Effects of Psychological Stress on Physiological Homeostasis and Disease Susceptibility

By

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Recommendations of the Viva Voce Board

As members of the Viva Voce Board, we certify that we have read the dissertation prepared by **Sushri Priyadarshini** entitled "**Effects of Psychological Stress on Physiology and Disease Susceptibility**" and recommend that it may be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to HBNI.
I hereby certify that I have read this dissertation prepared under my direction and recommend that it may be accepted as fulfilling the dissertation requirement.

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STATEMENT BY AUTHOR

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(Sushri Priyadarshini)

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree/ diploma at this or any other Institution / University.

(Sushri Priyadarshini)

List of Publications

Journal Publications

1. **S. Priyadarshini** and P. Aich (2012) Effects of psychological stress on innate immunity and metabolism in humans: a systematic analysis. PLoSONE7: e43232

2. P. Sood, **S. Priyadarshini** and P. Aich (2012) Psychological stressors as interventions: Good out of the evil. Front Biosci (Schol Ed), 4: 43-60

3. P. Sood, **S. Priyadarshini** and P. Aich (2013) Estimation of Psychological Stress in Humans: A Combination of Theory and Practice. PLoS ONE8, e63044.

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My PhD is not a simple story of hypothesis, research and evidence shaping into thesis; it is a journey that started with passion, lots of curiosity and spirit for exploring, with no clue of where I would meet ends. Thankfully, now I know how far I have come. This journey would not have been its worth, without the company of many individuals who kept it interesting enough to hold on to.

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Sushri Priyadarshini

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1. Name of the Student: Sushri Priyadarshini

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4. Title of the Thesis: Effects of psychological stress on physiological homeostasis and disease susceptibility

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PREAMBLE

All life forms constantly interact with the environment and show exceptional adaptive capability to sustain wide range of environmental changes. In order to survive, these changes are constantly sensed and the body responds accordingly to cope with the posing demands [1-3]. These demands or challenges are called stressors and are capable of triggering physiological reactions aimed at helping the body adapt to the changes [4]. These adaptive physiological reactions of the body comprise the stress response system and are critical for survival. Activation of the stress response system mobilizes body's energy resources to meet demands of the stressor, and can create an energy crisis if sustained for prolonged periods. Psychological stressors are causes that

are cognitively identified as threat by an organism and can trigger the stress response system [5]. Stress response systems exist in all organisms, however, only those organisms that have highly developed cognitive centers of the nervous system, are believed to perceive psychological stress (PS). Hence, most of the psychological stress studies are primarily focused on higher life forms. Sympathetic branch of the nervous system in higher life forms is responsible for the immediate stress or survival response, and provides the body with bursts of energy so that it can respond to perceived dangers quickly. The parasympathetic branch or the counter-balance creates a relaxation response, whose effects are those of slowing, restoration, and relaxation of the body after the threat has passed. Both of these components of nervous system are very crucial and responsible for mediating the large repertoire of neural and endocrine reaction cascades in a typical stress response system. Repeated and/or chronic activation of the stress response system creates an allostatic load on the physiology and over time takes a toll on the body. We speculated that perhaps psychological stress, hyper-activates some physiological processes that can provide energy, while slows down other non-essential functions such as reproduction, thereby disrupting the homeostatic balance. This homeostatic dis-balance, if stretched for long periods as in case of chronic stress makes the body prone to various disorders of mal-adaptation. Psychological stress has been reported to regulate physiology at different levels. PS has been implicated in a number of stress-associated disorders [6, 7] and enhanced disease susceptibility. Most of these reports evaluate physiological parameters associated with stress and stress-associated diseases, while very few studies provide insights to the underlying signaling events. Stress response is thus governed by multiple factors, and in turn regulates various physiological processes in different stages of its manifestation, most of which are probably functionally interlinked and cross-regulate each other. Discovering the entire gene-networking in a typical

stress response is, therefore, quite challenging. Although, studies in recent years have considerably expanded the molecular basis, however, it is not completely understood owing to the nature of studies carried out in this field and absence of holistic approaches. The current project is primary aimed at integrating data from scattered studies associated with psychological stress and reduce reported information from multiple physiological parameters to novel gene associations. In the current study, the existing gene-networking technologies in systematic analysis were explored and new methodologies were developed, to analyze the effects of psychological stress on physiological processes viz. metabolism, innate immunity, digestion, respiration, reproduction and development on human and few other species where data was available. Our results provided critical tools for identifying new gene-signaling clues to understand how psychological stress affects each of these processes and disease susceptibility which could predict cause-effect relations among them at molecular level. We carried out *in vitro* simulation of adipose tissue in a psychological stress micro-environment to validate some of the novel predictions from the systematic-analysis for effects of stress on immunity and metabolism. In order to consolidate all the stress associated data across species and various physiological processes, a database for stress was built.

