara S, Vitali R, Prete E, Costanzo M, Aloi M, Oliva S, Stronati L. NOD2 is regulated by mir-320 in physiological conditions but this control is altered in inflamed tissues of patients with inflammatory bowel disease. Inflammatory bowel diseases. 2016 Feb 1;22(2):315-26. Cao B, Zhou X, Ma J, Zhou W, Yang W, Fan D, Hong L. Role of miRNAs in Inflammatory Bowel Disease. Digestive Diseases and Sciences. 2017 Apr 8:1-3.
NOS	**miR-21,miR-221,miR-146A**	Zampetaki A, Dudek K, Mayr M. Oxidative stress in atherosclerosis: the role of microRNAs in arterial remodeling. Free Radical Biology and Medicine. 2013 Sep 9;64:69-77.
FOXO3	**miR-155**	Kong W, He L, Coppola M, Guo J, Esposito NN, Coppola D, Cheng JQ. MicroRNA-155 regulates cell survival, growth, and chemosensitivity by targeting FOXO3a in breast cancer. Journal of Biological Chemistry. 2010 Jun 4;285(23):17869-79.
β-Catenin	**miR-200**	Schmalhofer O, Brabletz S, Brabletz T. E-cadherin, β-catenin, and ZEB1 in malignant progression of cancer. Cancer and Metastasis Reviews. 2009 Jun 1;28(1-2):151-66.

CHAPTER 4: RESULTS

4.1 Model Construction

Inflammatory Bowel Disease is a complex system involving multiple proteins that are regulated by different miRNAs at distinct phases of disease progression. From extensive literature review and data mining of multiple pathway databases, we formulated an abstract pathway as shown in Figure **6.** To narrow our focus from a wide-range of elements involved in the highly interconnected network of disease pathway we selected four pivotal proteins namely NOD2, NOS, FOXO3, and B-catenin. The selection of these proteins is based on the function they perform such as apoptosis, cellular adhesion of epithelial membrane cells, cellular matrix integrity and inflammatory response. Our target proteins and their respective function are interconnected and expression or blockade of one can have impact on other key regulatory elements that lie in the downstream of the pathway. This disturbance caused by up blocking of our target proteins leading to disease state.

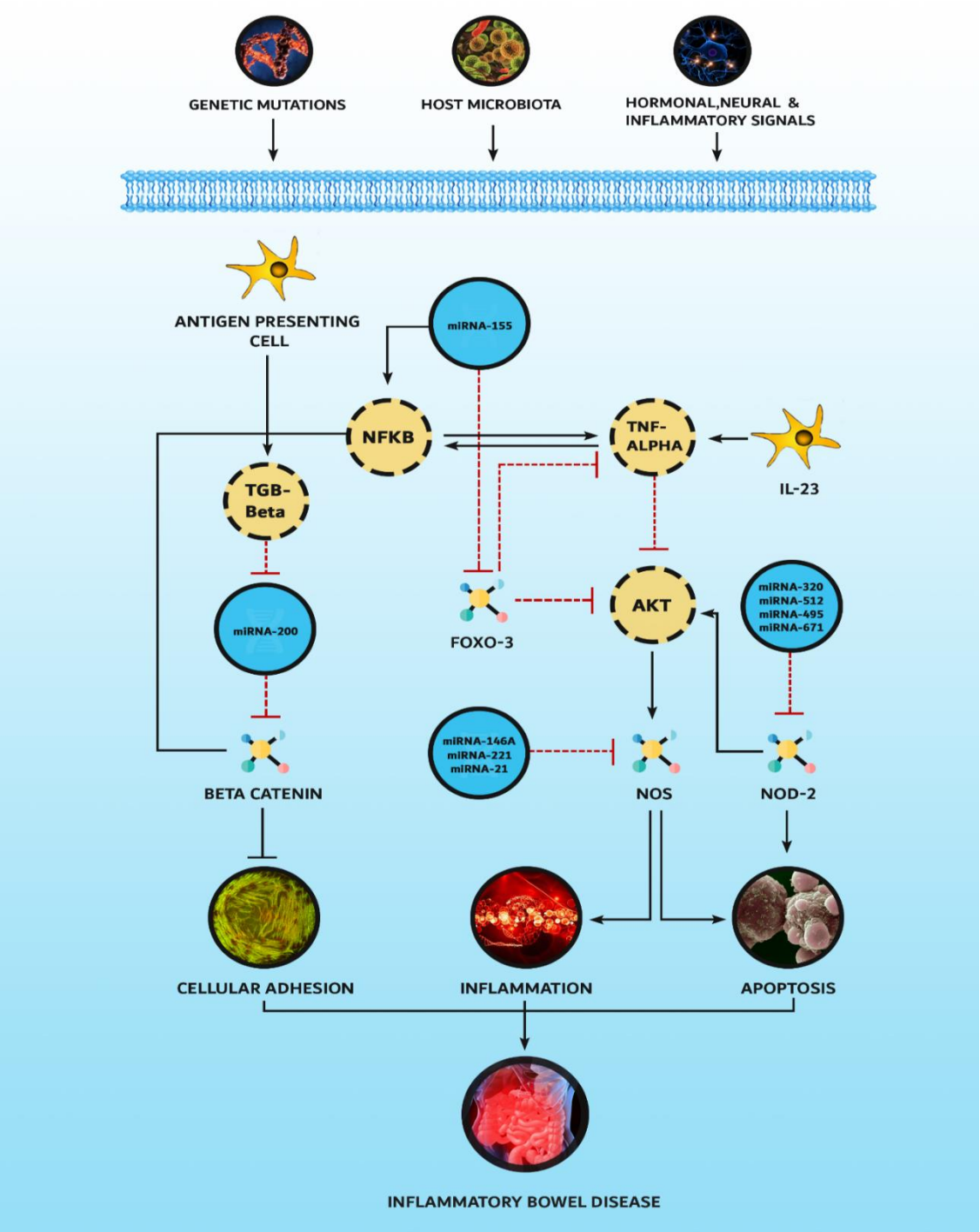

Figure 6 Abstracted Pathway of Inflammatory Bowel Disease with key regulatory proteins, their downstream targets and cellular processes controlled by them

4.2 Gut Pathways in homeostasis

The pathways in gut, which get affected by inflammatory bowel disease, are shown in Figure 7. The figure shows the entities of the involved pathways. Key proteins regulate the pathways such as the apoptosis pathway is regulated by NOD-2, NOS, and IL-23 signaling molecules. Similarly, β-catenin regulates extracellular matrix (ECM) integrity and cellular adhesion by playing vital structure role in tight junction proteins and cellular matrix scaffold. Inflammation is controlled by multiple signaling pathway regulated by numerous biological agents and for inflammatory bowel disease, NOS proteins is found to be highly involved in regulation of inflammatory responses. Our gut is under persistent stress from the internal and external environment including genetic mutations, microbiota changes, hormonal, neural and other stress signals. To study the changes that occur to homeostatic conditions under the inflammatory bowel disease we subjected our constructed model under normal conditions to Petri net modeling. Petri net model generation (Figure 8) was based on assumption that system is in continuous/dynamic state and all the entities of our model under normal as shown in Figure 7 conditions are already in play in maintaining homeostasis.

