ASSESMENT OF SERUM IP-10 AND CYSTATIN C LEVELS IN GENERALIZED ANXIETY DISORDER PATIENTS AND THEIR ASSOCIATION WITH ANXIETY SEVERITY: A CASE-CONTROL STUDY

By

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy

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Declaration

It is hereby declared that

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2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Approval

The project titled "Assessment of Serum IP-10 and Cystatin C Levels in Generalized Anxiety Disorder Patients and Their Association with Anxiety Severity: A Case-Control Study" submitted by Zahra Labiba Ahmed (21346006) of Summer, 2021 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on September.

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Ethics Statement

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the Guidelines of the Bangladesh Medical Research Council (BMRC). Ethical approval was obtained from the Research Ethics Committee (REC) of the University of Asia Pacific (UAP), Bangladesh, prior to commencement. Written informed consent was obtained from all participants after clear explanation of study objectives, procedures, risks, and benefits. Participants' confidentiality and privacy were strictly maintained, and they retained the right to withdraw at any stage without consequences. Blood samples were collected by qualified healthcare professionals using aseptic techniques, ensuring participant safety and comfort. All research procedures adhered to national and international ethical standards to protect the rights and well-being of all human subjects involved.

Abstract

Generalized Anxiety Disorder (GAD) is a common mental health disorder marked by persistent, excessive anxiety and physiological manifestations. This study examines the correlation between blood cytokine levels, specifically Cystatin C and IP-10, and the occurrence and severity of GAD. A total of 202 participants, comprising patients with GAD and healthy controls, were evaluated for body mass index (BMI), cytokine levels, and demographic variables. Statistical analysis indicated markedly increased levels of Cystatin C and IP-10 in patients with GAD relative to controls. Cytokine concentrations exhibited a strong correlation with disease severity, underscoring their possible involvement in the neuroinflammatory pathways associated with anxiety disorders. No notable variations in age or gender were detected between the groups, while BMI exhibited a substantial variance. The findings indicate that Cystatin C and IP-10 may function as biomarkers for the diagnosis and progression of GAD, providing novel insights into its pathophysiology and potential treatment targets. The limitations comprise the cross-sectional design and possible confounding variables, necessitating additional long-term studies.

Keywords: Generalized Anxiety Disorder, Biomarkers, Cystatin C, IP-10, Neuroinflammation, Cytokines.

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Dedication

This thesis is dedicated with all my love and gratitude to my family. Their unwavering faith, endless support, and invaluable lessons—especially that knowledge without humility is meaningless—have been my guiding light and strength through every challenge.

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List of Acronyms

GAD	Generalized Anxiety Disorder
BMI	Body Mass Index
IP-10	Interferon-Gamma-Inducible Protein 10
CST3	Cystatin C
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
HPA	Hypothalamic-Pituitary-Adrenal (axis)
NK	Natural Killer (cells)
CXCR3	C-X-C Chemokine Receptor type 3
ELISA	Enzyme-Linked Immunosorbent Assay
SSRIs	Selective Serotonin Reuptake Inhibitors
SNRIs	Serotonin-Norepinephrine Reuptake Inhibitors
CBT	Cognitive Behavioral Therapy

Chapter 1

Introduction

1.1 Generalized Anxiety Disorder (GAD)

A mental illness known as Generalized Anxiety Disorder (GAD) is defined by overwhelming, uncontrollable, and persistent worry about many different things, which can happen every day. A person with Generalized Anxiety Disorder (GAD) has uncontrollable anxiety that lasts for at least six months, according to the DSM-5 mental disorder manual. Problems with everyday life, like work, health, and relationships, are common sources of this unfounded anxiety. Physical symptoms of anxiety include lethargy, tense muscles, trouble sleeping, and irritability. A person's ability to function in social, occupational, and other crucial areas of life might be significantly impaired by Generalized Anxiety Disorder (GAD). While researchers have not yet pinpointed a single cause for Generalized Anxiety Disorder (GAD), they do believe that environmental factors, genetics, and psychological factors all have a role. (Symptoms and Causes of Generalized Anxiety Disorder, 2017)

1.2 Etiology of Generalized Anxiety Disorder (GAD)

Among various factors, genetic, neurological, environmental, and psychological factors have proven to interact the resultant of which is generalized anxiety disorder (GAD). These factors may greatly affect the development and sustainment of the disease which is why these factors have been the subject of much scientific investigation.

1.2.1 Genetic Factors

A substantial amount of evidence-based research suggests that GAD has a genetic component, and is often influenced by heredity. Research involving identical twins has shown that GAD has a heritability estimate between 30% and 40% (Hettema, Neale, & Kendler, 2001). Research has shown that the risk of developing Generalized Anxiety Disorder (GAD) increases for people who have a first-degree relative with a diagnosis of the disorder (McLeod et al., 2007). According to Lonsdorf et al. (2014), this hereditary predisposition may be associated with genes that regulate stress, particularly those that influence the 5-HTT serotonin transporter and the dopamine receptor.

1.2.2 Neurobiological Factors

Neuroimaging has revealed the role of the amygdala in generalized anxiety disorder. GAD sufferers frequently have an overactive amygdala, which processes worry. Etkin and Wager (2007) found that GAD patients' amygdalas are overactive when concerned. GAD is also linked to neurotransmitter systems, particularly GABA. People with aberrant brain GABAergic activity exhibit higher anxiety (Nuss, 2015). GAD and other anxiety disorders may be caused by serotonin and norepinephrine imbalances (Charney & Deutch, 1996).

1.2.3 Environmental Factors

Childhood trauma raises the likelihood of GAD. Childhood traumas such abuse, neglect, or family dysfunction are more likely to cause adult anxiety disorders (Green et al., 2010). In longitudinal research by Kendler et al. (2003), stressful life events, particularly those linked to health and relationships, were significant risk factors for GAD. Chronic stress or adversity may alter the brain's stress response, increasing the risk of anxiety disorders (McEwen, 2006).

1.2.4 Psychological Factors

Cognition and behavior affect GAD development and maintenance. Cognitive theories of anxiety suggest GAD sufferers have maladaptive thought patterns (Borkovec, Ray, & Stöber, 2004). These include overthinking, rumination, and catastrophizing. Cognitive mistakes increase anxiety by making people exaggerate unpleasant events. Studies show that attentional bias towards threat-related stimuli worsens GAD anxiety (Mathews & MacLeod, 2005). Avoiding stressful circumstances might make it harder for persons with Generalized Anxiety disease (GAD) to build good coping mechanisms, which can perpetuate the disease.

1.2.5 Personality and Temperament

GAD risk increases with neuroticism. Neurotic people are more likely to develop Generalized Anxiety Disorder (GAD), which causes anxiety and depression, according to Costa and McCrae (1992). Anxiety disorders are more likely among temperamental people, including those with behavioral limitation. Shy or reserved youngsters are more likely to have GAD (Kagan et al., 1987).

1.2.6 Cultural and Social Factors

Social and cultural factors affect GAD symptoms and progression. Cultures that value success may raise pressure and anxiety. Chiao et al. (2010) found that cultural values like individuality and collectivism can affect anxiety disorders in different cultures. GAD risk factors include socioeconomic status, lack of social support, recurrent interpersonal problems, and social isolation (Wilkins et al., 2011).

1.3 Subtypes of Generalized Anxiety Disorder (GAD)

Despite being considered a singular entity, generalized anxiety disorder (GAD) can manifest in several ways or be associated with specific subtypes based on symptoms, duration, and co-occurring disorders, according to research.

1.3.1 Unadulterated Generalized Anxiety Disorder

This is the most obvious sign that someone has GAD. People with this condition worry too much about a lot of things, often for no reason at all. People with this type often worry all the time about their health, job, and relationships with other people. A lot of people have trouble relaxing or handling stress because they can't control their anxiety. Most of the time, symptoms last for at least six months and can make it hard to do everyday things and work (American Psychiatric Association, 2013).

1.3.2 Generalized Anxiety Disorder with Comorbid Depression

A significant proportion of individuals with Generalized Anxiety Disorder exhibit symptoms of depression. This can make it harder to figure out what's wrong and how to treat it. It's often called comorbid generalized anxiety disorder and depression. Depression and anxiety are closely linked; indeed, the intensity of one disorder may exacerbate the other. Kessler et al. (2005) discovered that individuals with this variant of GAD may also endure grief, despair, fatigue, and heightened anxiety, which are symptoms prevalent in both conditions. Cognitive behavioral therapy (CBT) and antidepressant medication may be essential elements of a comprehensive approach to treating depression, which could exacerbate disability (Cuijpers et al., 2014).

1.3.3 Generalized Anxiety Disorder with Panic Disorder

Comorbid GAD and panic disorder affects people with both conditions. People with this form of GAD also have persistent anxiety and intense episodes of fear with physical symptoms such chest pain, difficulty breathing, and dizziness. People become more concerned about having another panic attack, making it harder to function (Craske et al., 2010). Due to their co- occurrence, GAD and panic attack treatment approaches must be individualized to each patient.

1.3.4 Generalized Anxiety Disorder with Social Anxiety

GAD with social anxiety causes chronic anxiety, especially in social situations. These GAD sufferers worry about others' opinions and assessments. It may make people awkward or avoid social situations. Heilberg et al. (2014) report that GAD sufferers may have greater social anxiety, which can isolate them and impair their functioning.

.1.3.5 Childhood Generalized Anxiety Disorder

Depending on the person, GAD in children and teens can take several forms. Generalized anxiety disorder (GAD) in children is characterized by excessive worry about school performance, parental approval, and health and safety. Often mistaken as a physical ailment, Generalized Anxiety Disorder (GAD) in children can cause cephalalgia or gut pain (Silverman & Albano, 1996). Diagnosing and treating GAD in children usually involves cognitive- behavioral therapies suited for their age.

1.3.6 Early-Onset Generalized Anxiety Disorder

Early-onset GAD occurs in adolescence or childhood. Lifelong, persistent anxiety may characterize this type. Studies show that early-onset GAD is related with more severe and persistent anxiety symptoms. It may also cause depression and addiction (Biederman et al., 2001). This group needs immediate attention to improve prognosis and prevent long-term damage.

1.3.7 Generalized Anxiety Disorder with Health Anxiety (Hypochondriasis)

People who fall into this category tend to be too worried about their health and exhibit symptoms typical of hypochondria, such as constantly monitoring their vitals for indications of disease or undergoing needless medical testing. Even when no symptoms are present, these people may live in constant terror of a major medical illness. According to Pinto-Meza et al. (2010), suffering from persistent worry over one's health might greatly impact one's ability to go about one's daily life.

1.4 Prevalence of Generalized Anxiety Disorder (GAD)

Generalized anxiety disorder (GAD) affects approximately 3.7% of the population permanently and 1.8% in the past year, according to the World Mental Health (WMH) surveys conducted by the World Health Organization (WHO) in 26 countries using DSM-5 criteria (Ruscio et al., 2017). U.S. epidemiological interviews show that 2.7% of adults had GAD in the past year and 5.7% throughout their lifetime. Women have greater rates than men (NIMH, n.d.). East Asia has lower WMH national estimations than many prosperous Western countries. The lifetime prevalence in Japan is 2.6% and the 12-month prevalence is 1.2%. Beijing/Shanghai has a lifetime prevalence of 1.0% and a 12-month prevalence of 0.6% (Ruscio et al., 2017). Not symptom ratings, these numbers are from DSM-5 diagnostic interviews for GAD. 2017 Ruscio et al. South Asia lacks extensive, nationally representative GAD diagnostic data. The National Mental Health Survey (NMHS) of India estimates a 12-month prevalence of 3.4% for any anxiety disorders among adults (Sagar et al., 2017), showing a considerable regional anxiety burden, not just GAD. In Bangladesh, 4.7% of individuals have anxiety disorders (general category, not GAD) according to the National Mental Health Survey of 2019. According to the Bangladesh Demographic and Health Survey (BDHS) 2022, 19.5% of ever-married women aged 15–49 had moderate-to-severe GAD-7 anxiety symptoms (Amin et al., 2025). An alternative national BDHS analysis (BMC Public Health, 2025) estimated 21% using a similar criterion (GAD-7 ≥6 or recent anxiolytic usage). GAD-7 scores represent current anxiety symptoms, not a clinical diagnosis of GAD.

Table 1: Prevalence of Generalized Anxiety Disorder

Region	Lifetime Prevalence (%)	12-Month Prevalence (%)
Global (WMH Survey)	3.70%	1.80%
United States (NIMH)	5.70%	2.70%
China (WMH Survey)	1.00%	0.60%
Japan (WMH Survey)	2.30%	1.20%
South Korea (WMH Survey)	2.70%	2.70%
India (NMHS)	3.50%	3.40%
Sri Lanka (Furukawa et al., 2013)	1.80%	1.80%
Bangladesh (National Mental Health Survey 2019)	4.70%	N/A
Bangladesh (BDHS 2022, women)	21% (moderate-severe anxiety)	18-21% (GAD-7 screening)

1.5 Diagnosis of Generalized Anxiety Disorder (GAD)

A clinical evaluation for GAD must adhere to the criteria outlined in the DSM-5 in order to be considered a diagnoseable mental disease. People who have Generalized worry Disorder (GAD) often feel tired, anxious, have tight muscles, and have trouble sleeping. The main sign of GAD is persistent, uncontrollable worry about many things that happen in life. To be labelled with Generalized Anxiety Disorder (GAD), these symptoms must have been present for at least six months and made it very hard to go about daily life.

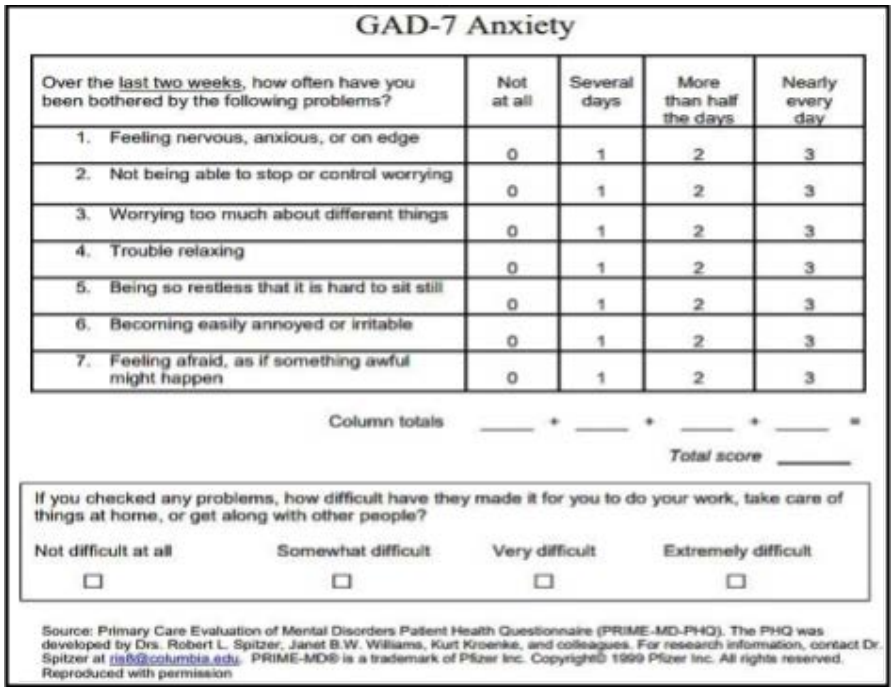
1.5.1 Severity of Generalized Anxiety Disorder

Common ways to measure how serious generalized anxiety disorder (GAD) is include how strong, long-lasting, and harmful the symptoms are to a person's personal, professional, and social life. People with generalized anxiety disorder (GAD) can have symptoms that are moderate or severe. This depends on things including how often the episodes happen, how bad the anxiety is, and how it affects daily life. The DSM-5 classifies the illness into mild, moderate, and severe categories according to the intensity of the symptoms and their impact on daily functioning.