The study was therefore designed with the following objectives:

1. To identify and predict important gene-associations that might be involved in mediating stress effects on physiology and disease susceptibility
    - Develop systematic-analysis methodologies that can reduce physiological parameter-based data to gene-associations
    - Using the established methodologies for analysis and prediction of gene-associations in physiological processes namely immunity, metabolism, ageing,

digestion, respiration, reproduction, development and diseases that might have role in stress response system

2. To test the robustness of the predicted gene-associations based on systematic-analysis methodology
    - Validate the involvement of HTRs in modulating immunity and metabolism during stress response in an *in vitro* co-culture of adipocytes and macrophages treated with stress hormone cortisol and serotonin
3. To develop an integrated platform for mining and analysis of stress associated data and its disease/phenotype correlation

1. Development of systematic-analysis methodologies

1.1 Effect of psychological stress on innate immunity and metabolism: A systematic analysis

We first attempted to develop gene-networking based systematic analysis in the context of psychological stress association with immunity and metabolism. In the absence of extensive direct studies which explore the relationship between immunity and metabolism, we used an indirect association-based approach which sorts data to build a model based on the annotation profile of training-set genes that have already reported associations with each of these processes. Endeavour was used for identifying the candidate genes from whole human genome based on their similarity/association with the training set using the model. Top ranking candidate genes were then used for text mining and association network generation each for immunity and metabolism using Agilent's Literature Search plugin in Cytoscape. These gene-association networks were then clustered using MCODE clustering algorithm, followed by network topology and Gene-Ontology (GO) enrichment analysis [8].

1.2 Understanding effects of Psychological Stress on physiology and disease through human stressome - An integral algorithm.

In order to understand the effect of psychological stress on other physiological processes and diseases, gene-association networks were generated for all the above mentioned physiological processes in the similar manner. A text-mining based gene-association network was generated for psychological stress using entire human genome (human stressome) as keywords. Disease Ontology (DO) enrichment [9] analysis of the human stressome showed that the significantly enriched DO terms could broadly be grouped into following 5 categories: Infectious, Metabolic, Autoimmune, Neurodegenerative and Neurophysiological (total of 140 disease networks). Analysis of such huge number of disease networks together with psychological stress was a huge problem and needed to be integrated in way that would reflect the over-representation status of each of the diseases along with associated genes in a single a comprehensive integrated disease-gene network. So, a network condensation algorithm was developed for analysis of association between diseases and psychological stress as well as for analysis of relationship between stress associated genes, physiological functions and diseases. These condensed association networks helped us identify the disease genes and physiological functions that might be most susceptible targets of psychological manifestation in body.

1.3 Results

Analysis of gene-association networks for immunity and metabolism allowed us to identify enriched cytokine and serotonin receptor clusters that led to prediction of possible role of serotonin receptors on regulation of immunity and metabolism. It also indicated that endo-cannabinoid system and serotonergic system might be important players regulating inflammation and energy-efficiency as counter-regulatory stress response mechanisms, based on which a probabilistic model for effects of short-term and long-term effects of psychological stress on innate immunity and metabolism was proposed

The GO enrichment profile of stressome network showed that stress was associated with steroid hormone biosynthesis along with increased redox reactions, isoprenoid biosynthesis and nitric oxide biosynthesis process. PDGH (Prostaglandin dehydrogenase) is a stress gene which is highest represented in Stress-diseases network. This revealed the importance of lipid metabolism/turnover and resulting accumulation of oxidized products as one of the major causes of stress-associated diseases. Enrichment profile of stress-disease network showed enrichment of calidiol 1-monooxygenase activity and eicosanoids (ICs), prostaglandins (PGs) secretion hinting that stress mediated diseases might result due to accumulation of ICs and PGs in the body.