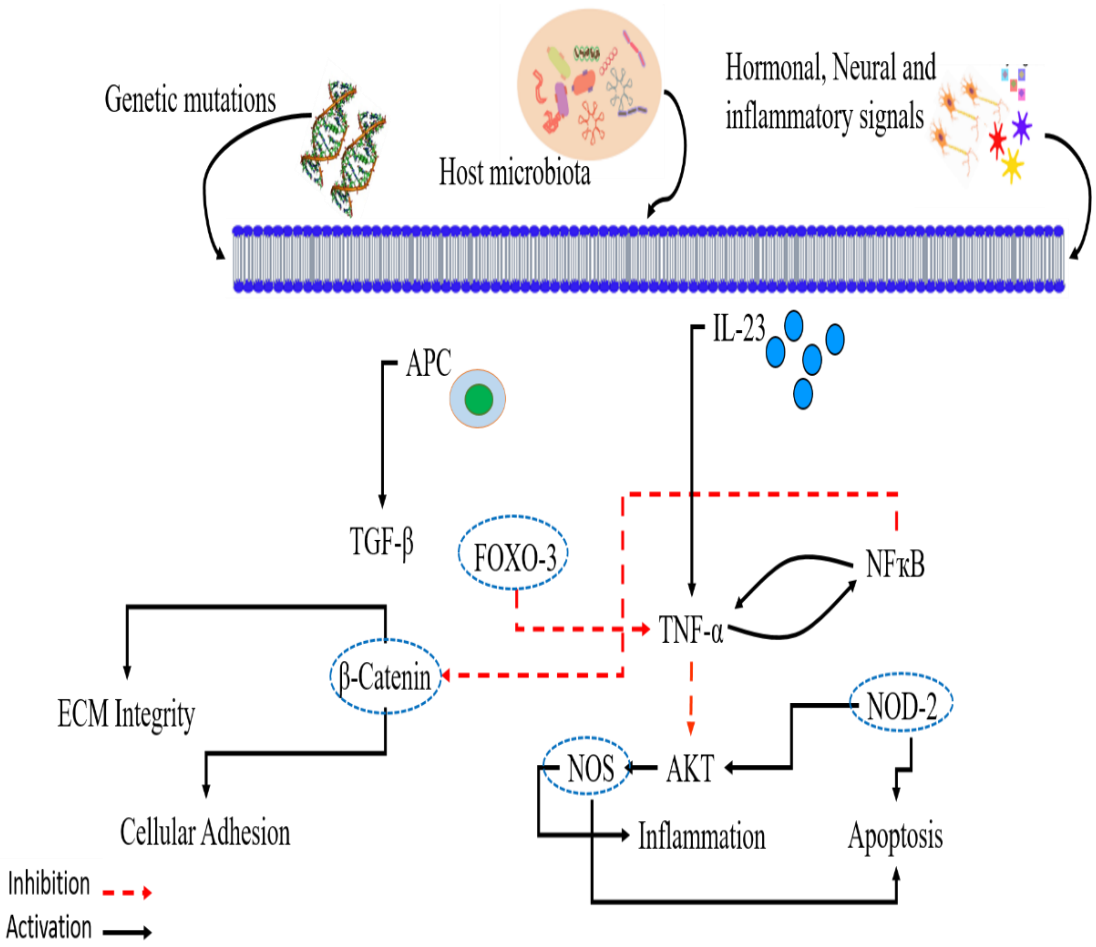

Figure 7: Normal pathways involved in gut before the imbalance caused by miRNAs leading to Inflammatory Bowel Disease

4.3 Petri net model of Gut Pathways in homeostasis

Our normal gut pathway from Figure 7 was subjected to Petri net modeling to analyze the relative quantitative dynamics of the system as shown in Figure 8. We call our normal model as base model as it will serve as standard to future alterations and interceptions. In Figure 8 our proteins in focus or target proteins are colored in blue circles. The red lines show inhibitory links whereas black arrowhead lines show activation.

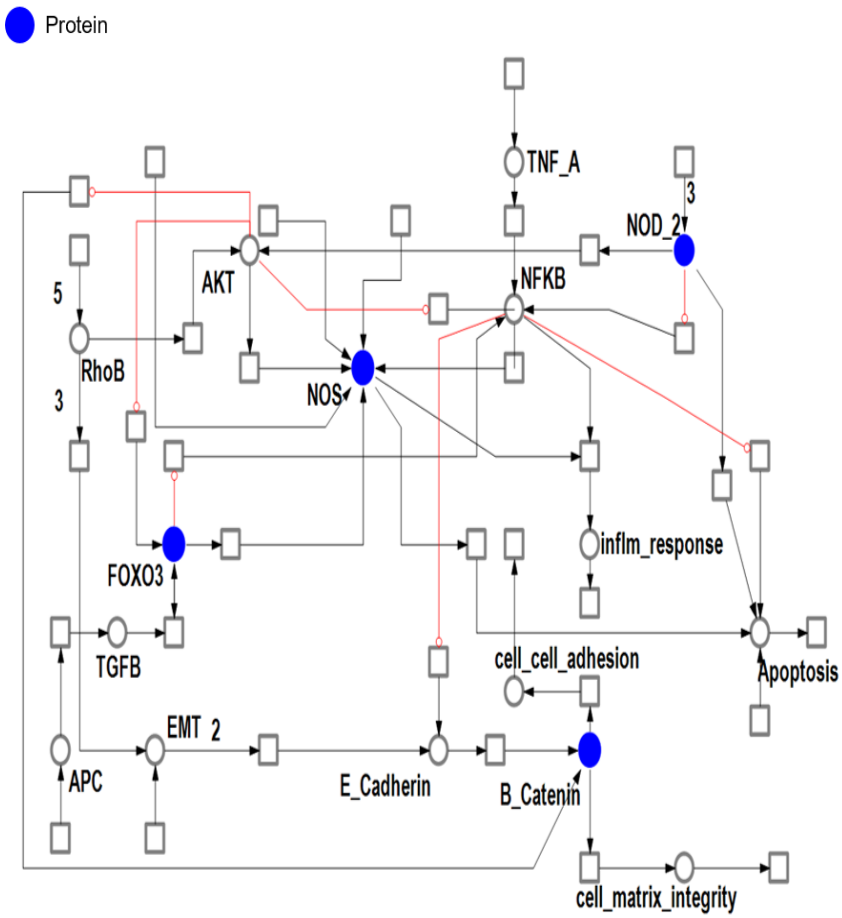

Figure 8 Illustration of Petri net model of base model representing key signaling proteins involved in regulation of pathophysiological pathways involved in homeostasis

Petri net model simulations Figure 9 shows the relative concentration levels of involved 9 entities including our 4 key regulatory proteins during homeostasis. These concentration levels give us a platform to relate the magnitude changes that occur to involved entities during disease condition and under therapeutic interventions. From the graph, it is evident the high levels of entities such as RhoB and β-catenin which are responsible for maintaining extracellular matrix integrity and cellular adhesion whereas the inflammatory agents such as TNF-α and NOS levels are low as it should be during homeostasis.