Mildly anxious individuals will not notice many of the symptoms, and will not be obstructed thereof. In a moderate case, there are a number of symptoms and an impairment that is noticeable but tolerable. In a severe case, there are many symptoms, problems with daily life, and discomfort.

1.5.2 Diagnostic Tools for Generalized Anxiety Disorder

For the purpose of determining the severity of anxiety symptoms in patients suspected of having Generalized Anxiety Disorder-7, the GAD-7 is a popular self-assessment instrument. The symptoms of anxiousness are covered in seven questions on the exam. On a scale from 0 (rarely) to 3, each question is evaluated on a daily basis. Anxiety symptoms are quantified by adding up the total score, which can range from 0 to 21 (Spitzer et al., 2006). When it comes to screening and severity evaluation, the GAD-7 has extensive validation. Interpretation of Scores are- 0-4 (Negligible anxiety), 5-9 (Mild anxiety), 10-14 (Moderate anxiety), 15-21 (Severe anxiety).



GAD-7 Anxiety

Over the last two weeks, how often have you been bothered by the following problems?	Not at all	Several days	More than half the days	Nearly every day
1. Feeling nervous, anxious, or on edge	0	1	2	3
2. Not being able to stop or control worrying	0	1	2	3
3. Worrying too much about different things	0	1	2	3
4. Trouble relaxing	0	1	2	3
5. Being so restless that it is hard to sit still	0	1	2	3
6. Becoming easily annoyed or irritable	0	1	2	3
7. Feeling afraid, as if something awful might happen	0	1	2	3

Column totals _____ + _____ + _____ + _____ =

Total score _____

If you checked any problems, how difficult have they made it for you to do your work, take care of things at home, or get along with other people?

Not difficult at all ☐ Somewhat difficult ☐ Very difficult ☐ Extremely difficult ☐

Source: Primary Care Evaluation of Mental Disorders Patient Health Questionnaire (PRIME-MD-PHQ). The PHQ was developed by Drs. Robert L. Spitzer, Janet B.W. Williams, Kurt Kroenke, and colleagues. For research information, contact Dr. Spitzer at rls6@columbia.edu. PRIME-MD® is a trademark of Pfizer Inc. Copyright© 1999 Pfizer Inc. All rights reserved. Reproduced with permission

Figure 1.1: GAD-7 Questionnaire

Anxiety Rating Scale developed by Hamilton (HAM-A) Medical professionals utilize the HAM-A to look for 14 distinct mental and bodily symptoms of anxiety. For the purpose of gauging the severity of GAD, it is commonly used in therapeutic settings (Hamilton, 1959).

1.5.2 Limitations of Diagnosis

Generalized Anxiety Disorder (GAD) diagnosis is complicated by many factors. GAD sufferers may delay treatment since they don't understand their symptoms. Physical illness and stress can also promote irritability and restlessness (Wittchen et al., 2010). When combined with depression, social anxiety disorder, panic disorder, or others, GAD is hard to distinguish. This comorbidity delays diagnosis and therapy (Kessler et al., 2005). Anxious people may not reveal their symptoms in stigmatized mental health societies. Due to underreporting, anxiety disorders may take longer to diagnose and treat in non-Western countries (Chavira et al., 2008). Generalized Anxiety Disorder (GAD) symptoms including tense muscles and altered sleep patterns might mimic thyroid and chronic fatigue syndrome symptoms. This issue can delay mental health care or lead to misdiagnosis, according to Wittchen et al. (2010).

1.6 Signs and Symptoms of Generalized Anxiety Disorder (GAD)

1.6.1. Cognitive Symptoms

A person with generalized anxiety disorder (GAD) worries about their health, career, and relationships. The American Psychiatric Association (2013) reports widespread severe anxiety. Generalized Anxiety Disorder (GAD) sufferers know their worries are overwhelming yet struggle to control them (Spitzer et al., 2006). Many people with Generalized Anxiety Disorder predict disasters even when there is little proof (Wittchen et al., 2010). Overthinking—"the pattern of worrying thoughts that refuse to go away"—may prevent relaxation and other activities (Borkovec et al., 2004).

1.6.2. Emotional Symptoms

Restlessness and agitation define generalized anxiety disorder (GAD). This increased attentiveness may make it hard to relax or focus (APA, 2013). Generalized Anxiety Disorder sufferers often experience increased anxiety before stressful events, even when there is little threat. This fear has considerable mental and emotional consequences (Behar et al., 2009). Since their worries consume them, anxious people struggle to focus (Wittchen et al., 2010).

1.6.3. Physiological Symptoms

Chronic anxiety can be mentally demanding. This symptom arises when the body stays attentive for too long (APA, 2013). GAD often causes back, shoulder, and neck tension. Stress is often linked to this (Spitzer et al., 2006). Generalized Anxiety Disorder (GAD) often causes sleeplessness. Lack of mental or physical relaxation at night can make falling asleep difficult (Wittchen et al., 2010). Stress and anxiety can cause headaches, stomachaches, nausea, and diarrhea (APA, 2013).

1.6.4. Behavioral Symptoms

GAD sufferers may avoid anxiety triggers to relieve symptoms. This can cause serious personal and public concerns (Borkovec et al., 2004). GAD sufferers may avoid social situations because of fear of criticism or judgment. According to Chavira et al. (2008), this may cause loneliness and isolation.

1.6.5 Concurrent symptoms

Kessler et al. (2005) say GAD is typically seen with depression, panic disorder, social anxiety disorder, and panic disorder. Multiple conditions hinder identifying and treating Generalized Anxiety Disorder (GAD) due to clinical overlap.

1.7 The pathophysiology of GAD (generalized anxiety disorder)

Genetic, neurological, and environmental variables all play complex roles in the pathogenesis of GAD. Although the precise processes are unclear, recent research has pinpointed important brain areas, neurotransmitter systems, and genetic components that contribute to the development and maintenance of GAD.

1.7.1 Neurobiological Factors

The amygdala processes emotions, especially fear, and contributes to GAD pathogenesis. GAD sufferers have high emotional reactions due to an overactive amygdala. Functional magnetic resonance imaging (fMRI) studies suggest that GAD patients have more amygdala activity than healthy people (Etkin & Wager, 2007). Hyperactivity worsens sickness symptoms include higher emotional reactivity and concern, according to studies (Huang et al., 2016). The amygdala and prefrontal cortex (PFC) regulate our emotions. GAD patients may have faulty amygdala-prefrontal cortex connections. Goldin et al. (2009) suggest that the prefrontal cortex (PFC) fails to regulate the hyperactive amygdala, causing anxiety to remain. Bishop et al. (2004) link low prefrontal cortex (PFC) activity to poor executive performance and anxiety and stress regulation.

1.7.2 Neurotransmitter Systems

GAD is linked to serotonin, a mood-controlling neurotransmitter. People with reduced serotonin activity are more anxious. Serotonin decreases anxiety by regulating stress and emotions. Generalized anxiety disorder (GAD) patients often take serotonin-boosting

medications like SSRIs (Meyer & Quenzer, 2013). GABA, the brain's main inhibitory neurotransmitter, helps manage anxiety. GABAergic transmission is impaired in GAD patients, making it harder to inhibit hyperactive neurons. This increases anxiety and alertness. GABA receptor dysfunction contributes to GAD and other anxiety disorders, according to Nuss (2015). Norepinephrine controls arousal, alertness, and stress response. Norepinephrine activity in Generalized Anxiety Disorder (GAD) patients may increase alertness and arousal (Charney & Deutch, 1996). Excess norepinephrine causes agitation and hypervigilance.

1.7.3 Genetic Variables

The genetics of GAD are increasingly supported by this work. Twin studies link many anxiety disorders, including GAD, to genetics. According to Hettema et al. (2001), heritability is 30%– 40%. GAD may be more common in patients with genetic differences in GABA, dopamine, and serotonin systems. Anxiety disorders are also linked to the corticotropin-releasing factor (CRF) gene and other stress-regulating genes (Mick et al., 2008).

1.7.4 Environmental and Psychological Factors

Chronic stress, especially childhood stress, may disrupt brain development and raise GAD risk. Stressful childhood experiences and trauma may affect brain shape and function, say researchers. These areas are strongly linked to emotional regulation, according to McEwen (2006). Changes like this may increase senior anxiety disorders like GAD. Psychological theories claim that people with generalized anxiety disorder (GAD) may have cognitive sensitivity to anxiety, which causes excessive worry and catastrophic thinking. According to mathews and macleod (2005), GAD sufferers tend to focus on potentially dangerous information, which raises their anxiety and makes it difficult to relax.

1.7.5 The Stress Response System

A component of the pathophysiology of GAD is the hypothalamic-pituitary-adrenal (HPA) axis, which controls the reaction of the body to stress. It has been demonstrated that individuals with GAD exhibit abnormalities in the HPA axis, leading to an exaggerated response to stress. According to Gold et al. (2002), this disruption could lead to elevated levels of cortisol, the stress hormone, which could worsen physical symptoms and anxiety.

1.8 Management of Generalized Anxiety Disorder (GAD)

In most cases, a mix of medication and therapy is used to treat Generalized Anxiety Disorder (GAD). The long-term goals of both methods are the same: to alleviate symptoms, enhance everyday functioning, and teach patients to manage their anxiety. Individual differences in disease severity, co-occurring disorders, and patient preferences all play a role in developing individualized treatment plans.

1.8.1 Pharmacotherapy

Buspirone, antidepressants, and benzodiazepines are used to treat GAD. The main GAD treatment is selective serotonin reuptake inhibitors (SSRIs). Example: paroxetine, escitalopram, sertraline. These medications increase brain serotonin, regulating anxiety and mood. Clinical research show that SSRIs reduce GAD symptoms and have fewer negative effects than alternative treatments (Bandelow et al., 2017). SNRIs venlafaxine and duloxetine treat GAD. These drugs raise serotonin and norepinephrine levels, affecting mood and stress. SNRIs like venlafaxine have helped treat GAD, especially in severe cases (Kennedy et al., 2014).

Benzodiazepines including diazepam, lorazepam, and alprazolam relieve anxiety quickly. Due to their dependence-inducing qualities, they are mainly used in acute situations or as supplements to longer-term treatments (Baldwin et al., 2014). For long-term GAD treatment, doctors use non-benzodiazepine anxiolytic buspirone. Buspirone is a good long-term drug because it doesn't cause sleepiness or dependence like benzodiazepines (Muench & Hamer, 2010).

1.8.2 Psychotherapy

CBT is the best psychotherapy for GAD. It aims to identify and address illogical ideas and acts that worsen anxiety. CBT helps people manage stress and anxiety. Cognitive-behavioural treatment (CBT) has consistently reduced anxiety symptoms and improved functioning (Hofmann et al., 2012). Exposure treatment is essential to CBT. For fear and avoidance reduction, it systematically exposes people to stressful situations in a controlled context. MBCT uses cognitive therapy and mindfulness to help people become more aware of their thoughts and feelings without reacting. Emotional modulation and rumination reduction are effective treatments for Generalized Anxiety Disorder (Hofmann et al., 2010).

1.8.3 Lifestyle Modifications and Self-Help Strategies

Regular exercise reduces anxiety. Exercise releases endorphins and serotonin, which improve mood and reduce stress (Asmundson et al., 2013). GAD sufferers benefit from aerobic exercise including walking, jogging, and swimming. Deep breathing, progressive muscle relaxation, and meditation help reduce anxiety's physical effects (Chesney et al., 2014). GAD sufferers often lack sleep, which can worsen symptoms. Go to bed and wake up at the same time every day and avoid coffee and electronics before bed to sleep better and reduce anxiety (Taylor et al., 2017). People with GAD may benefit from outside help. Support groups let people share their stories, feel less alone, and learn how to solve challenges from others in similar situations.

1.8.4 Obstacles to Treatment

Despite successful therapies, various hurdles may prevent people from seeking care. Due to mental health stigma, many people may not seek GAD treatment. Fear of criticism or miscommunication might postpone therapy and worsen symptoms (Corrigan, 2004). Mental health care may be tougher to receive in rural and low-income communities due to restrictions. Lack of skilled specialists and long therapeutic wait periods can make getting aid difficult (Patel et al., 2018). Therapy and drugs can be expensive, especially for uninsured or low-income patients. This cost burden may deter patients from starting or continuing treatment (González et al., 2010).

1.9 Generalized Anxiety Disorder biomarkers

Genes, proteins, and other substances are used as biomarkers to diagnose and track disease. Biomarkers for Generalized Anxiety Disorder (GAD) could improve diagnosis and treatment. The hunt for GAD biomarkers is early, although brain, genetic, and hormone research have showed promise.

Table 2: Types of Biomarkers in GAD

Biomarker Type	Description
Neuroimaging Biomarkers (Amygdala Hyperactivity)	Increased activation of the amygdala in response to anxiety-provoking stimuli. (Etkin & Wager, 2007; Huang et al., 2016)
Neuroimaging Biomarkers (Prefrontal Cortex Dysfunction)	Reduced activity in the prefrontal cortex, impairing emotional regulation. (Goldin et al., 2009)
Genetic Biomarkers (Serotonin Transporter Gene)	Genetic variations in the serotonin transporter gene (5-HTTLPR) are linked to increased anxiety. (Lonsdorf et al., 2014)
Genetic Biomarkers (GABA Receptors)	Genetic polymorphisms in GABA receptors are associated with GAD susceptibility. (Nuss, 2015)
Neurochemical Biomarkers (Corticotropin-Releasing Factor)	Elevated CRF levels are associated with dysregulation of the stress response in GAD. (Zorilla et al., 2001)
Neurochemical Biomarkers (Cortisol)	Chronic elevation of cortisol in response to stress is observed in individuals with GAD. (Gold et al., 2002)
Neurochemical Biomarkers (Serotonin & Norepinephrine)	Imbalances in serotonin and norepinephrine systems contributing to heightened anxiety. (Charney & Deutch, 1996)
Electrophysiological Biomarkers (EEG/Theta Waves)	Individuals with GAD display abnormal brain wave patterns, particularly an increase in theta waves. (Thayer et al., 2011)
Electrophysiological Biomarkers (Heart Rate Variability)	GAD patients exhibit low heart rate variability (HRV), which indicates impaired autonomic regulation. (Thayer et al., 2011)

1.10 Cytokines and Their Role in Generalized Anxiety Disorder (GAD)

Small proteins called cytokines regulate inflammation, immunological responses, and cell signaling. Recent research suggests that cytokines may have a role in GAD and other psychiatric diseases. Researchers are studying cytokines like IP-10 and cystatin C to see if they affect mood and anxiety.