Liver is the major site for breakdown of fats and also detoxifies foreign substances or toxins (including micro-organisms), especially from the gut, with major percentage of blood being filtered is from the portal vein. In fact, it has been shown that UGTs (Uridine 5'-Diphospho Glucuronosyl Transferase enzymes, highly enriched in the stress network) are responsible for cholesterol homeostasis in liver. During sustained stress response, when there is continuous release of esterified fatty acids from body's fat depots, liver is under constant detoxification pressure and might eventually malfunction due to over-activity. A functional analysis of the GO enrichment profile of human thus prioritizes liver as one of the major organs to be affected during stress response. Accordingly, Hepatitis C was also seen to be maximum affected by stress and contribution of PDGH (stress associated gene) to the stress disease network was largest.

2. Validation of predictions of text-mining based systematic-analysis using gene-association networks

Based on the results discussed above, we assumed that serotonin might be involved in the inflammatory and/or metabolic stress response system, via HTRs receptors (as seen in the enriched cluster). A detailed back-ground search on the endocrine role of serotonin revealed that, gut is the major site of serotonin production, and serotonin content in inflamed adipose tissue is

very high [10], which also correlates well with increased visceral obesity [6, 11, 12]. Apart from fat/lipid storage, adipose tissue acts as an endocrine organ that can control metabolism [12] is capable of secreting inflammatory cytokines [13-15]. So, we designed an *in vitro* simulation of adipose tissue to understand the effects psychological stress on the balance between immunity and metabolism. This was achieved by doing an indirect co-culture of 3T3L1 adipocytes cells and RAW 264.7 macrophage cells in a stress micro-environment, where they were treated with cortisol (the established stress hormone [16]) and serotonin as a candidate compensatory regulatory hormone [17, 18].

2.1 Role of HTR5a and HTR2c in cortisol-induced peripheral serotonergic signaling in adipocytes during stress.

Standardization experiments identified 48h time point as optimum for gene-expression expression profiling and cytokine profiling of adipocytes and macrophages grown individually as well as in indirect co-culture under treatment of 100 µM cortisol and serotonin. Adipogenesis assays done to test effects of each these hormones on adipocytes showed that after 48h of treatment, maximum adipogenesis was seen when adipocytes were treated with combination of both cortisol and serotonin at 100 µM. mRNA transcriptional profiling done through RT-PCR revealed when treated with both cortisol and serotonin (100 µM) together, expression of serotonin receptors HTR5a , HTR2c and transporter (SERT) along with adipogenic transcription factor (PPARγ) and adipogenesis differentiation marker (AP2) were up-regulated. When adipocytes were co-cultured with macrophages, the expression of HTR5a, HTR2c, PPARγ2 and AP2 in co-cultured adipocytes was further enhanced.

To check whether signaling through HTR5a and HTR2c is responsible for increased adipogenesis in adipocytes, they were treated with HTR5a antagonist and HTR2c antagonist. It was observed that adipogenesis decreased with increasing dosage of these antagonists (unlike

cortisol and serotonin). Transcriptional profiling of the adipogenic markers in adipocytes treated with 10 µM HTR5a antagonist and 10 µM HTR2c antagonist showed that blocking signaling through HTR5a suppresses adipogenesis differentiation marker AP2 (thereby hampering adipocyte differentiation) very strongly; while blocking signaling through HTR2c showed that adipogenic transcription factor PPARγ2 and scavenger receptor OLR1 and CD36 were suppressed, showing that HTR2c signaling is important for transcription of adipogenic genes and scavenger receptors. SERT was not affected significantly by blocking HTR5a and HTR2c signaling.

To find out whether this pronounced adipogenic effect in co-cultured adipocytes due to serotonin and cortisol was additive or synergistic effect, transcriptional profiling of co-cultured adipocytes was done after treating the co-cultured adipocytes individually with serotonin(S), cortisol(C) as well as both(CS). It was seen that HTR5a and HTR2c were synergistically up-regulated under the effect of both cortisol and serotonin. Similar effects were seen on OLR1, CD36 and PPARγ2. Cortisol however appeared to singularly contribute to adipocyte differentiation (AP2), though serotonin also slightly up-regulated this gene. It was also observed that macrophages co-cultured with adipocytes too accumulated higher amount of lipids on treatment with both cortisol and serotonin. However, this was not mediated by signaling through HTR5a and HTR2c, since cortisol and serotonin treatment suppressed them. OLR1, CD36 and PPARγ2 were up-regulated in these macrophages, indicating that, most probably reason for increased lipid content of macrophages was due to increased scavenging of oxidized lipids.