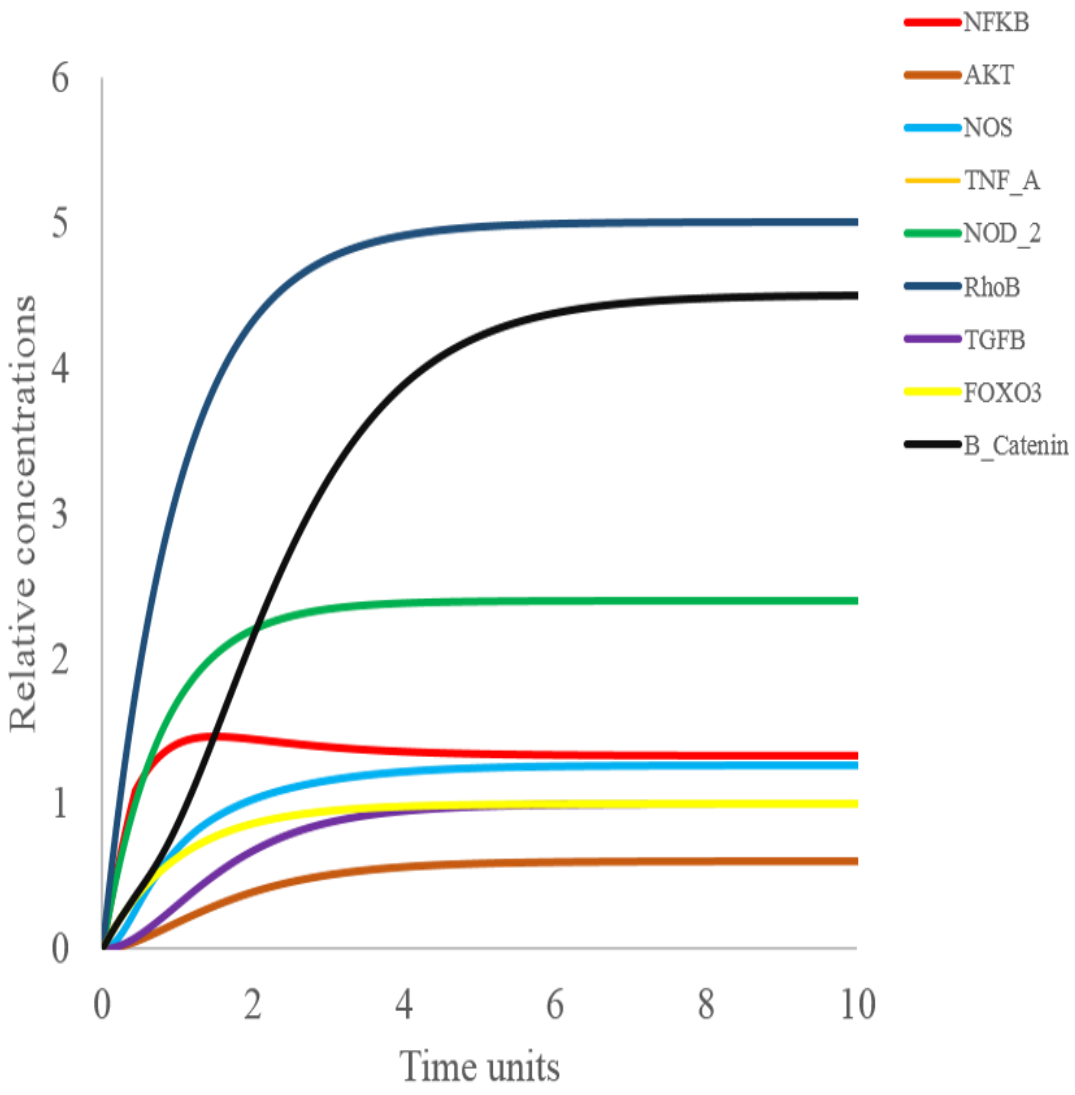

Figure 9: The graph shows the relative concentration of involved 9 entities in our base model under homeostatic conditions

4.4 Gut Pathways in inflammatory bowel disease

Our base model from Figure 7 was modified with addition of miRNA that are affecting our regulatory proteins to generate a disease model as shown in Figure 10. The disease model shows our regulatory proteins are being regulated by the expression of miRNA due to stress resulting in disruption of homeostatic conditions and taking the system towards the inflammatory bowel disease. From the model we can see the β-catenin is regulated by one miRNA (miR-200), similarly, FOXO-3 is also controlled by single miRNA (miR-155). Whereas NOS is governed by 3 miRNAs (miR-21, miR-221, and miR-146A) and NOD-2 by 4 miRNAs (miR-320, miR-495, miR-512, miR-671). The incursion of these miRNAs causes disruption of base model regulated pathways causing change in expression levels of our regulatory proteins that leads the system towards inflammatory bowel disease. This can be seen in our disease model below in Figure 10.

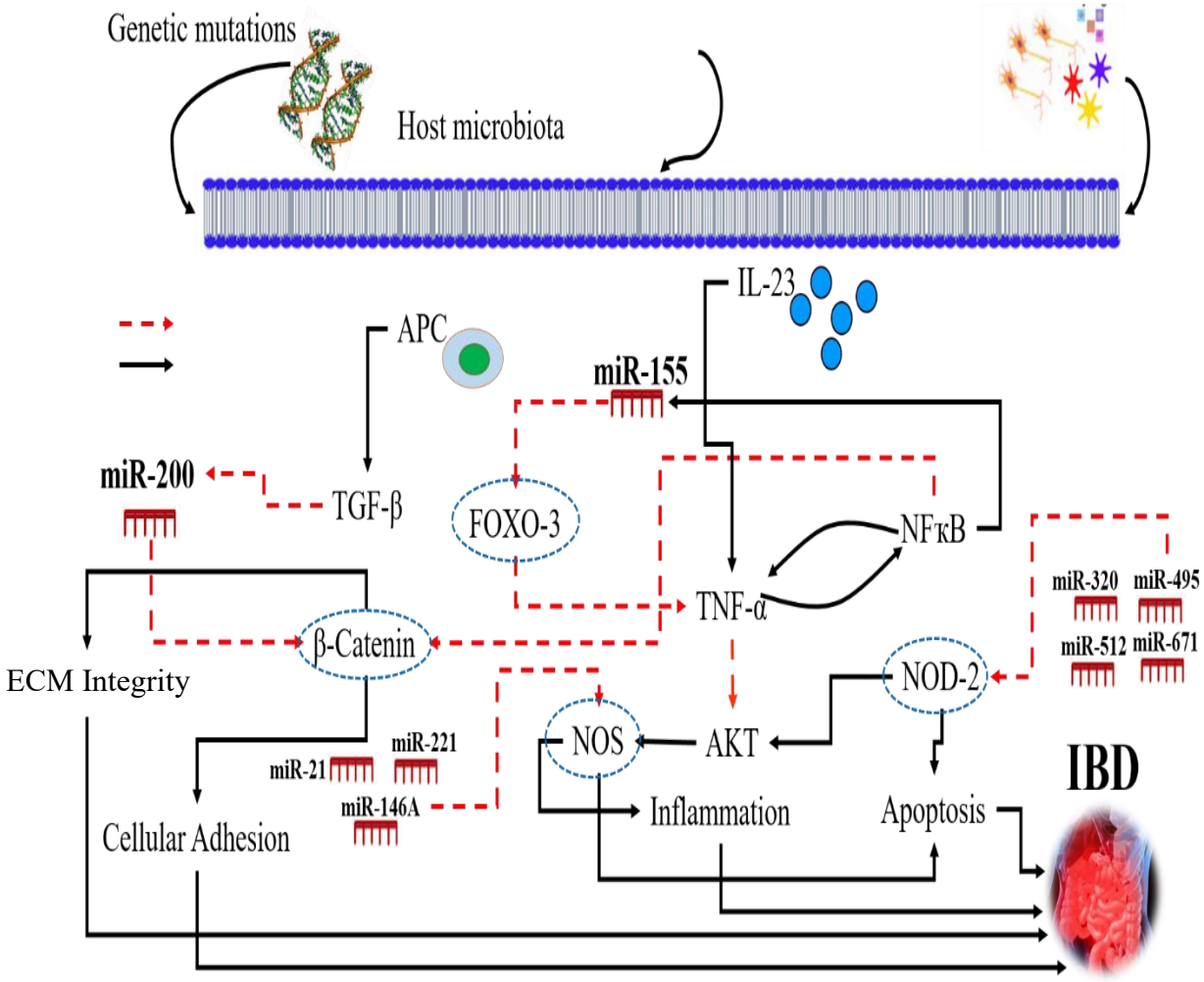

Figure 10 Pathways involved in inflammatory bowel disease under the imbalance caused by the miRNA expression under the stress

4.5 Petri net model of Gut Pathways in inflammatory bowel disease

Our disease model from Figure 10 was subjected to Petri net modeling to analyze the relative quantitative dynamics of the system as shown in Figure 11. miRNAs are shown by red circles which are affecting our target proteins shown with blue circle.