1.10.1 Cytokine Effect on GAD

The immune system and central nervous system (CNS) are interconnected, with cytokines affecting cerebral function and leading to anxiety disorders like GAD. Many studies demonstrate that GAD patients have increased inflammatory cytokines. This shows immune system issues may cause and maintain anxiety symptoms. Individuals with GAD exhibit elevated levels of pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β. These cytokines influence the hypothalamic-pituitary-adrenal (HPA) axis and serotonin and dopamine transmission, modifying the brain's stress-response mechanism. Chronic inflammation may alter neurotransmitter systems, causing anxiety-like symptoms (Miller & Raison, 2016).

Immune system activity, as shown by higher cytokine levels, may affect the amygdala and prefrontal brain, which regulate emotions. These modifications may worsen Generalized Anxiety Disorder symptoms and increase anxiety (Müller et al., 2015).

1.10.2 IP-10 (Interferon-Gamma-Inducible Protein 10)

IP-10 (CXCL10) is a pro-inflammatory cytokine that regulates immune function and inflammation. This protein is produced in response to interferon-gamma (IFN-γ) and plays a vital part in chemotaxis, where immune cells migrate towards infection or injury areas..

Structure: One member of the CXC chemokine family is the diminutive IP-10 protein. Ten kDa is its mass. A short N-terminal section and a conserved C-terminal domain are essential for receptor engagement; the molecule has a typical chemokine structure (Yoon et al., 2003).

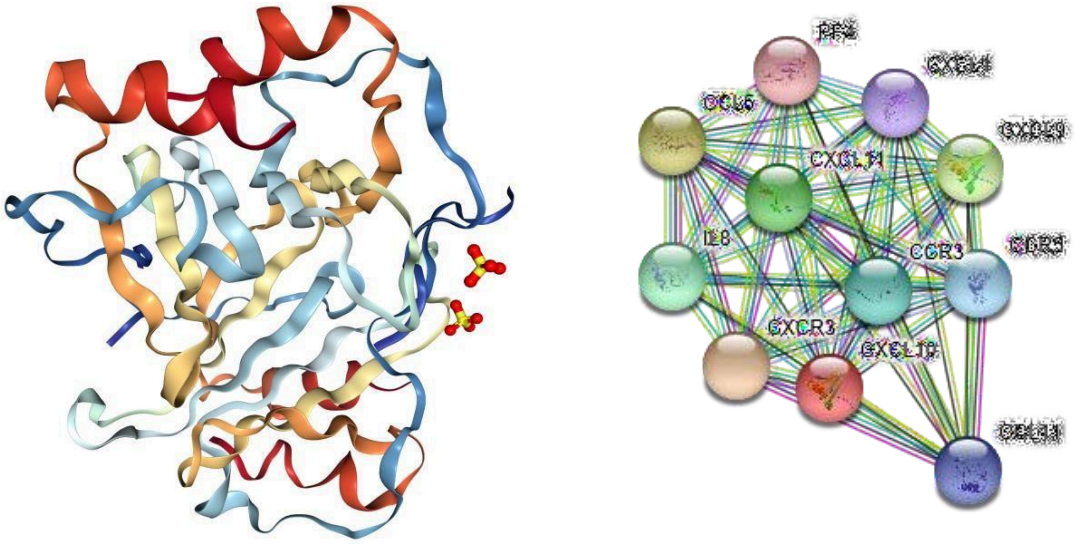


Figure 1: Human IP-10/CXCL10 protein Secondary structure and protein interaction (*IP-10/CXCL10 General Information | Sino Biological*, n.d.)

Biological Activities and Signaling: To exert its actions, IP-10 binds to CXCR3, a receptor present on some neuronal cells, natural killer (NK) cells, and T-cells. The activation of CXCR3 by IP-10 facilitates the migration of immune cells to sites of inflammation. According to Bing et al. (2014), IP-10 has the ability to influence neuronal circuits linked to anxiety and emotional processing via modulating neuroinflammation and neuroimmune interactions in the brain.

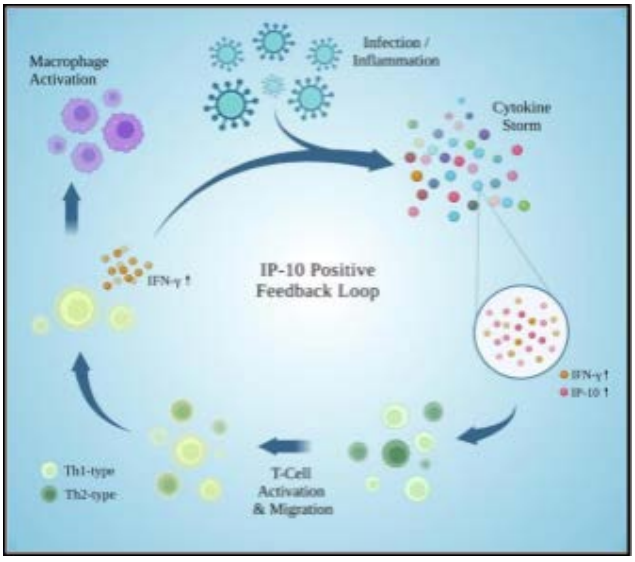


Figure 2: Biological activities of IP-10

Association with Psychiatric Disorders: Certain mental diseases, such as schizophrenia and major depressive disorder (MDD), have been found to have elevated IP-10 levels, which may indicate that immune response dysregulation plays a role in these situations (Tartaglia et al., 2012). While there has been limited research on IP-10 and GAD, the potential link between the two anxiety disorders is worth exploring further due to IP-10's involvement in inflammation and immunological activation.

1.10.3 Cystatin C

The cysteine protease inhibitor cystatin C plays a critical role in modulating the immune response and tissue remodelling by regulating the activity of proteases. It aids in immune system stability and is present in various tissues, including cerebrospinal fluid (CSF) and blood.

Structure: The single-chain inhibitory structure of 13-kDa cystatin C is typical. It inhibits cysteine proteases like cathepsins, which break down proteins and cause inflammation (Beynon et al., 2001).

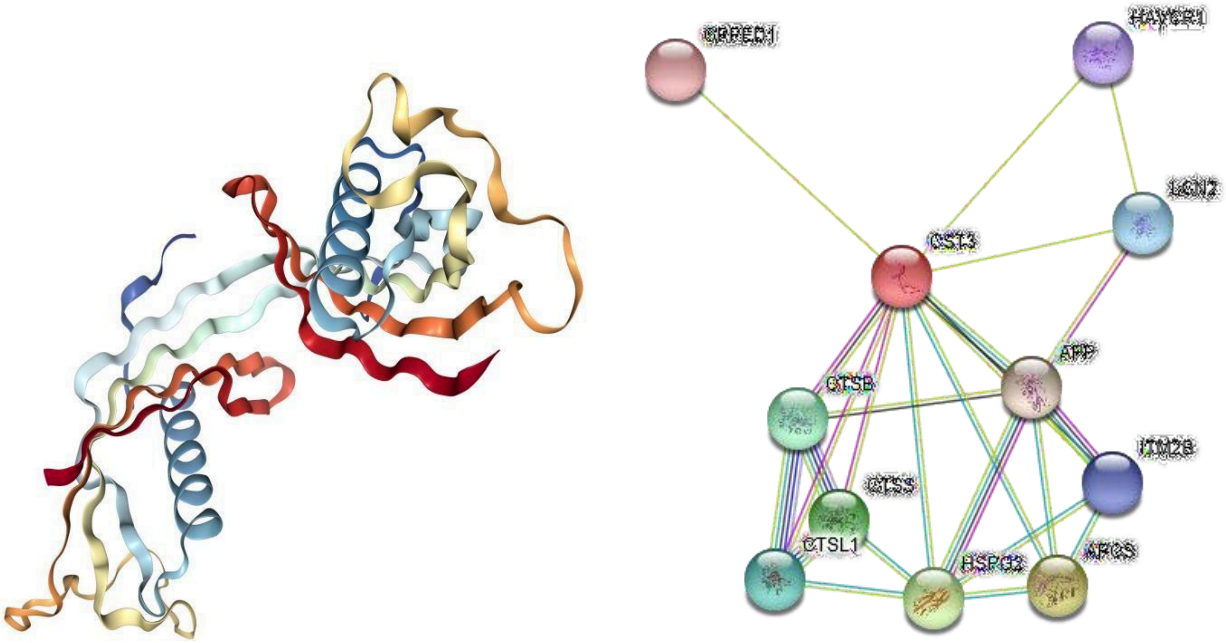


Figure 3: Human Cystatin C Protein Secondary structure (left) & Cystatin C Protein Interaction (Right) (*Cystatin C General Information | Sino Biological*, n.d.)

Biological Activities and Signaling: Cystatin C inhibits proteases that destroy tissue during immunological activation to control inflammation. It controls the blood-brain barrier (BBB), conserves neurons, and prevents neuroinflammation. Cystatin C activates microglia, central nervous system immune cells, and the hypothalamic-pituitary-adrenal axis, which regulate stress and anxiety (Sultana et al., 2015).

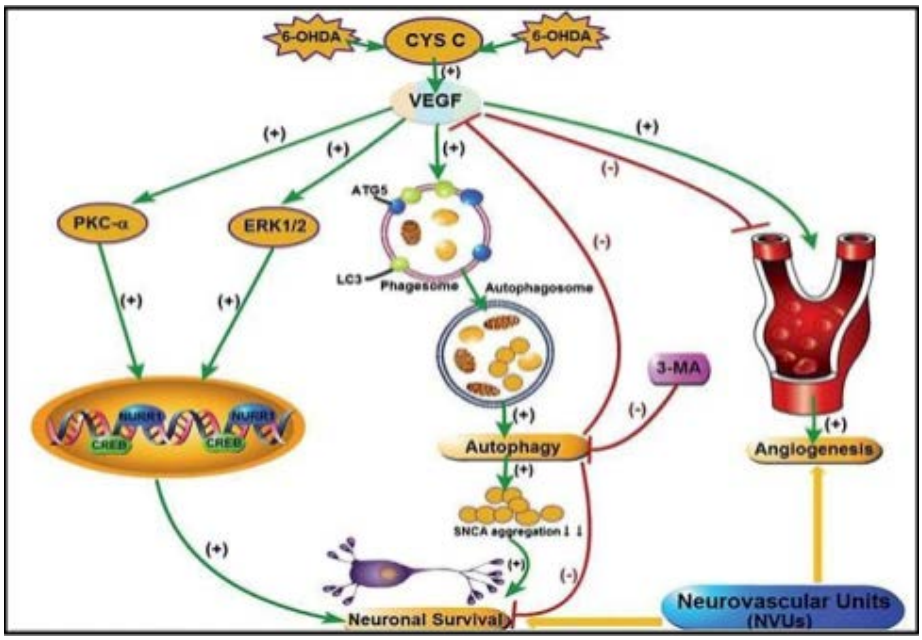


Figure 4: Schematic signaling mechanism underlying the vascular dual functions of CYS C

Association with Psychiatric Disorders: Cystatin C abnormalities are associated to several mental diseases. Elevated cystatin C levels may cause neuroinflammation and cognitive impairments in mood disorders and neurodegenerative diseases (Aro et al., 2013). Cystatin C's association with neuroinflammation and cognitive functioning implies it may be involved in anxiety disorders like GAD, albeit few studies have examined it.

1.10.4. Possible Mechanisms Connecting Cytokines to GAD

Neuroinflammatory mechanisms involve IP-10 and cystatin C. Neuroinflammation may influence the amygdala, prefrontal cortex, and hippocampus, which regulate mood and anxiety, due to persistent immune system activation and high cytokine levels. Inflammation may worsen or initially produce GAD symptoms (Müller et al., 2015). Cytokines like IP-10 and cystatin C may affect the stress-response HPA axis. Chronic cytokine activation may dysregulate the HPA axis, exacerbating GAD anxiety. Inflammation and anxiety may worsen with high cortisol levels (Gold et al., 2002).

1.11 Rationale of the Study

GAD is a serious mental disorder characterized by excessive anxiety about a variety of events. It creates sadness, social anxiety, and disrupts daily life. GAD is common, but we don't fully understand it, making it difficult to develop focused treatments. Recent research suggests that neuroinflammation and immune system dysregulation may contribute to GAD, highlighting the importance of cytokines and immunological biomarkers (Miller & Raison, 2016; Müller et al., 2015).

The study compares serum levels of IP-10 and Cystatin C in patients with Generalized Anxiety Disorder (GAD) to those in a healthy control group, investigates their link to GAD symptoms as evaluated by clinical assessments and standardized anxiety metrics, and assesses biomarker potential. Examining these cytokines and their relationship to Generalized Anxiety Disorder (GAD) will contribute to research linking immune system dysregulation and neuroinflammation to mental health issues, particularly anxiety disorders. IP-10 and Cystatin C are serum biomarkers that may aid in the diagnosis and treatment of GAD. It could also encourage immune-based treatment research to improve GAD psychotherapy and medication.

Chapter 2

2. Materials and Methods

2.1 Study Design and Participants

This prospective case-control study enrolled 101 participants diagnosed with Generalized Anxiety Disorder (GAD) and 101 healthy controls (HCs). All participants were aged between 18 and 60 years and were assessed using the GAD-7 scale. The study was conducted from June 23, 2025, to August 3, 2025, at the National Institute of Mental Health, Bangladesh. Participants were selected from various regions of Bangladesh, and both male and female subjects were included.

The GAD-7 tool was utilized to screen participants, and those who scored above 4 were considered GAD patients, while those scoring below 4 were categorized as healthy controls. The GAD patients and healthy controls were matched by age and sex to minimize confounding variables in the analysis. All participants underwent a structured clinical interview following the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) criteria for GAD diagnosis. The study aimed to analyze the socio-demographic and biographical characteristics of the participants and to measure the severity of GAD.

2.1.1 Criteria for Acceptance

1. This study included both male and female participants.
2. Participants aged between 18 and 60 years were eligible to participate.
3. Participants who were willing to voluntarily provide blood samples and other required information were included.
4. Only individuals who scored higher than 4 on the GAD-7 scale were included in the GAD patient group.

5. GAD patients and healthy controls were matched by age and sex to ensure comparability between the groups.
6. A structured clinical interview was conducted, and socio-demographic information was collected through a pre-designed questionnaire.