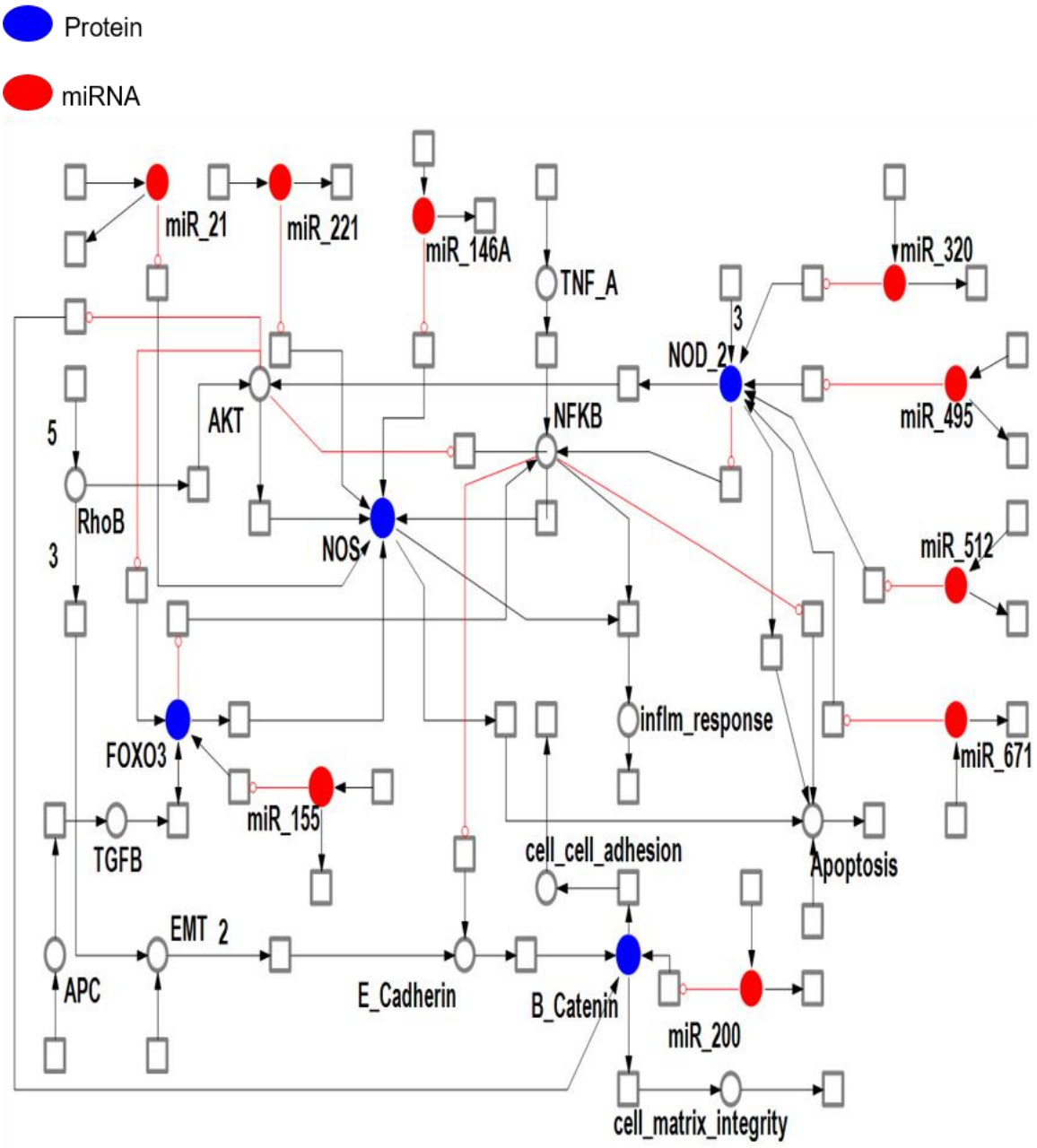

Figure 11 Illustration of Petri net model of disease model representing key signaling proteins involved in regulation of pathophysiological pathways disrupted by miRNA incursion during inflammatory bowel disease

Petri net model simulation of disease model from Figure 11 shows the relative concentration levels of involved 11 entities including our 4 key regulatory proteins during inflammatory bowel disease. These concentration levels are then compared to concentration levels of the same entities from baseline model and their relative change is observed as shown in Figure 12. It is observed that entities from base model mentioned in the previous results namely RhoB and β-catenin show drops in their magnitudes whereas levels of entities such as TNF-α and NOS are increased from the base model simulations.

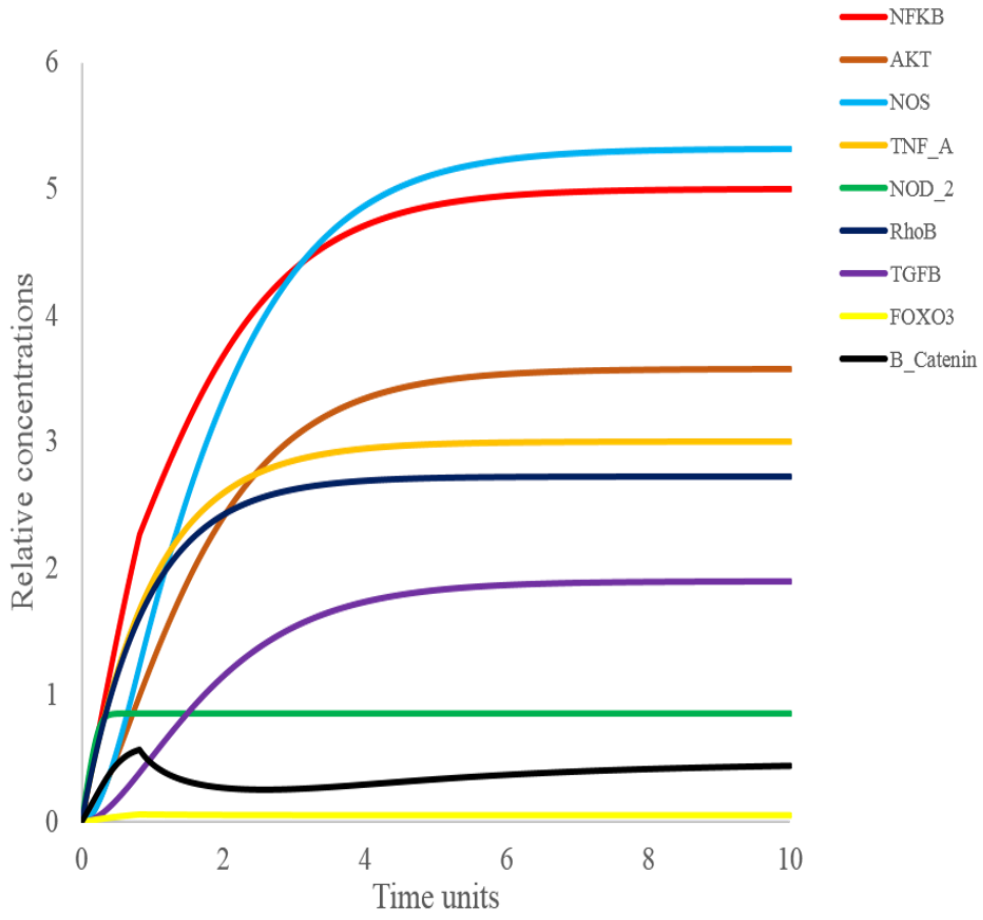

Figure 12 The graph shows the relative concentration of involved 9 entities in our base model under inflammatory bowel disease

4.6 Comparing the expression levels of regulatory proteins in baseline and disease model

We further analyze the relative changes in magnitudes occur from our baseline model to disease model to our target proteins which are key regulatory agents of vital pathophysiological pathways. In the following graphs, the green line indicates levels during homeostasis conditions whereas the red line depicts the levels during inflammatory bowel disease.

From Figure 13 it is evident that NOS which is involved in inflammation and apoptosis as per our model has low expression levels during homeostasis and its level rises significantly during inflammatory bowel disease.

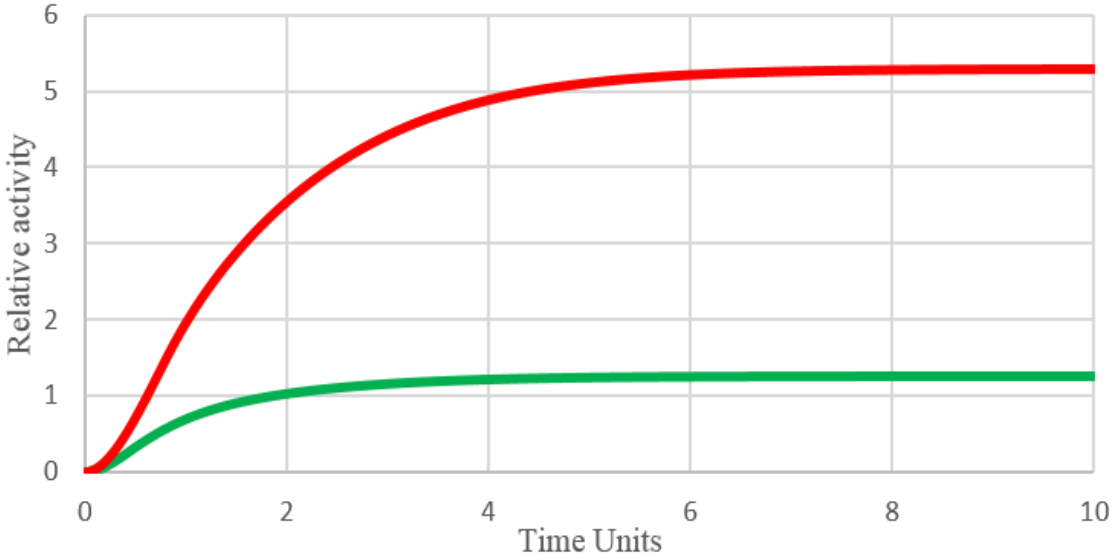

Figure 13 Relative expression levels of NOS during homeostasis (base line model) and inflammatory bowel disease (disease model)

Figure 14 shows the relative expression levels of NOD-2 which is involved in apoptosis and inflammation, indicates drop of levels resulting in hindrance in apoptosis of worn out cells and hike in inflammation in combination with rise of NOS levels.

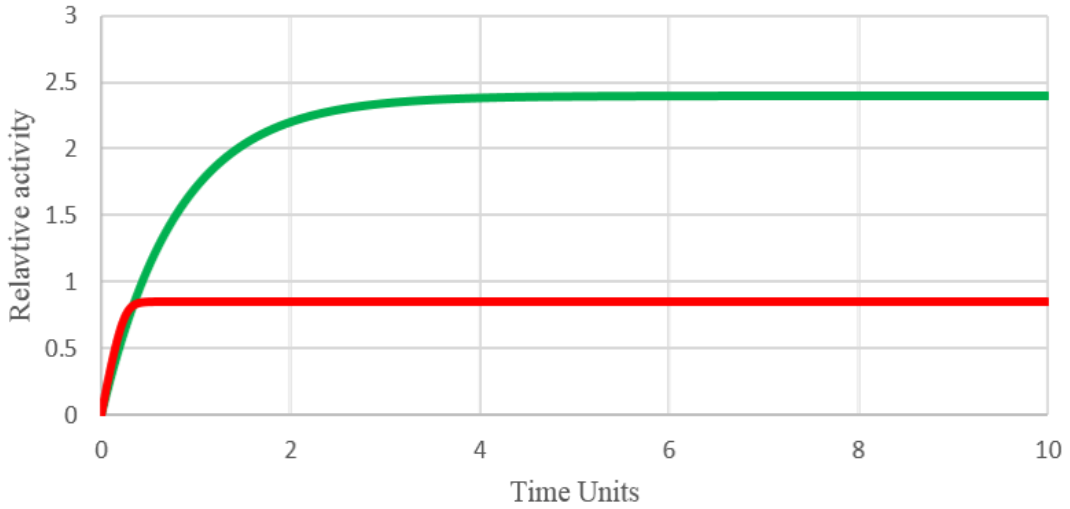

Figure 14 Relative expression levels of NOD-2 during homeostasis (base line model) and inflammatory bowel disease (disease model)

Figure 15 shown the relative expression of FOXO-3 protein which is involved in playing multiple regulating roles by interacting with NFκB, TGF-β, and TNF-α. It can be seen that during inflammatory bowel disease the expression of FOXO-3 is reduces to almost 0. This causes disruption of downstream pathways maneuvered by NFκB, TGF-β, and TNF-α such as apoptosis, inflammation and cellular adhesion.

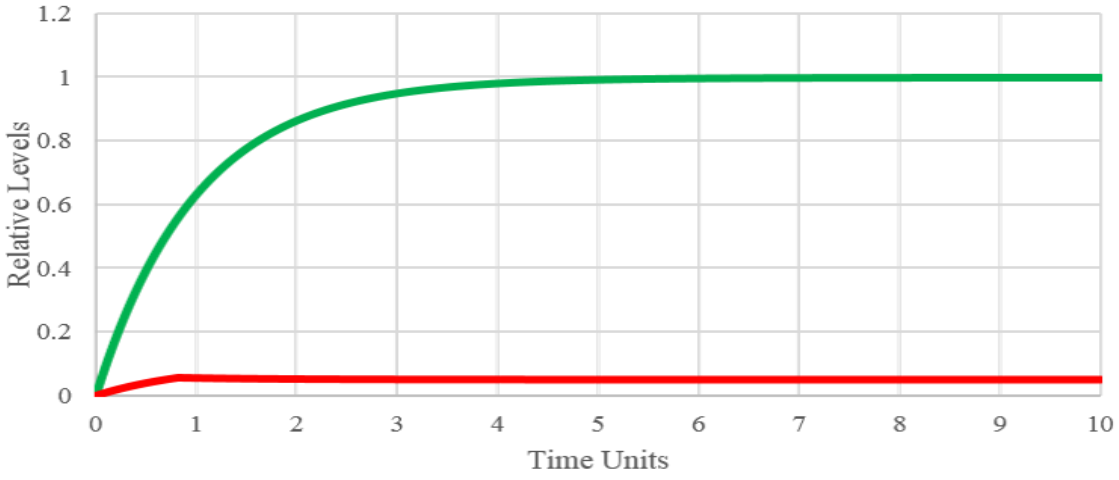

Figure 15 Relative expression levels of FOXO-3 during homeostasis (base line model) and inflammatory bowel disease (disease model)

Figure 16 shows the relative expression levels of β-catenin, which is key regulatory protein due to its important role in both extracellular matrix integrity and cellular adhesion. β-catenin is structural component of both cellular matrix scaffolds and tight junctions of membrane elements. Petri net simulations show significant drop in its levels during inflammatory bowel disease. This drop is understandable as during inflammatory bowel disease cellular adhesion is at low causing invasion of foreign particles into gut membrane and loss of extracellular matrix integrity leading to loss of environment that holds the repair elements.

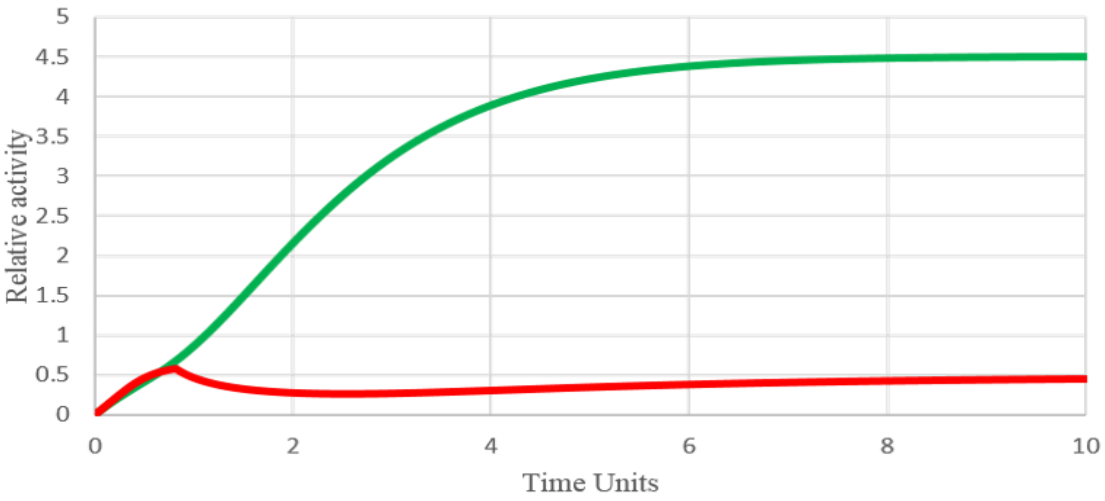

Figure 16 Relative expression levels of β-catenin during homeostasis (base line model) and inflammatory bowel disease (disease model)

4.7 Gut Pathways in antagomir therapy in inflammatory bowel disease

Our baseline model from Figure 7 was molded to disease model Figure 10 by the inclusion of miRNAs affecting our target regulatory proteins. Disease model served as a base for us to apply therapeutic interventions. Such interventions include the silencing action of antagomirs on the miRNAs, which disrupted our baseline model and caused inflammatory bowel disease as a result. Figure 17 show our therapeutic model involving antagomirs acting on miRNAs, which are inhibiting our regulatory proteins of homeostasis. The application of antagomirs breaks the inhibitory links of miRNAs with our target proteins resulting in getting back our baseline model and ultimately setting the whole system into recovery mode from inflammatory bowel disease to homeostasis.