2.1.2 Exclusion Criteria

1. Subjects having cognitive impairments or those unwilling to participate in the study were excluded.
2. Serious medical conditions or psychiatric disorders apart from GAD, which could interfere with the study, was a major exclusion criteria.
3. Participants who scored 4 or below on the GAD-7 scale were considered healthy.
4. Those taking medications that could interfere with the concentrations of IP-10 and Cystatin-C or with a history of severe organic conditions, infectious diseases, or substance abuse were excluded.
5. Pregnant women were excluded from the study.
6. Participants who had a history of other psychiatric disorders or were unable to comply with the study requirements, including providing necessary biological samples, were excluded.

2.2 Kit Components and Quantity

2.2.1 Kit Specifications

This research utilized two high-sensitivity ELISA kits from Boster Biological Technology to assess critical biomarkers linked to Generalized Anxiety Disorder (GAD):

1. Human CXCL10/IP-10 ELISA Kit, EZ-Set™ (Catalog Number EZ0735): This kit allows you to create sandwich ELISA experiments and quantify human CXCL10 (IP-10) in a variety of sample types, including cell culture supernatants, serum, plasma

(heparin or EDTA), saliva, and urine. The kit captures CXCL10 using rabbit anti-human polyclonal antibodies and detects it using biotinylated goat anti-human polyclonal antibodies. The test has a detection range of 31.2 pg/ml to 2,000 pg/ml, with a sensitivity of <1 pg/ml.

2. Human Cystatin C ELISA Kit EZ-Set™ (Catalog Number EZ0678): This sandwich ELISA kit assays human Cystatin C (CST3) in cell culture supernatants, serum, plasma (heparin or EDTA), saliva, urine, and human milk. The kit utilizes monoclonal mouse anti-human CST3 as the capture antibody and polyclonal goat anti-human CST3 as the detection antibody. The assay exhibits a detection range from 312 pg/ml to 20,000 pg/ml, with a sensitivity of under 10 pg/ml.

Both kits are offered as DIY antibody pairs, comprising necessary components including capture and detection antibodies, standards, and buffers, thereby enabling the creation of tailored ELISA assays for the precise quantification of CXCL10 and Cystatin C in human biological specimens.

Figure 5: Bosterbio ELISA Kit EZ-Set™

2.2.2 Provided Components and Quantity

Table 3: Overview of Kit (IP-10/Human CXCL10)

Size	5 plates/kit
Range	31.2 pg/ml - 2,000 pg/ml
Specificity	Natural and recombinant Human CXCL10
Immunogen	Expression system for standard: E.coli; Immunogen sequence: V22-P98
Cross-Reactivity	There is no detectable cross-reactivity with other relevant proteins.
Storage Instructions	Store at 4°C for 6 months or at -20°C for 12 months. Avoid multiple freeze-thaw cycles. (Ships with gel Ice can be stored for up to 3 days at room temperature. Freeze upon receiving.)

Table 4: Component Details (IP-10/Human CXCL10)

Catalog number	Description	Quantity	Storage of opened/reconstituted material
EZ0735-CA	Rabbit anti-human CXCL10 polyclonal antibody (Capture Antibody)	500 µl, 0.4 mg/mL (recommended dilution 1:100)	Store undiluted at 4°C for 1 month or at -20°C for 3 months provided this is within the expiration date of the kit.
EZ0735-DA	Biotinylated goat anti-human CXCL10 Polyclonal antibody (Detection Antibody)	500 µl, 10 µg/mL (recommended dilution 1:100)	

AR1103	Avidin-Biotin-Peroxidase Complex (ABC)	500 µl (recommended dilution 1:100)	
EK0735-ST	Lyophilized recombinant human CXCL10 standard	10 ng/tube×3	Discard the standard stock solution after 12 hours at 4°C. May be stored at -20°C for 48 hours provided this is within the expiration date of the kit.

Table 5: Overview of Kit (Cystatin-C/Human CST3)

Size	5 plates/kit
Range	312 pg/ml - 20,000 pg/ml
Specificity	Natural and recombinant Human CST3
Immunogen	Expression system for standard: NS0; Immunogen sequence: M1-A146
Cross-Reactivity	There is no detectable cross-reactivity with other relevant proteins.
Storage Instructions	Store at 4°C for 6 months or at -20°C for 12 months. Avoid multiple freeze-thaw cycles. (Ships with gel Ice can be stored for up to 3 days at room temperature. Freeze upon receiving.)

Table 6: Component Details (Cystatin-C/Human CST3)

Catalog number	Description	Quantity	Storage of opened/reconstituted material
EZ0678-CA	Rabbit anti-human CST3 polyclonal antibody (Capture Antibody)	500 µl, 0.4 mg/mL (recommended dilution 1:100)	Store undiluted at 4°C for 1 month or at -20°C for 3 months provided this is within the expiration date of the kit.
EZ0678-DA	Biotinylated goat anti-human CST3 polyclonal antibody (Detection Antibody)	500 µl, 2.3 µg/mL (recommended dilution 1:100)	
AR1103	Avidin-Biotin-Peroxidase Complex (ABC)	500 µl (recommended dilution 1:100)	
EK0678-ST	Lyophilized recombinant human CST3 standard	20 ng/tube×3	Discard the standard stock solution after 12 hours at 4°C. May be stored at -20°C for 48 hours provided this is within the expiration date of the kit.

2.2.3 Other Materials & Solutions Required but Not Provided

1. Microplate reader in standard size.
2. Automated plate washer.
3. Incubator.
4. Adjustable pipettes and pipette tips. Multichannel pipettes are recommended in the condition of large number of samples in the detection.
5. Clean tubes and Eppendorf tubes.
6. 96 well microplate (Cat# AR1100)
7. Plate Sealers.
8. Capture Antibody Diluent: PBS.
9. Reagent Diluent: 1% BSA in PBS, pH 7.2-7.4, 0.2 um filtered.
10. Color Developing Reagent: Tetramethylbenzidine (Cat# AR1104)
11. Stop Solution: 2 N H2SO4 (Cat# AR1105)
12. Wash Buffer (PBS and PBS-T).

PBS: 8g NaCl, 0.2g KCl, 1.15g Na2HPO4, 0.2g KH2PO4, adjust the total volume to 1 L with distilled water, pH 7.2-7.4, 0.2 um filtered.

PBS-T: 0.1% Tween? 20 in PBS, pH 7.2-7.4.

*Item 6 – 12 are included in the EZ Set Accessory Kit (EZA001)

2.2.4. Blood Collection Accessories

1. Syringe (10 mL)
2. Butterfly syringe (27G X ½" size)
3. Cotton
4. Hexisol
5. Rubber tourniquet with buckle

6. Falcon tube
7. Green Strip
8. Ice Box

2.3 Blood Sample Collection & Storage

Each participant had a venipuncture performed between 9 and 11 am to extract 5 ml of blood from their cephalic vein, which was then transferred into a Falcon tube devoid of anticoagulants. Subsequent to collection, the blood samples were maintained at ambient temperature for one hour without disturbance, permitting clot formation. The clotted blood samples were centrifuged at 1000xg for 15 minutes.

Following centrifugation, serum samples were obtained in Eppendorf tubes. Subsequently, serum samples were preserved at -80°C for further examination.

2.4 Serum Analysis

This section delineates the methodology employed for serum analysis with the BosterBio EZ-Set ELISA Kits to quantify IP-10 (CXCL10) and Cystatin-C (CST3) concentrations in human serum samples.

2.4.1 Required Materials & Reagents

1. IP-10 ELISA Kit (Catalog Number: EZ0735)
2. Cystatin-C ELISA Kit (Catalog No: EZ0678)
3. Microplate reader operating at a wavelength of 450 nm
4. Automated plate washer or manual cleaning implements
5. Adjustable pipettes (including tips)
6. 96-well microplates (AR1100)
7. Plate sealing apparatus

8. Antibody Diluent (PBS)
9. Reagent Diluent (1% Bovine Serum Albumin in Phosphate-Buffered Saline, pH 7.2-7.4)
10. Colorimetric Developing Reagent (Tetramethylbenzidine, TMB)
11. Stop Solution (2N H2SO4)
12. PBS and PBS-T (PBS supplemented with 0.1% Tween 20)

2.4.2 Preparation of Reagents

2.4.2.1 Capture Antibody Solution

1. The Rabbit anti-human CXCL10 polyclonal antibody (capture antibody) was diluted to a 1:100 working concentration with the Capture Antibody Diluent (PBS)
2. The Rabbit anti-human CST3 polyclonal antibody (capture antibody) was diluted to a 1:100 working concentration using Capture Antibody Diluent (PBS).

2.4.2.2 Detection Antibody Solution

1. The biotinylated goat anti-human CXCL10 polyclonal antibody (detection antibody) was diluted to a working concentration of 1:100 in reagent diluent (1% BSA in PBS).
2. The biotinylated goat anti-human CST3 polyclonal antibody (detection antibody) was diluted to a working concentration of 1:100 in reagent diluent (1% BSA in PBS).

2.4.2.3 Avidin-Biotin-Peroxidase Complex (ABC) Solution

The Avidin-Biotin-Peroxidase Complex (ABC) was diluted at a ratio of 1:100 in Reagent Diluent.

2.4.3 Standard Preparation

2.4.3.1 IP-10 (CXCL10) Standard Preparation

1. **Preparation of Stock Solution**: The lyophilized recombinant human CXCL10 standard (EK0735-ST) was reconstituted with 1 mL of Reagent Diluent to produce a stock solution of 10,000 pg/mL. The vial was carefully shaken and permitted to rest for 10 minutes with gentle agitation.
2. **Preparation of Standards (Final Concentration Range: 8–500 pg/mL)**: The standards were generated through repeated dilution of the 10,000 pg/mL stock solution
    a) Tube 1: 200 µL of stock solution (10,000 pg/mL) was combined with 800 µL of reagent diluent to achieve a concentration of 500 pg/mL.
    b) Tube 2: 300 µL of the solution from Tube #1 was combined with 300 µL of Reagent Diluent to achieve a concentration of 250 pg/mL.
    c) Tube 3: 300 µL of the solution from Tube #2 was combined with 300 µL of Reagent Diluent to achieve a concentration of 125 pg/mL.
    d) Tube 4: 300 µL of the solution from Tube #3 was combined with 300 µL of Reagent Diluent to achieve a concentration of 62.5 pg/mL.
    e) Tube 5: 300 µL of the solution from Tube #4 was combined with 300 µL of Reagent Diluent to achieve a concentration of 31.25 pg/mL.
    f) Tube 6: 300 µL of the solution from Tube #5 was combined with 300 µL of Reagent Diluent to achieve a concentration of 15.63 pg/mL.
    g) Tube 7: 300 µL of the solution from Tube #6 was combined with 300 µL of Reagent Diluent to achieve a concentration of 8 pg/mL.
    h) Tube 8: This served as the blank (0 pg/mL).

2.4.3.2 Cystatin C (CST 3) Standard Preparation

1. **Preparation of Stock Solution:** The lyophilized recombinant human Cystatin-C standard (EK0678-ST) was reconstituted with 1 mL of Reagent Diluent to provide a stock solution of 20,000 pg/mL. The vial was delicately rotated and permitted to rest for 10 minutes with mild agitation.
2. **Preparation of Standards (Final Range: 78–5000 pg/mL):** The standards were generated through repeated dilution of the 20,000 pg/mL stock solution:
    a) Tube 1: 200 µL of stock solution (20,000 pg/mL) was combined with 800 µL of reagent diluent to achieve a concentration of 5,000 pg/mL.
    b) Tube 2: 250 µL of the solution from Tube #1 was combined with 250 µL of Reagent Diluent to achieve a concentration of 2,500 pg/mL.
    c) Tube 3: 250 µL of the solution from Tube #2 was combined with 250 µL of Reagent Diluent to achieve a concentration of 1,250 pg/mL.
    d) Tube 4: 250 µL of the solution from Tube #3 was combined with 250 µL of Reagent Diluent to achieve a concentration of 625 pg/mL.
    e) Tube 5: 250 µL of the solution from Tube #4 was combined with 250 µL of Reagent Diluent to achieve a concentration of 312.5 pg/mL.
    f) Tube 6: 250 µL of the solution from Tube #5 was combined with 250 µL of Reagent Diluent to achieve a concentration of 156.25 pg/mL.
    g) Tube 7: 250 µL of the solution from Tube #6 was combined with 250 µL of Reagent Diluent to achieve a concentration of 78.125 pg/mL.
    h) Tube 8: This served as the blank (0 pg/mL).

2.4.4 Assay Protocol

1. Coating of Plates: 100 µL of the diluted capture antibody solution was introduced into each well of the 96-well microplate. The plate was sealed and treated overnight at 4°C to facilitate antibody binding.
2. After overnight incubation, the plate cover was removed, and the antibody solution was discarded. 200 µL of Reagent Diluent was added to each well, and the plate was incubated at room temperature for 2 hours to block nonspecific binding sites.
3. The wells were rinsed thrice with PBS to eliminate surplus chemical residue. Following the final wash, the plate was delicately blotted to eliminate any residual PBS.

Figure 6: PBS Buffer

4. According to our plate design, 100 µL of the standards, samples, or controls was added to the corresponding microplate wells.
5. After being sealed, the plate was left to incubate for two hours at room temperature.
6. The plate was rinsed again after incubation. Each well received 100 µL of the diluted biotinylated detection antibody solution. For 90 minutes, the plate was incubated at room temperature.

7. After washing, 100 µL of Avidin-Biotin-Peroxidase Complex was added to each well. The plate was incubated for 40 minutes at room temperature.