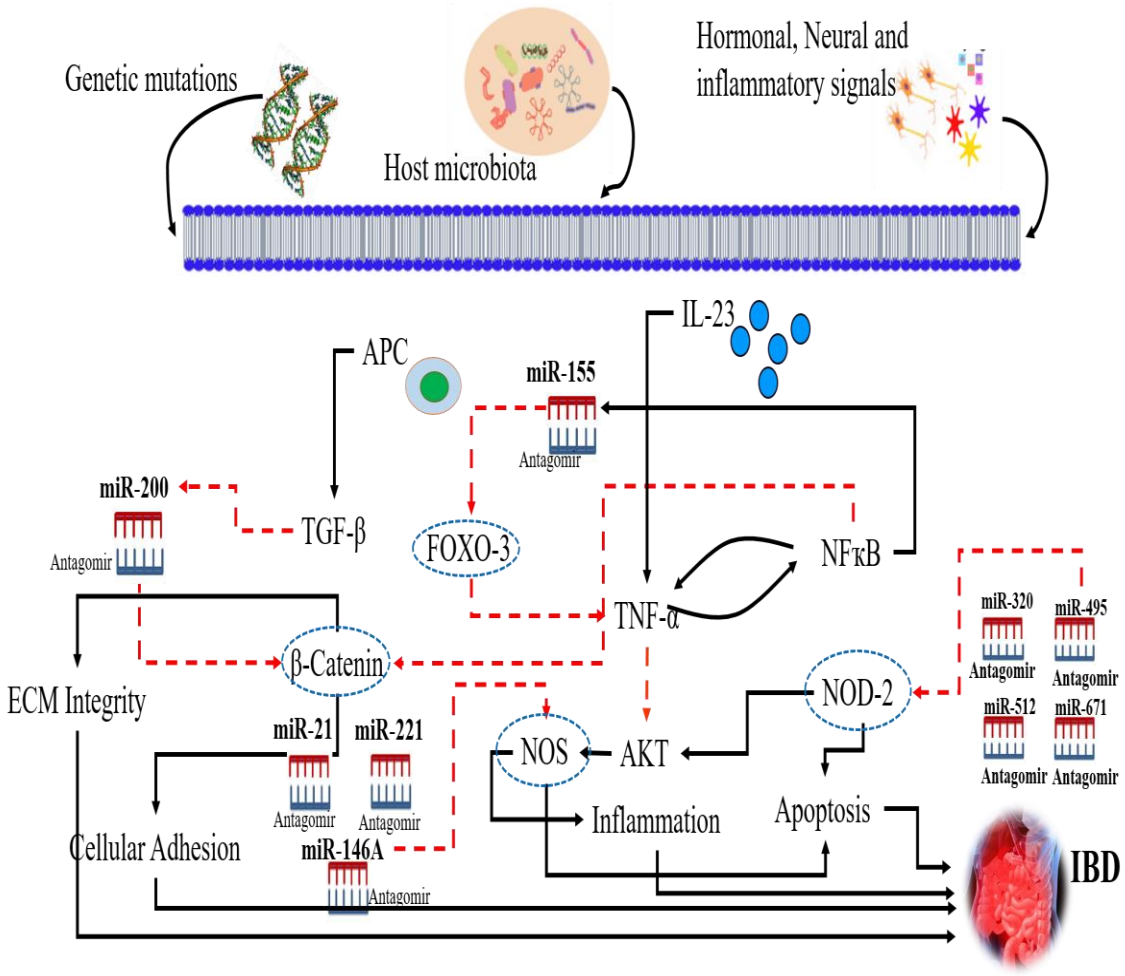

Figure 17 Antagomir therapy applied to disease model of inflammatory bowel disease.

4.8 Petri net model of Gut Pathways in antagomir therapy in inflammatory bowel disease

Our antagomir therapy model Figure 17 was subjected to Petri net modeling to analyze the impact of antagomirs on our target proteins levels from disease model Figure 18. Our target proteins are shown in blue circles, red circles show miRNAs and green circles show antagomirs.

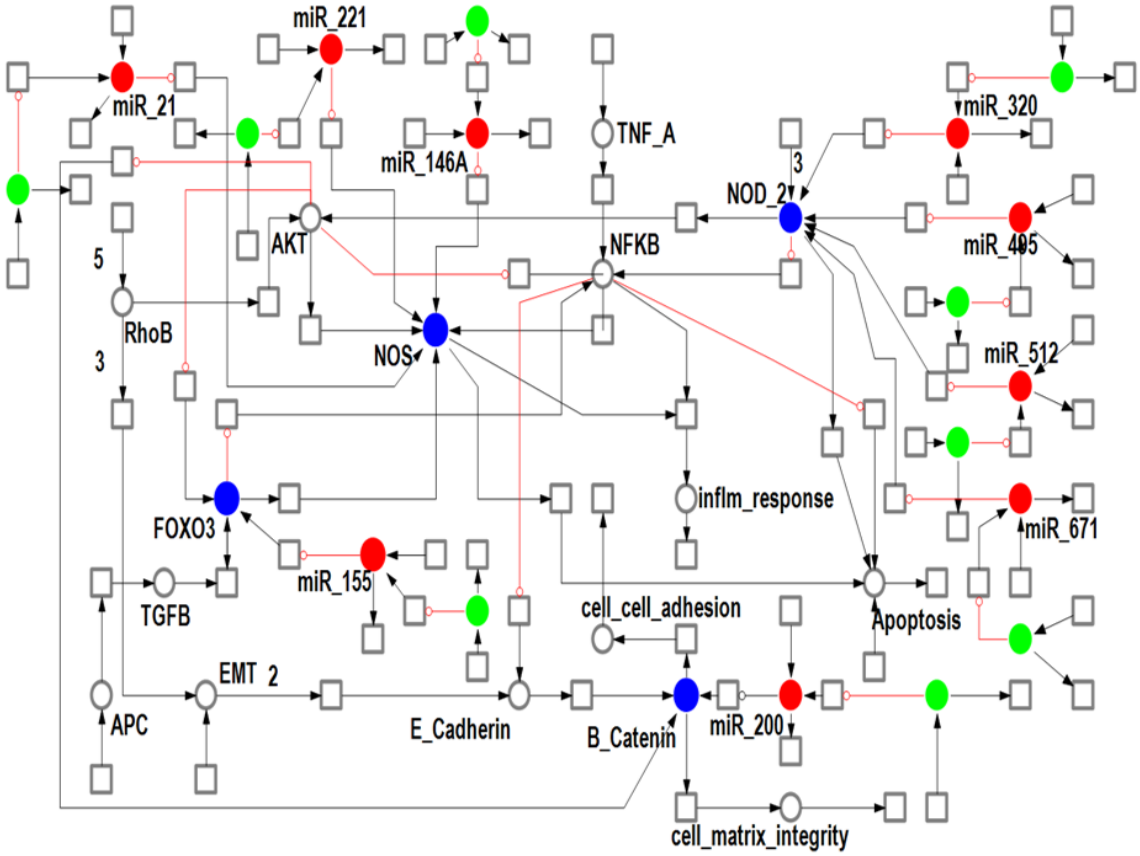

Figure 18 Illustration of Petri net model of antagomir therapy model representing key signaling proteins involved in regulation of pathophysiological pathways disrupted by miRNA incursion during inflammatory bowel disease and antagomirs acting on miRNAs

Petri net model simulation of antagomir therapy model from Figure 18 shows the relative concentration levels of involved 11 entities including our 4 key regulatory proteins during inflammatory bowel disease. These concentration levels are then compared to concentration levels of the same entities from antagomir therapy model and their relative change is observed as shown in Figure 19. It is observed that entities from disease model mentioned in the previous results namely RhoB and β-catenin show recovery in their magnitudes whereas levels of entities such as TNF-α and NOS are decrease from the disease model simulations.

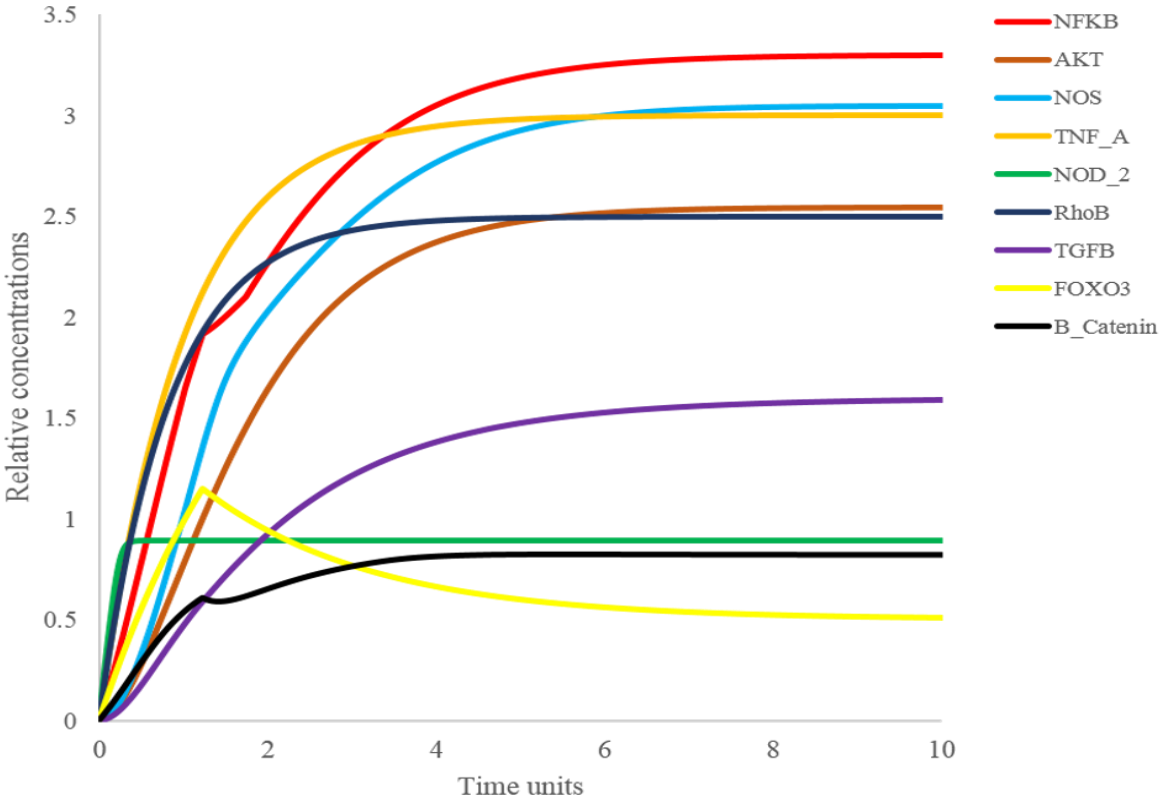

Figure 19 The graph shows the relative concentration of involved 9 entities in our disease model under antagomir therapy

4.9 Comparing the expression levels of regulatory proteins in baseline model, disease model, and antagomir therapy model

We further analyze the relative changes in magnitudes occur from our disease model to antagomir therapy model on our target proteins which are key regulatory agents of vital pathophysiological pathways. In the following graph, the blue line shows levels after applying antagomirs whereas the red line depicts the levels during inflammatory bowel disease. From Figure 20 it is evident that NOS which is involved in inflammation and apoptosis as per our model has high expression level during inflammatory bowel disease and its level recovers significantly during antagomir therapy.

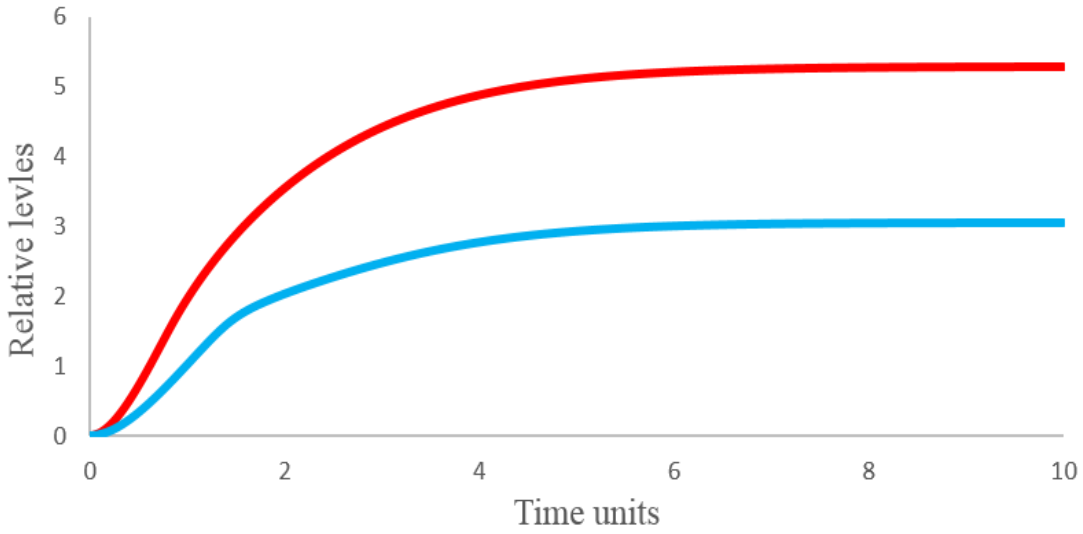

Figure 20 Relative expression levels of NOS during inflammatory bowel disease (disease model) and antagomir therapy

Figure 21 shows the relative expression level of NOD-2 that is involved in apoptosis and inflammation, indicates slight recovery towards the normal level as compared to disease model level.

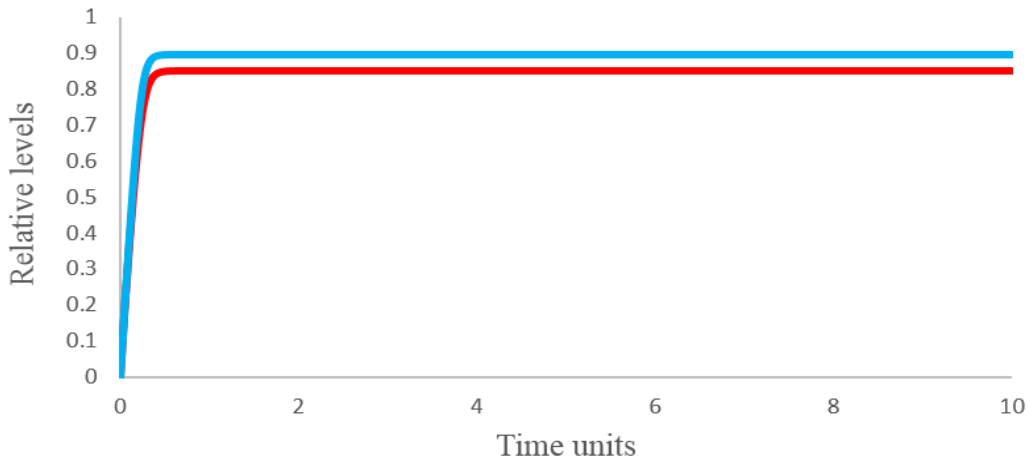

Figure 21 Relative expression levels of NOD-2 during inflammatory bowel disease (disease model) and antagomir therapy

Figure 22 shown the relative expression of FOXO-3 protein which is involved in playing multiple regulating roles by interacting with NFκB, TGF-β, and TNF-α. It can be seen that after application of antagomirs FOXO-3 levels rises which during inflammatory bowel disease was reduced to almost 0. This causes recovery in the disruption of downstream pathways maneuvered by NFκB, TGF-β, and TNF-α such as apoptosis, inflammation and cellular adhesion.