Plate 1 (Cystatin)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Std-1	Std-2	Std-3	Std-4	Std-5	Std-6	Std-7	Blank	Patient	Patient	Patient	Patient
B	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
C	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
D	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
E	Patient	Patient	Patient	Patient	Control	Control	Control	Control	Control	Control	Control	Control
F	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
G	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
H	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control

Figure 7: Plate Design

Plate 2 (Cystatin)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Std-1	Std-2	Std-3	Std-4	Std-5	Std-6	Std-7	Blank	Patient	Patient	Patient	Patient
B	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
C	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
D	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
E	Patient	Patient	Patient	Patient	Control	Control	Control	Control	Control	Control	Control	Control
F	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
G	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
H	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control

Figure 8: Plate Design

Plate 3 (IP-10)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Std-1	Std-2	Std-3	Std-4	Std-5	Std-6	Std-7	Blank	Patient	Patient	Patient	Patient
B	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
C	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
D	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
E	Patient	Patient	Patient	Patient	Control	Control	Control	Control	Control	Control	Control	Control
F	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
G	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
H	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control

Figure 9: Plate Design

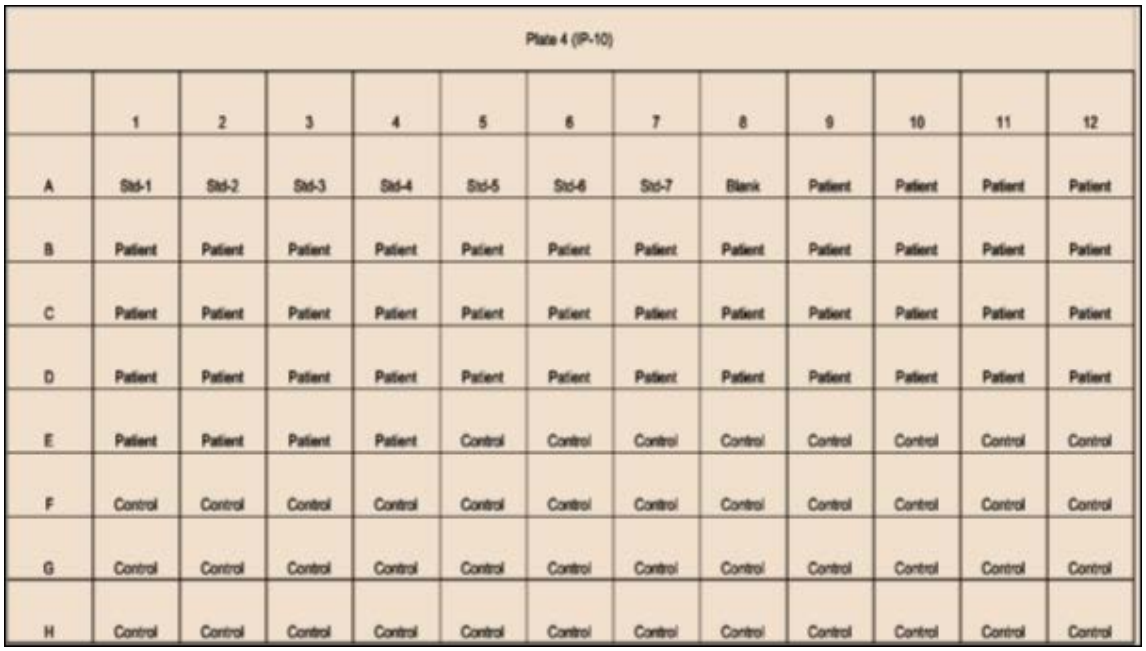


Plate 4 (IP-10)												
	1	2	3	4	5	6	7	8	9	10	11	12
A	Std-1	Std-2	Std-3	Std-4	Std-5	Std-6	Std-7	Blank	Patient	Patient	Patient	Patient
B	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
C	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
D	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient
E	Patient	Patient	Patient	Patient	Control	Control	Control	Control	Control	Control	Control	Control
F	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
G	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
H	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control	Control

Figure 10: Plate Design

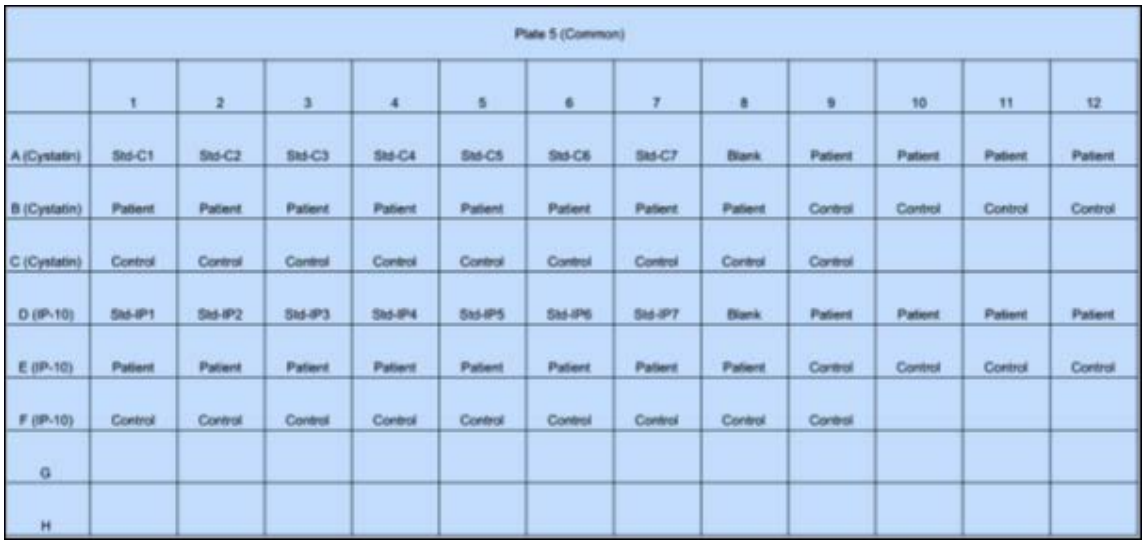


Plate 5 (Common)												
	1	2	3	4	5	6	7	8	9	10	11	12
A (Cystatin)	Std-C1	Std-C2	Std-C3	Std-C4	Std-C5	Std-C6	Std-C7	Blank	Patient	Patient	Patient	Patient
B (Cystatin)	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Control	Control	Control	Control
C (Cystatin)	Control	Control	Control	Control	Control	Control	Control	Control	Control			
D (IP-10)	Std-IP1	Std-IP2	Std-IP3	Std-IP4	Std-IP5	Std-IP6	Std-IP7	Blank	Patient	Patient	Patient	Patient
E (IP-10)	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Patient	Control	Control	Control	Control
F (IP-10)	Control	Control	Control	Control	Control	Control	Control	Control	Control			
G												
H												

Figure 11: Plate Design

8. Each well received 90 µL of color-developing reagent (TMB) following the last wash. To enable color development, the plate was incubated at room temperature for 30 minutes in the dark. Some wells turned blue; the intensity of the color varied based on the concentration of the cytokines.

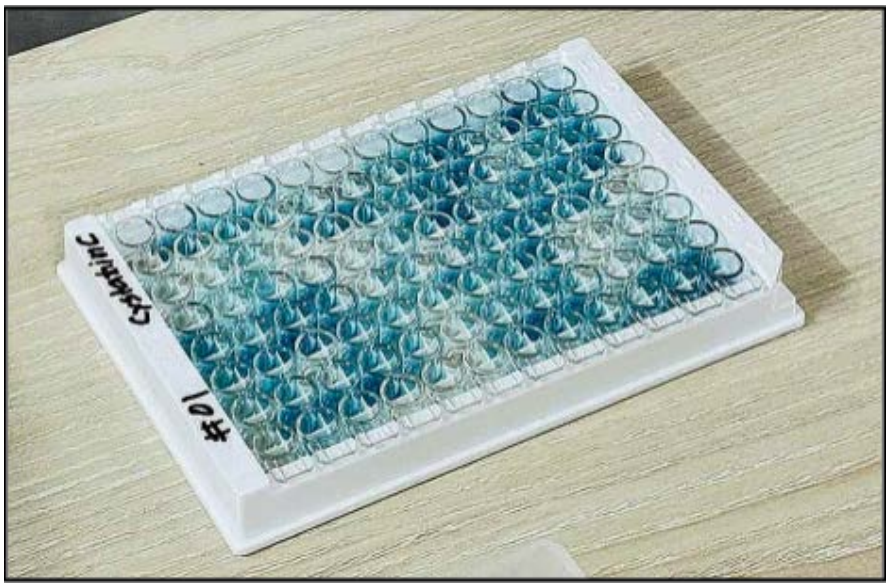


Figure 12: Color change of plate after adding coloring agent and incubation

9. Each well received an addition of 100 µL of Stop Solution (2N H_2SO_4). The hue transitioned instantaneously to yellow.

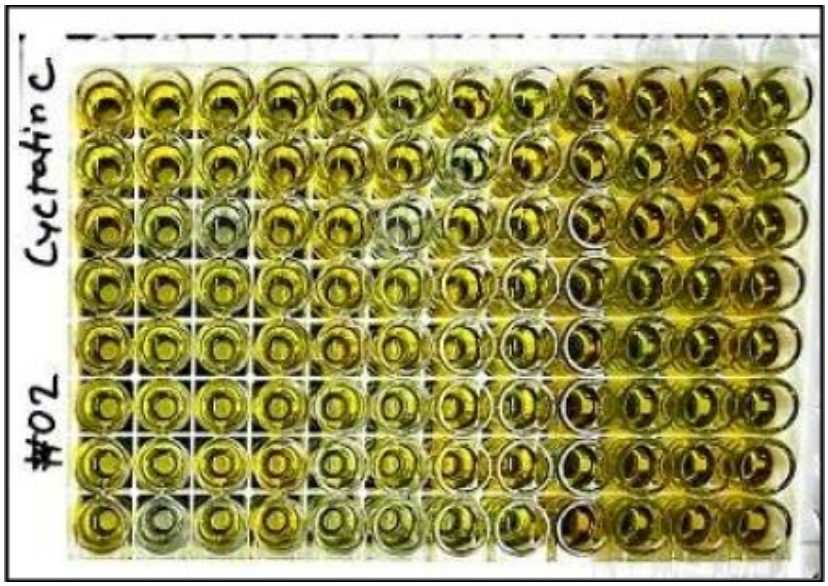


Figure 13: Color change of Plate after adding stop solution

10. At last, absorbance of the plate was measured by an ELISA microplate reader and recorded.

Figure 14: ELISA Microplate reader

2.4.5 Statistical Analysis

IP-10 and Cystatin-C were measured in serum. The data distribution was mean-centered with low skewness. GAD patients and healthy controls were compared using an independent sample t-test. The mean standard error of the mean (mean SEM) was used to measure blood IP-10 and Cystatin-C. The cytokines' association to GAD severity was analyzed using Spearman's correlation and scatter plot graphs. Box plot graphs showed serum cytokine differences between groups. P-values < 0.05 were statistically significant for each analysis. SPSS 23.0

(IBM Corp., Armonk, NY) was used for the investigation. IP-10 and Cystatin-C had minimal detectable ranges of <1 pg/mL and <10 pg/mL, respectively, with no cross-reactivity with other serum cytokines. The operators processed all samples without knowing the participants' clinical status.

Chapter 3

3. Result

3.1 Results

A range of parameters are taken into consideration in order to categorize the study participants according to their current state. In this study, gender, level of education, occupation, financial condition, marital status, residence, smoking history, and family history are all evaluated. The socio-demographic characteristics of study participants are provided in table.

Table 7: Socio-demographic profile of the study population.

Parameters	GAD Patients (n=101)			Healthy Controls (n=101)		
	n	%	Mean ± SEM	n	%	Mean ± SEM
BMI			33.666666666 6667 ± 15.677			33.6666666666667 ± 15.7726908857613
Below 18.5	5	4.95%		11	10.89%	
18.5-25	59	58.42%		64	63.37%	
above 25	37	36.63%		26	25.74%	
Education Level			25.25 ± 8.1789873048 7665			25.25 ± 13.4497521662421
Illiterate	4	3.96%		0	0	
Primary Level	21	20.79%		4	3.96%	

Secondary Level	36	35.64%		49	48.51%	
Graduate	40	39.60%		48	47.52%	
Occupation			16.8333333333333 ± 5.11479335957095			16.8333333333333 ± 10.1732219303643
Business	8	7.92%		0	0.00%	
Service	36	35.64%		34	33.66%	
Housewife	11	10.89%		7	6.93%	
Unemployed	18	17.82%		60	59.41%	
Student	2	1.98%		0	0.00%	
Others	26	25.74%		0	0.00%	
Economic Impression			33.6666666666667 ± 11.4649223479466			33.6666666666667 ± 9.83756970891581
High	12	11.88%		14	13.86%	
Medium	51	50.50%		43	42.57%	
Low	38	37.62%		44	43.56%	
Smoking Habit			50.5 ± 18.5			50.5 ± 30.5
Yes	32	31.68%		20	19.80%	
No	69	68.32%		81	80.20%	

Residence			50.5 ± 29.5			50.5 ± 26.5
Urban	80	79.21%		85	84.16%	
Rural	21	20.79%		16	15.84%	
Previous History of GAD			50.5 ± 26.5			50.5 ± 50.5
Yes	24	23.76%		0	0.00%	
No	77	76.24%		101	100.00%	
Family History of GAD			50.5 ± 24.5			50.5 ± 18.5
Yes	26	25.74%		32	31.68%	
No	75	74.26%		69	68.32%	

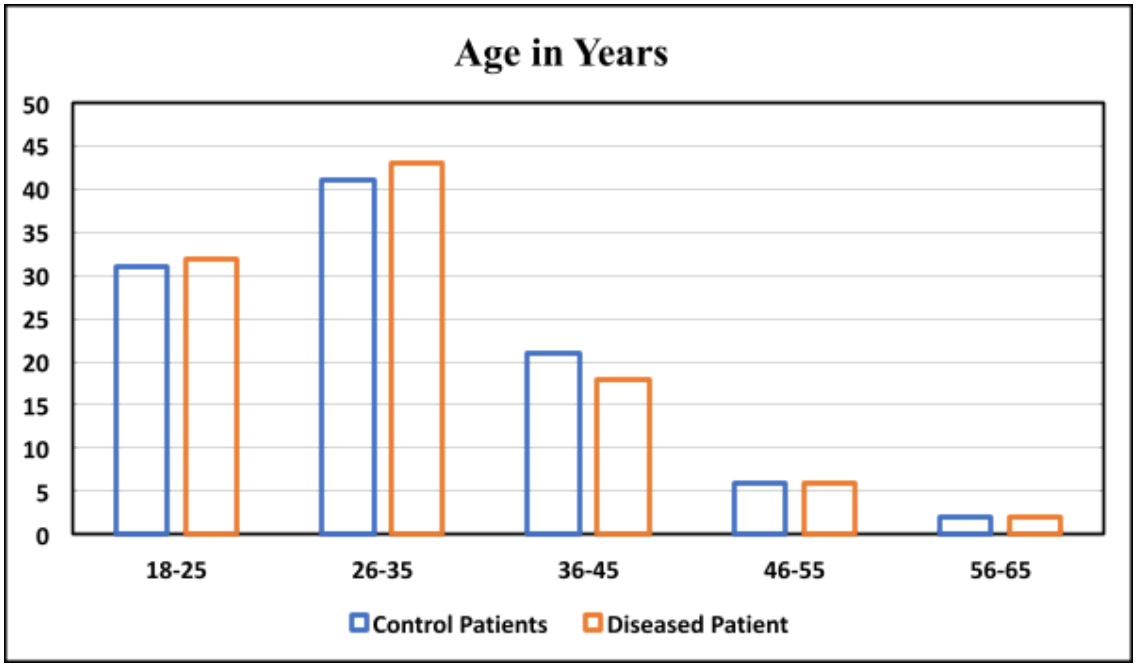


Figure 15: Distribution of study population according to age in years.