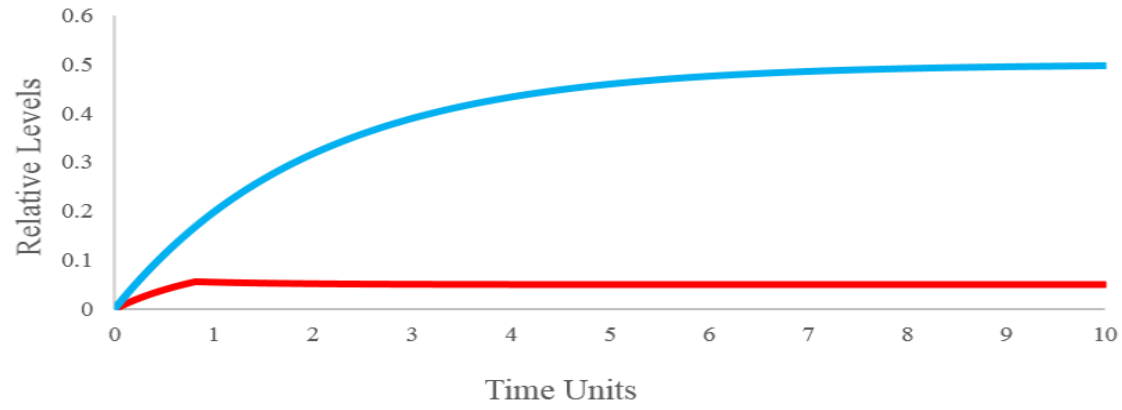

Figure 22 Relative expression levels of FOXO-3 during inflammatory bowel disease (disease model) and antagomir therapy

Figure 23 shows the relative expression levels of β-catenin, which is key regulatory protein due to its important role in both extracellular matrix integrity and cellular adhesion. β-catenin is structural component of both cellular matrix scaffolds and tight junctions of membrane elements. Petri net simulations show significant drop in its levels during inflammatory bowel disease. This drop in the levels is recovered after antagomirs interventions.

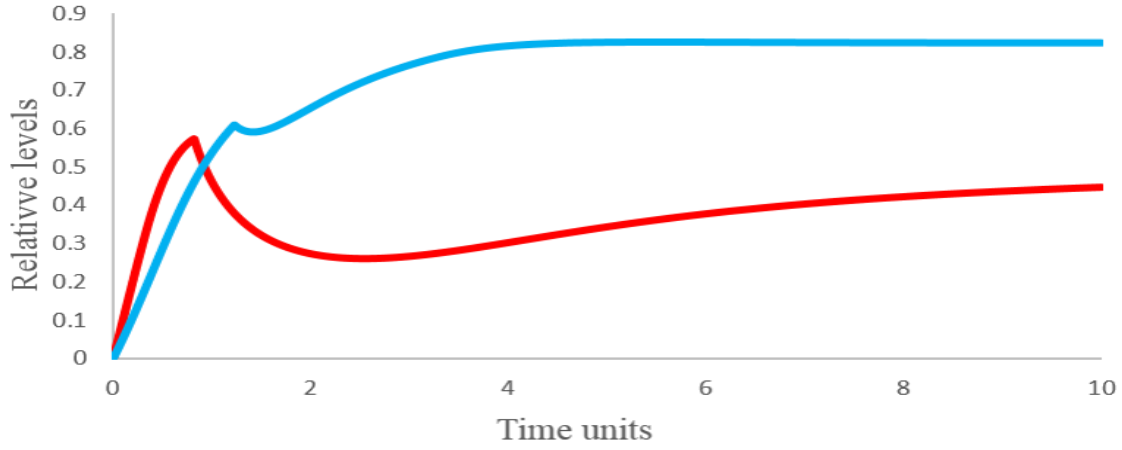

Figure 23 Relative expression levels of β-catenin during inflammatory bowel disease (disease model) and antagomir therapy

CHAPTER 5: DISCUSSION

Inflammatory bowel disease is a modern disease of modern world and people from all age groups are prone to it. It has equal prevalence in both genders of the population but the age group of 15-35 are at higher risk. Inflammatory bowel disease is a multi-factorial disease. Both phenotypes UC and CD have overlapping symptoms which are customarily treated using anti-inflammatory, anti-biotic, immune-suppressants and in some chronic cases with surgery. About 2.5 Million in Europe and about 1 Million in United States of America are currently affected by inflammatory bowel disease (Kaplan, 2015). Pakistan is still in the uncharted territory due to lack of incident reporting and difficult trace of medical data. Genetic changes are among the factors involved in the onset of inflammatory bowel disease and it includes changes at protein expression levels. MicroRNAs (miRNAs) are in vivo non-coding RNAs with nucleotide length of 22 to 25. They can regulate the protein expression by controlling gene transcription as post transcriptional controllers of mRNA (Auyeung, Ulitsky, McGeary, & Bartel, 2013; Bartel, 2009). Due to their small size they can degrade mRNA when bind in perfect base pair manner or halt translation due to imperfect base pairing against multiple genes. One miRNA can bind to multiple mRNA transcripts and vice versa (Bartel, 2009). Consequently, miRNAs can edict multiple cell process such as cell death, cell cycle regulation, cell proliferation and their differentiation (Pillai, Bhattacharyya, & Filipowicz, 2007). Apoptosis have been linked with the development of inflammatory bowel diseases in humans depends on several factors such as the presence or absence of reactive oxygen species (ROS) and nuclear factor kappa B (NFκB) activation, among others (Nunes, Bernardazzi, & de Souza,

2014). Similarly, epithelial barrier dysfunctionality, loss of extracellular matrix integrity and inflammation are also highly related with inflammatory bowel disease (Cader & Kaser, 2013; Matricon, Barnich, & Ardid, 2010b; Silva, Rodrigues, Ayrizono, & Leal, 2016). Hence, the intricacy of inflammatory bowel is evidently complex and involves many entities at different levels simultaneously. There is also a need to find new effective alternate therapeutics to treat inflammatory bowel disease. miRNA is considered as emerging and more targeted agents of therapeutics. miRNA levels have been reported to be deregulated during inflammatory bowel disease (Bischoff et al., 2014; Cao et al., 2017; Kalla et al., 2014; Liang, Ridzon, Wong, & Chen, 2007; Manuscript, n.d.). Furthermore, formal modeling approaches are extensively applicable because of their computational capacity of testing exhaustively. These approaches are being used successfully over a few years for the purpose of modeling and validation of intricate systems in biology (Kitano, 2002b). Petri nets can provide discrete, hybrid or continuous approximations (René David & Alla, 2010). Since our base model has 11 entities which will grow in the disease and antagomir therapy models. Petri nets serve as the most efficient and effective method of modeling and analysis. Petri nets provides a generic description principle which can be applicable to any level of abstraction (Sackmann et al., 2006).

Form the constructed models for homeostasis, disease, and therapy and their simulations it is well established that miRNAs play crucial role in the regulation of key signaling pathways. Antagomirs can serve as effective alternative therapeutic agents for personalized medicine. In our models also established that we need to cater

all the key entities involved in the onset of inflammatory bowel disease simultaneously to set the whole system into recovery mode effectively. Our generated model also serves as platform for further integrations of key entities and more pathways involved in the inflammatory bowel disease progression. Our models can analyze the impact overall system caused by change in one entity or more than one separately or simultaneously. Further wet lab-based experiments are required to explore the inhibitory effects of antagomirs on miRNAs in inflammatory bowel disease in gut microenvironment.

CHAPTER 6: CONCLUSION AND FUTURE PROSPECTS

Inflammatory bowel disease is a growing global health problem. Apoptosis, cellular adhesion, extracellular matrix integrity and inflammation implications in inflammatory bowel disease has been elucidated in the previously conducted studies with details discreetly. Similarly, miRNAs deregulated levels are also associated with the disease. In this study, we integrate all the pathophysiological pathways, their regulatory proteins and their disruptive miRNAs to develop a model. With the designed model, we can study the disease dynamics before the onset, during progression, and after therapeutic interventions. Our results showed antagomirs as promising molecular therapeutic agents. Altogether, our findings could aid in the development of alternative treatment strategies that are focused on genetics for effective and definitive remedy. Our constructed model could aid in further understanding of the dynamics of the disease by adding and exploring other entities (genes/proteins/miRNA) involved in inflammatory bowel disease. Furthermore, the model can be extended with necessary perturbations and interventions to observe the effect of the entities on expression patterns.

CHAPTER 7: REFERENCES

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