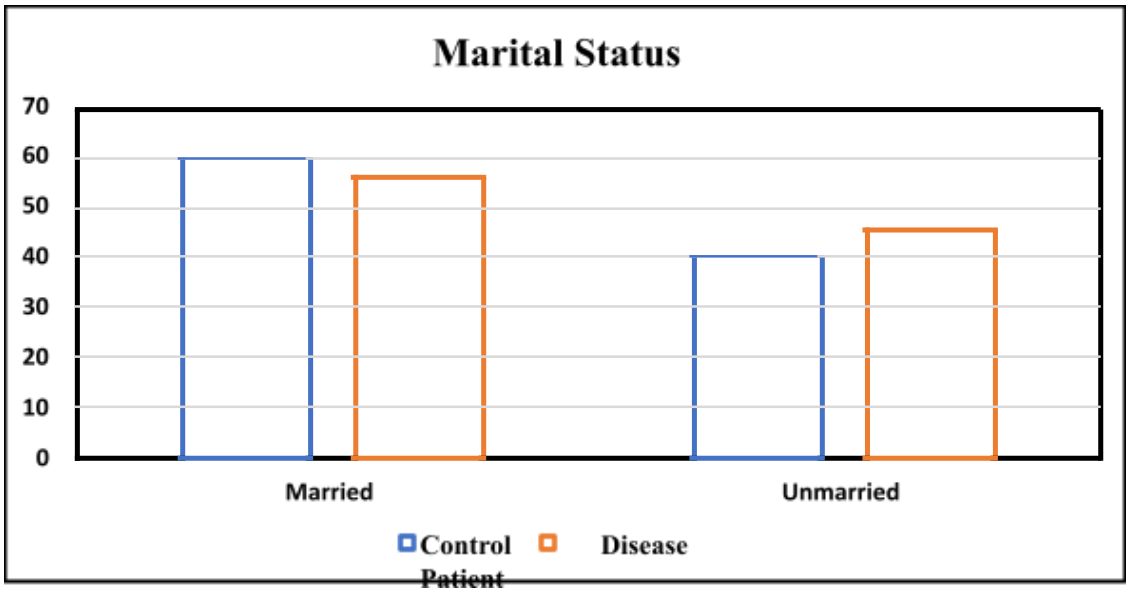


Figure 16: Distribution of study population according to marital status.

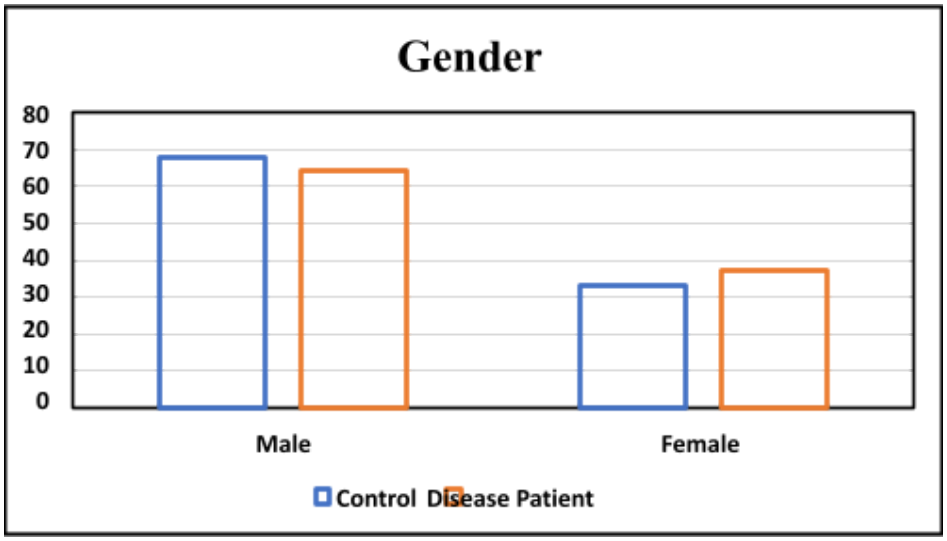


Figure 17: Distribution of study population according to gender

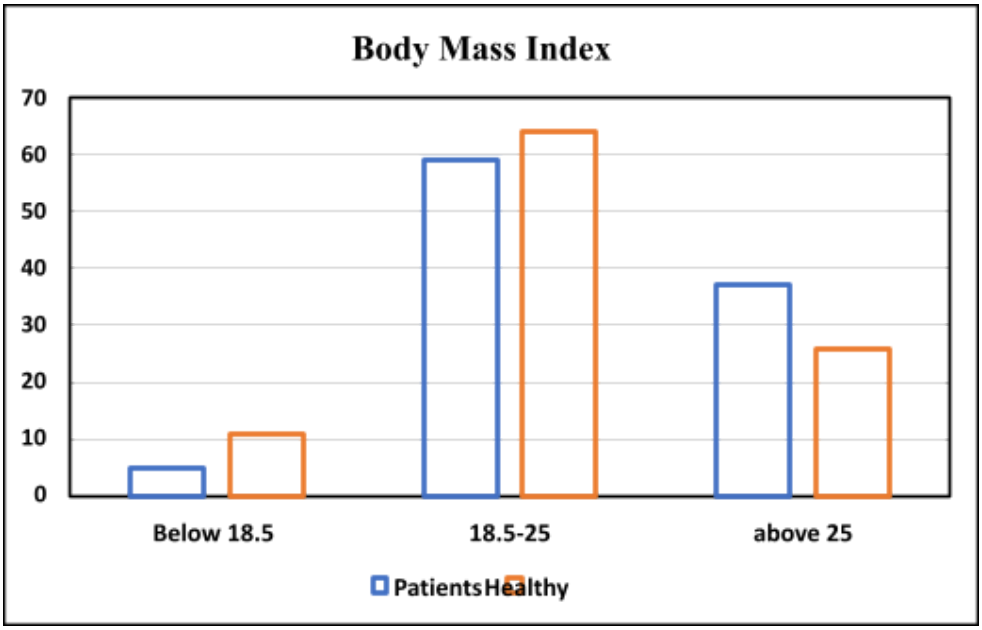


Figure 18: Distribution of study population according to BMI

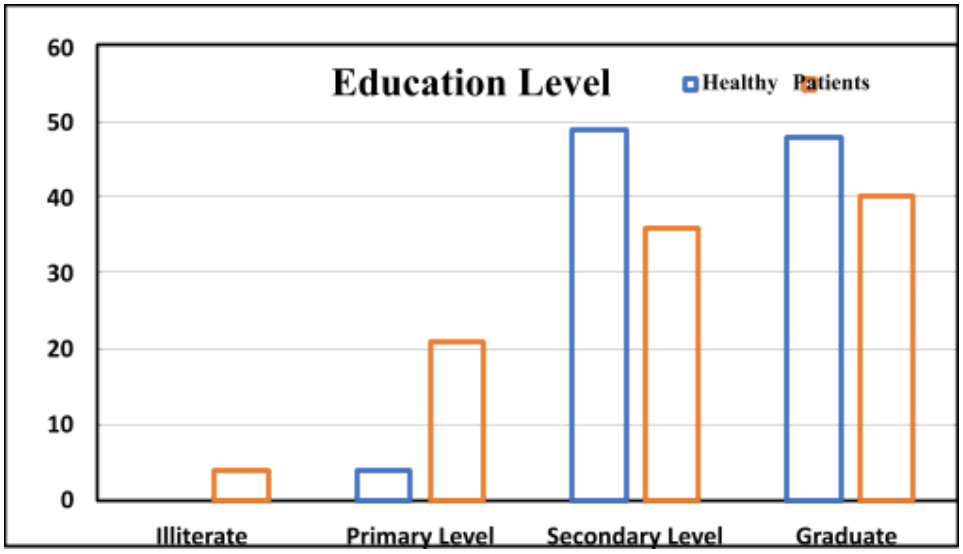


Figure 19: Distribution of study population according to education level

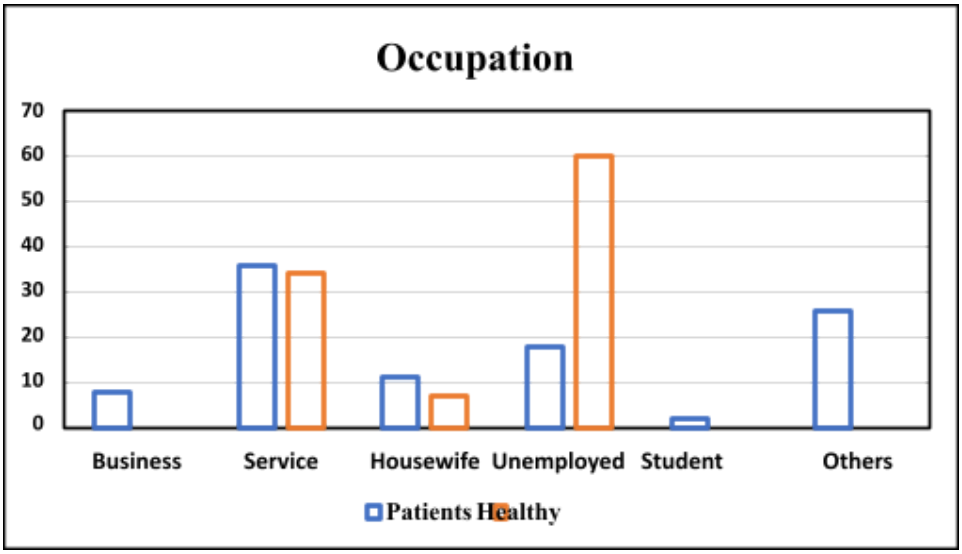


Figure 20: Distribution of study population according to occupation

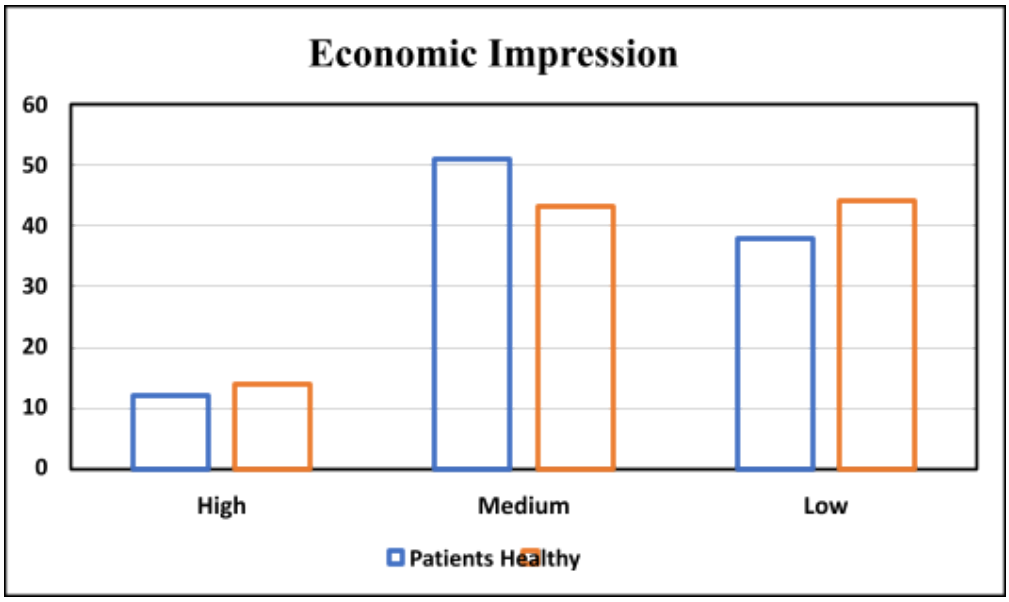


Figure 21: Distribution of study population according to economic impression

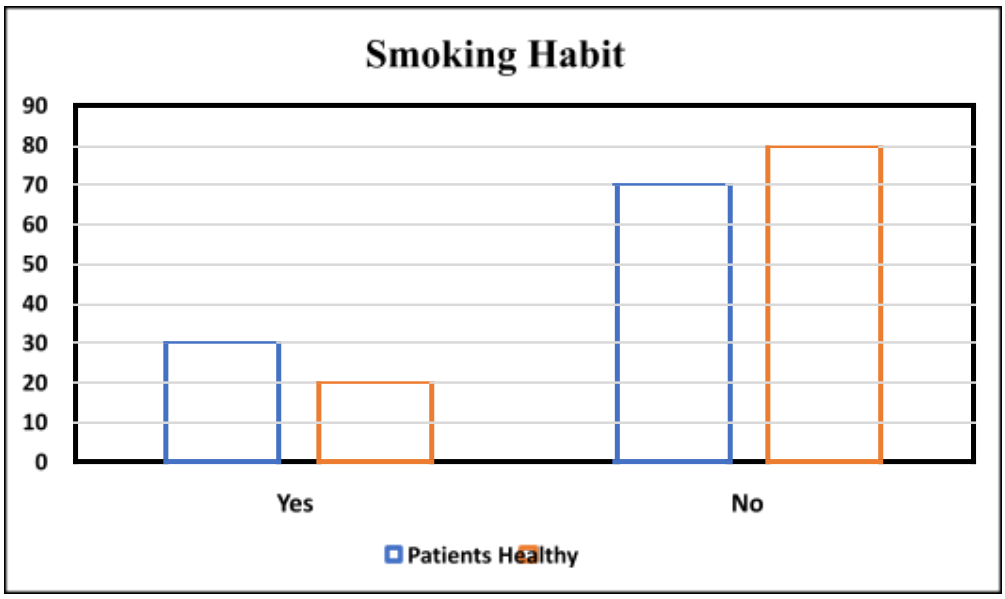


Figure 22: Distribution of study population according to smoking habit

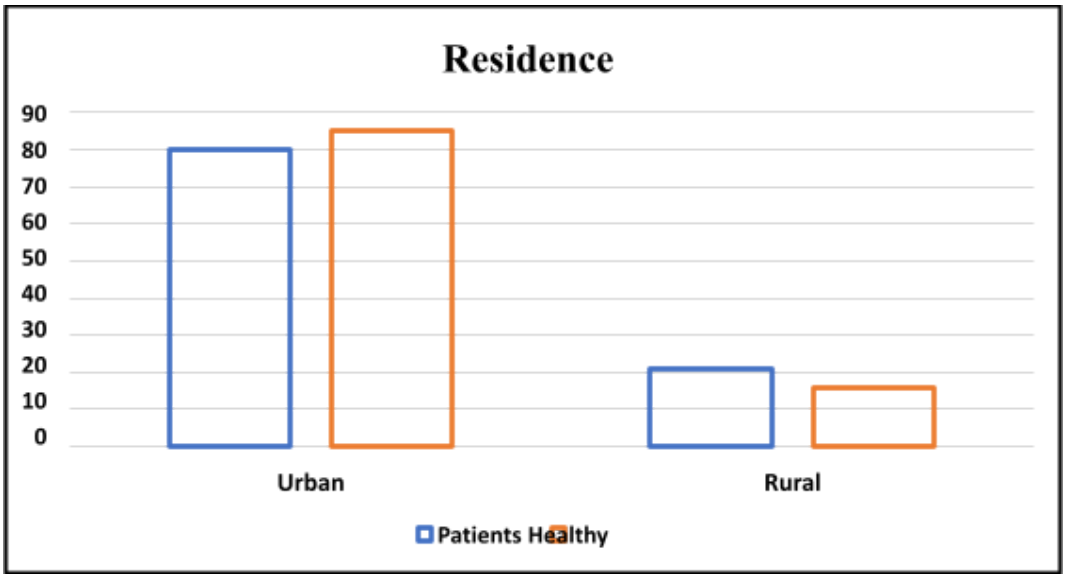


Figure 23: Distribution of study population according to residency

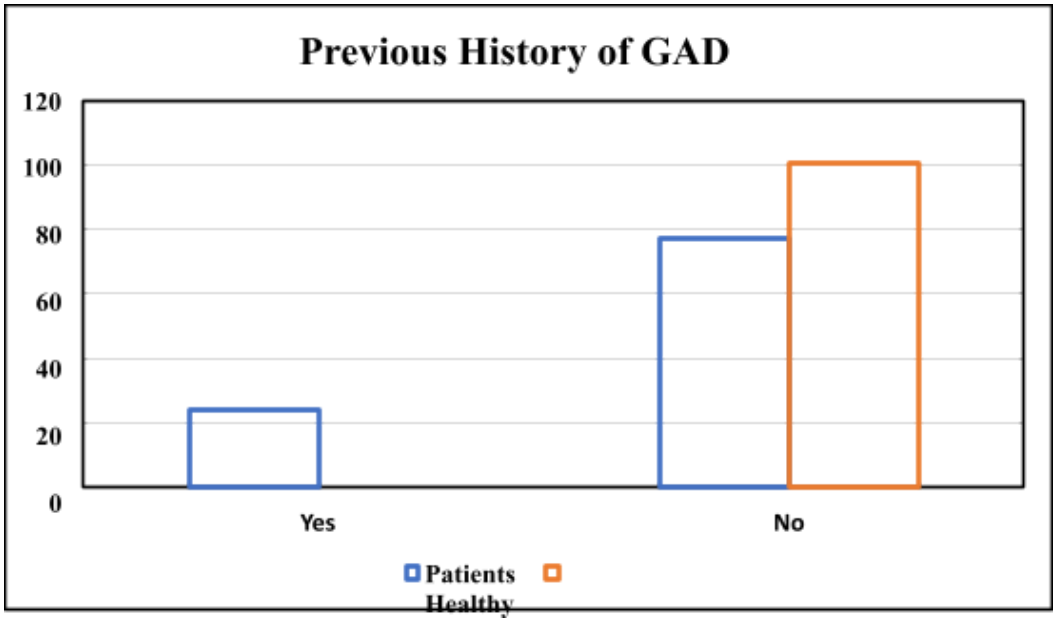


Figure 24: Distribution of study population according to previous history of GAD

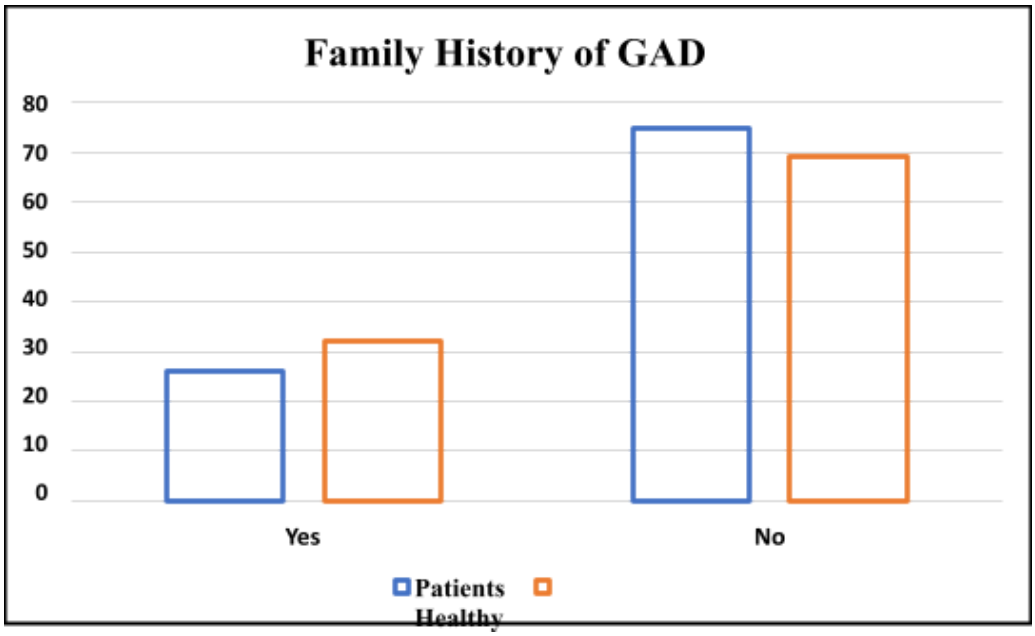


Figure 25: Distribution of study population according to family history of GAD

3.1.2 Absorbance of IP-10 and Cystatin-C

Microplate absorbance reader had been used to quantify the absorbance of IP-10 and cystatin-C from the serum sample of patients and controls. It is displayed in table 3.2 & 3.3.

Table 8: IP-10 Standard Concentration

Standard	Std. Conc (pg/mL)	Std. Abs
1	500.0	1.3239
2	250.0	0.5245
3	125.0	0.1362
4	62.5	0.0593
5	31.2	0.0467
6	15.6	0.0501
7	7.8	0.0455
Blank	0.0	0.0462

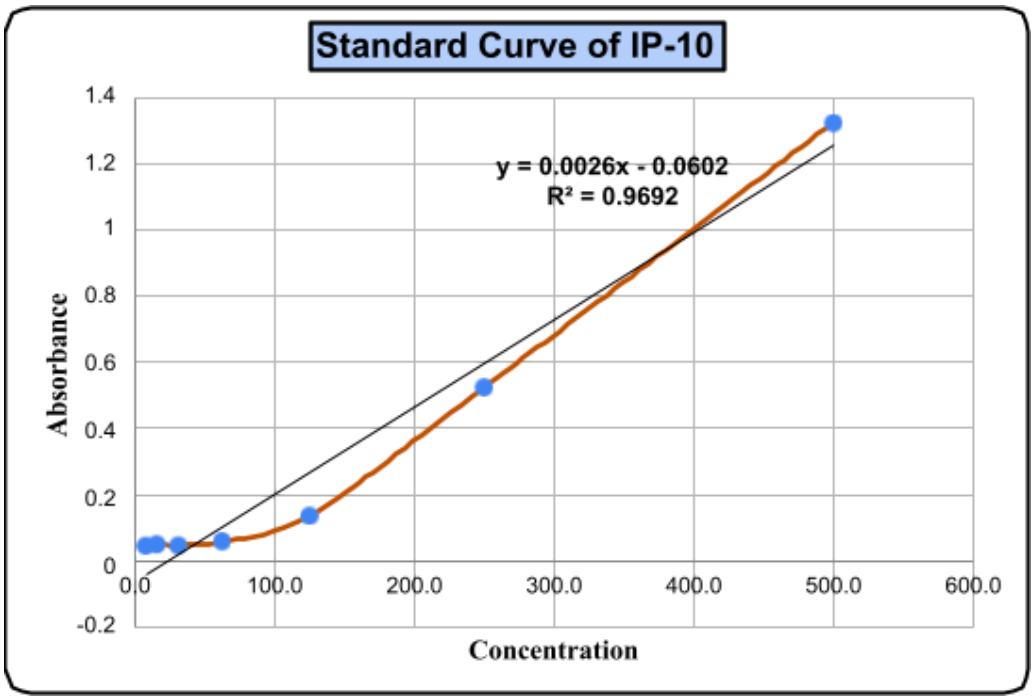


Figure 26: Standard curve of IP-10

Table 9: Cystatin-C Standard Concentration

Standard	Std. Conc (pg/mL)	Std. Abs
1	5000.0	2.8613
2	2500.0	2.1071
3	1250.0	1.4062
4	625.0	0.7708
5	312.5	0.3588
6	156.3	0.2207
7	78.1	0.0681
Blank	0.0	0.0462

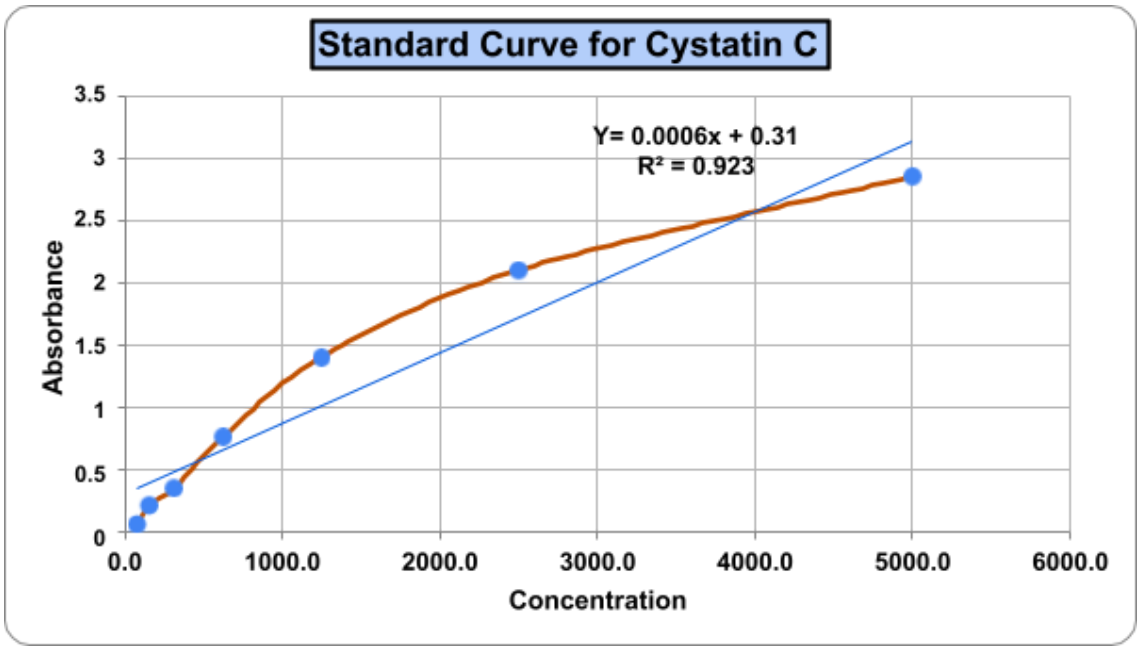


Figure 27: Standard curve for Cystatin C

Table 10: Concentration(mg/L) of IP-10 & Cystatin-C in HCs

Subject No.	Conc Cystatin C mg/L	Conc IP-10 mg/L
C- 02	0.18	93.50
C- 03	4.49	93.46
C- 05	3.09	88.73
C- 06	3.15	73.15
C- 07	3.03	93.58
C- 08	0.89	91.08
C- 100	0.52	92.85
C- 101	2.93	92.04
C- 102	3.60	92.04
C- 103	1.82	93.15
C- 104	4.11	91.92
C- 105	2.94	95.31
C- 106	1.29	90.62
C- 107	3.17	85.88
C- 108	1.21	91.58
C- 109	0.52	73.15
C- 11	2.11	91.92
C- 110	0.41	92.69
C- 112	4.36	91.31
C- 113	2.21	92.69
C- 115	1.57	93.04
C- 116	0.43	91.88
C- 117	2.28	86.96
C- 118	4.26	92.77
C- 119	1.27	93.65
C- 120	0.87	92.62
C- 121	1.68	90.38
C- 122	0.41	92.35
C- 123	0.52	91.04

C- 124	1.34	92.31
C- 125	0.22	88.19
C- 126	0.18	90.88
C- 13	1.03	93.15
C- 14	4.83	91.27
C- 15	3.99	66.19
C- 18	0.39	91.96
C- 19	0.02	95.38
C- 22	3.84	93.81
C- 23	0.57	89.15
C- 26	0.66	91.62
C- 27	0.42	91.19
C- 30	0.18	93.50
C- 31	0.33	90.35
C- 32	2.95	87.88
C- 33	1.07	92.46
C- 35	0.07	91.62
C- 36	3.76	40.62
C- 38	0.37	87.96
C- 40	0.34	91.88
C- 42	0.40	87.00
C- 43	0.05	91.69
C- 45	2.26	92.62
C- 46	0.99	92.08
C- 47	0.14	92.46
C- 48	4.39	87.42
C- 50	1.39	90.65
C- 51	0.10	57.73
C- 52	4.49	91.58
C- 53	0.03	93.00

C- 54	3.04	93.73
C- 55	3.51	87.81
C- 56	0.15	90.73
C- 57	0.17	90.81
C- 58	3.23	92.88
C- 59	0.13	91.50
C- 60	4.49	90.92
C- 61	0.51	90.38
C- 62	1.57	92.12
C- 63	0.52	73.15
C- 64	0.52	73.15
C- 65	1.83	88.69
C- 66	0.55	50.27
C- 67	1.57	91.38
C- 68	2.99	90.88
C- 69	2.02	91.62
C- 70	0.50	92.46
C- 71	3.41	56.62
C- 72	1.96	73.15
C- 73	2.51	88.69
C- 74	1.66	92.69
C- 75	0.44	91.62
C- 76	0.50	90.81
C- 78	4.06	94.31
C- 79	0.52	73.15
C- 80	0.18	87.81
C- 81	0.08	91.38
C- 82	4.83	89.42
C- 83	1.26	90.92
C- 84	2.03	93.50

C- 85	3.37	91.23
C- 86	0.02	93.00
C- 87	3.31	91.08
C- 88	0.77	92.00
C- 89	0.02	91.96
C- 90	1.65	92.46
C- 91	0.14	92.77
C- 92	1.93	90.88
C- 95	3.53	90.77
C- 96	0.38	92.81
C- 98	0.22	94.27
C- 99	0.43	90.92

Table 11: Concentration (mg/L) of IP-10 & Cystatin-C in GAD Patients

Patient No.	Conc Cystatin C mg/L	Conc IP-10 mg/L
G- 01	2.30	99.69
G- 02	0.59	90.85
G- 03	0.44	123.54
G- 04	2.87	89.96
G- 05	3.50	93.19
G- 06	3.17	91.38
G- 07	2.58	91.15
G- 08	3.51	90.81
G- 09	6.06	116.62
G- 10	4.72	97.04
G- 100	1.42	137.46
G- 101	0.96	91.35
G- 11	1.44	92.54

G- 12	2.07	94.27
G- 13	3.50	91.35
G- 14	4.26	133.73
G- 15	0.92	92.04
G- 16	2.64	91.38
G- 17	1.15	94.46
G- 18	6.27	92.58
G- 19	0.44	90.04
G- 20	1.46	92.19
G- 21	3.10	188.54
G- 22	1.62	101.96
G- 23	1.44	93.23
G- 24	1.83	91.92
G- 25	0.39	92.46
G- 26	1.63	91.81
G- 27	0.68	91.65
G- 28	3.39	886.35
G- 29	5.26	93.69
G- 30	2.06	92.65
G- 31	2.27	93.38
G- 32	2.86	93.00
G- 33	4.15	91.15
G- 34	1.90	259.12
G- 35	2.28	90.85
G- 36	0.36	91.50
G- 37	1.65	90.85
G- 38	1.28	89.62
G- 39	3.20	92.12
G- 40	1.87	91.85
G- 41	4.16	225.00
G- 42	0.38	91.92

G- 43	0.35	288.85
G- 44	1.59	93.31
G- 45	0.61	92.54
G- 46	3.92	94.15
G- 47	4.22	91.73
G- 48	3.53	91.31
G- 49	2.37	92.96
G- 50	3.55	93.00
G- 51	2.43	93.15
G- 52	4.20	388.96
G- 53	3.30	91.69
G- 54	3.29	94.38
G- 55	2.40	89.88
G- 56	2.40	91.92
G- 57	1.13	94.35
G- 58	2.95	219.15
G- 59	5.99	113.23
G- 60	1.88	116.54
G- 61	0.36	93.23
G- 62	2.03	90.00
G- 63	4.20	92.58
G- 64	0.43	95.54
G- 65	5.07	92.15
G- 66	4.97	96.85
G- 67	1.01	113.38
G- 68	3.10	104.27
G- 69	3.30	137.88
G- 70	5.49	90.69
G- 71	1.96	91.62
G- 72	1.00	92.00

G- 73	1.95	95.96
G- 74	5.65	151.38
G- 75	0.45	91.23
G- 76	1.90	91.35
G- 77	2.79	94.08
G- 78	0.78	92.23
G- 79	5.06	92.19
G- 80	5.46	89.65
G- 81	0.35	91.73
G- 82	3.79	90.50
G- 83	4.15	100.12
G- 84	2.12	334.62
G- 85	0.62	110.15
G- 86	0.53	154.35
G- 87	0.75	156.54
G- 88	0.42	92.62
G- 89	0.40	308.38
G- 90	1.10	125.81
G- 91	3.51	91.12
G- 92	2.71	90.15
G- 93	0.89	157.12
G- 94	3.99	499.31
G- 95	5.49	92.54
G- 96	1.69	217.46
G- 97	2.30	93.77
G- 98	3.09	92.38
G- 99	5.46	91.81

Table 12: Gender cross-tabulation

Gender	Control	Patient	Total

Female	33	37	70
Male	68	64	132
Total	101	101	202

Table 13: Chi-Square Tests for independent (between gender)

Column 1	Value	df	Asymptotic Significance (2- sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3.50^a	1	0.554		
Continuity Correction	0.197	1	0.657		
Likelihood Ratio	3.5	1	0.554		
Fisher's Exact Test				0.658	0.329
N of Valid Cases	202				

Interpretation: A chi-square test for independence was conducted in SPSS utilizing group statistics to analyze the relationship between gender (female and male) and group membership (Healthy Controls vs. GAD Patients).

The Pearson chi-square test produced a p-value of 0.554, exceeding the conventional significance threshold of 0.05. The findings suggest a lack of statistically significant association between gender and group membership within the study population.

The Fisher's Exact Test and the likelihood ratio yielded non-significant results ($p = 0.658$ and $p = 0.554$, respectively), reinforcing the conclusion that the distribution of males and females is not significantly different between the two groups.

Table 14: Relationship between different groups

	Group	N	Mean	Std. Error Mean	P Value
Age	Patients	101	30.74	.868	0.860
	Controls	101	30.97	.955	
BMI	Patients	101	23.849308831374440	.346448901069854	0.032
	Controls	101	22.815275755818526	.332184604282487	
Conc. Cystatin C. mg/L	Patients	101	2.519608910891089	.160907255442014	>0.001
	Controls	101	1.649617161716172	.146569184723755	
Conc. IP-10	Patients	101	125.862147753236850	10.085795429986728	>0.001
	Controls	101	88.551363290175120	.930273357545384	

Interpretation: Independent sample t-tests were conducted using SPSS, utilizing data derived from the group statistics. The tests aimed to compare the means of Age, BMI, Concentration of Cystatin C, and Concentration of IP-10 between two independent groups: Healthy Controls and GAD Patients. No significant difference in age was observed between the groups ($p = 0.860$), suggesting that the ages were comparable. The difference in BMI was statistically significant ($p = 0.032$), indicating variability between patients and controls. The concentration of Cystatin C, exhibited a highly significant difference ($p < 0.001$), indicating a notable variation between the groups. The concentration of IP-10, exhibited a highly significant difference ($p < 0.001$), indicating distinct group variations. Levene's test for equality of variances was conducted for all variables to assess the assumption of equal variances. The findings demonstrate significant differences in BMI, Cystatin C, and IP-10

concentrations between healthy controls and GAD patients, whereas age does not exhibit notable variation between the groups. This indicates a potential association between the biomarkers and BMI with the disease condition being investigated.

Table 15: Severity of disease & cytokine concentration.

Status	n= 202	Conc Cystatin C mg/L	Conc IP-10
Mild	47	2.631277	107.3319
Moderate	22	2.669545	144.8919
Severe	32	2.251875	141.6303
Non-existent	101	1.679902	88.61257

Interpretation: The table indicates that cytokine concentrations escalate with the severity of the condition. Moderate cases have the greatest levels of Cystatin C (2.67 mg/L) and IP-10 (144.89 pg/mL), whereas severe cases demonstrate marginally lower Cystatin C levels but consistently elevated IP-10 levels. Healthy controls, a non-existent group, exhibit the lowest values. This signifies a positive association between cytokine concentration and disease severity, with healthy patients categorized as non-existent (n=202 total). The relationship between cytokines and severity of disease is presented graphically in figure 28 & 29.

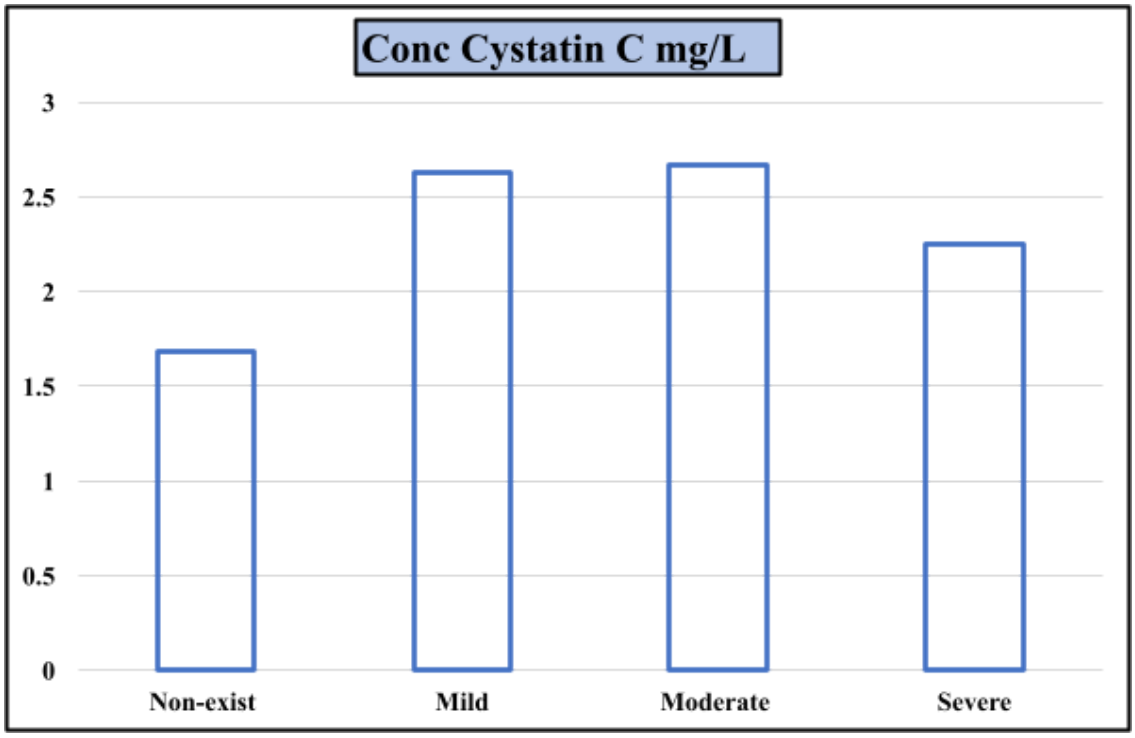


Figure 28: Severity of disease and their co-relation with cytokine concentration.

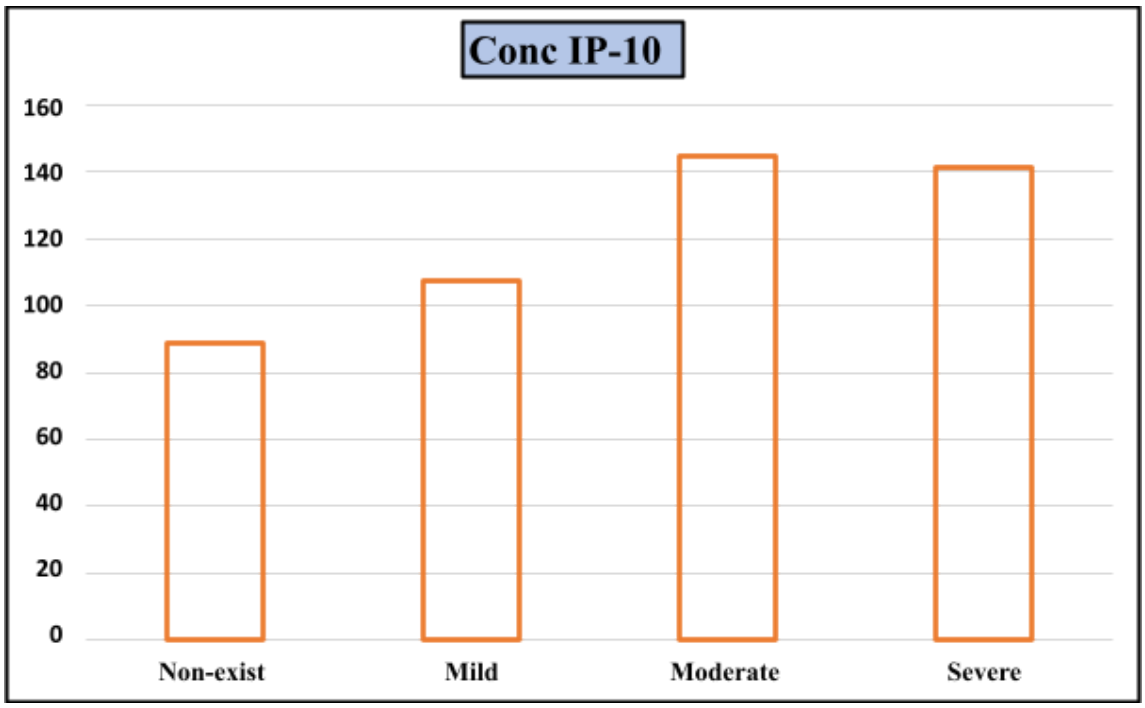


Figure 29: Severity of disease and their co-relation with cytokine concentration.

Chapter 4

Discussion

The purpose of this study was to look into the relationship between generalized anxiety disorder (GAD) and serum levels of the cytokines IP-10 and Cystatin C. Body mass index (BMI), Cystatin C, and IP-10 levels were significantly different between GAD patients and healthy controls, according to independent samples t-tests; however, age was similar across the groups. The increased levels of IP-10 and Cystatin C in GAD patients point to a possible involvement of neuroinflammation and immune system dysregulation in the disease's etiology.

The absence of variation in age distribution reduces confounding influences from this demographic variable, hence reinforcing the identified correlation between cytokine levels and disease state. In line with earlier studies that connected metabolic changes to mental illnesses, the notable rise in BMI seen in GAD patients might point to lifestyle or metabolic changes linked to anxiety disorders (Bandelow et al., 2017).

Neurodegenerative and mood problems have been linked to cystatin C, a recognized regulator of neuroinflammation (Aro et al., 2013). As demonstrated here, increased inflammatory activation and neuroimmune interactions with anxiety pathophysiology may be the cause of its elevated blood levels in GAD patients. Similarly, GAD patients had considerably greater levels of IP-10, a pro-inflammatory chemokine involved in immune cell trafficking and neuroinflammation. This supports the idea that IP-10 is involved in anxiety disorders and is in line with studies that have found a link between high IP-10 levels and mental illnesses (Tartaglia et al., 2012; Müller et al., 2015).

Cystatin C and IP-10 levels generally increased from absent (healthy controls) to moderate severity, based on the analysis of cytokine concentrations in relation to disease severity. At this point, Cystatin C levels went down a little, but IP-10 levels stayed high in severe cases. This is an intriguing observation. As anxiety symptoms worsen, this pattern could imply distinct regulatory mechanisms or compensatory reactions. These findings, which are corroborated by previous research, could be explained by neuroinflammatory pathways involving cytokines and dysregulated hypothalamic-pituitary-adrenal (HPA) axis activity (Gold et al. 2002; Müller et al. 2015). The lack of a significant relationship between group membership and gender implies that the disease's existence and cytokine changes in this population are unaffected by gender. These data imply that serum IP-10 and Cystatin C could be useful biomarkers for diagnosing and assessing GAD severity. In addition to classic neurotransmitter-centric approaches, their significance in the inflammatory environment sheds light on additional therapeutic targets and mechanisms (Miller & Raison, 2016). Despite the importance of the findings, this study has many limitations. The sequential association between cytokine levels and GAD advancement is still uncertain due to the constraints of the cross-sectional design, which prevents decisive conclusions. Furthermore, potential confounding factors such as lifestyle variables and body mass index fluctuations, which were not thoroughly controlled, may have influenced cytokine concentrations. The sample size is appropriate, but it may limit the findings' applicability to bigger populations or specific demographic groupings.

Chapter 5

Conclusion

This study illustrates that GAD patients had higher serum Cystatin C and IP-10 levels than healthy controls, which correlates with disease severity. These findings reinforce the emerging data linking neuroinflammation and immunological dysregulation to GAD. The discovered cytokines may be diagnostic and severity biomarkers, providing novel insights beyond neurotransmitter-based models. This study advances GAD understanding and management, but causality and therapeutic applications need more study.

Future Prospects

Future research should incorporate longitudinal designs to monitor cytokine variations over time and their predictive value for the onset and progression of GAD. The underlying mechanisms of neuroimmune interactions in anxiety disorders could be clarified by expanding biomarker panels and integrating neuroimaging or genetic data. Additionally, the advancement of treatment development may be facilitated by the investigation of the therapeutic potential of targeting cytokine pathways.

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