2.2 Modulation of the metabolic and immune balance in adipose tissue by stress hormones: An in vitro simulation.

Whole genome transcription analysis of adipocytes grown alone as well as co-cultured with macrophages was done through microarray at three different time-points 6h, 24h and 48h. It was observed that at 48h the number of differentially regulated genes increased approximate by 3 folds. Hence microarray of co-cultured macrophages was also done at 48h. It was observed that macrophages did not show strict M1 or M2 polarisation due to treatment with cortisol and serotonin. Inflammatory cytokine profiling through ELISA at 24h and 48h was done to check the effect of these hormones on IL6, IL1b, IL12, TNFa, IL10 and TGFb in macrophages grown alone as well as co-cultured. Protein profiling through ELISA showed that both pro (IL6, IL1b, IL12, TNFa) and anti (IL10, TGFb) inflammatory cytokines were affected by both the hormones, thereby confirming the microarray results. It was also observed that the inflammatory secretion of macrophages dropped at 48h. Temporal genome wide transcription analysis showed that co-culturing activates inflammatory TNF pathway, which later gets suppressed at 48h due to negative feed-back regulation. It was also observed that with increasing time, transcription of pro-inflammatory cytokines and chemokines dropped and anti-inflammatory cytokines increased. Transcriptional activation oxidative enzymes like PTGS2 (COX2), Aldehyde dehydrogenase, Cytochrome oxidase and Scavenger receptors of oxidized lipids (OLR1) also increased with time due to co-culturing. This indicated that lipid oxidation and resulting oxidative by-product accumulation in adipocyte-macrophage co-culture increased with time. While fatty-acid metabolism was more pronounced due to cortisol, serotonin caused higher transcription of enzymes coding for fatty–acid elongation.

2.3 Discussion and Conclusion

Overall, the current results showed that cortisol is able to cause up-regulation of serotonin receptors HTR5a and HTR2c and induce activation of adipogenic signaling through these receptors in adipocytes. This signaling is even more strongly induced in adipocytes co-cultured with macrophages. HTR5a receptor mediated signaling contributes mostly towards adipocyte differentiation, while HTR2c contributes towards higher expression of scavenger receptors, which in return also promote higher expression of the adipogenic transcription factor PPARγ2 and hence adipogenic genes. Thus in a stress endocrine environment created by cortisol, serotonergic signaling can alter the metabolic status of adipocytes as well as macrophages, making them more efficient in lipid storing. In fact adipocyte-macrophage co-culture subjected to stress hormones leads to higher transcription of enzymes that catalyze oxidative reactions which further increase with time. This indicates that there might be increasing accumulation of oxidative by-products such as prostaglandins, leukotrienes, etc, which are also natural ligands for PPARγ [19] and thus, in turn might lead to higher adipogenesis. This probably explains the synergistic action of both the hormones on PPARγ2 transcription. Thus, we see that with increasing time, adipocyte-macrophage co-culture tends to accumulate more lipids under the action of stress hormones, and gradually takes an anti-inflammatory profile. These results provide evidence for the predictions from the systematic analysis discussed earlier and also support studies that report high oxidative stress in animal models of psychological stress [20, 21]. Excessive lipid accumulation, higher lipid oxidation leading to accumulation of oxidative by-products and suppression of pro-inflammation observed in this system might also easily make body susceptible to diseases like obesity, diabetes, arteriosclerosis [22-28] and other disorders of mal-adaptation [29].

3. Psychological Stress associated Data Integration

Psychological stress associated genes were mined for the following species: mouse, rat, cattle, cattle and pig using the above established methodology and gene-association networks were created where ever sufficient data was available in literature. Gene association networks for ageing, metabolism, immunity, respiration, digestion and reproduction were also created for each of these species. In order to consolidate all these PS associated data across species and various physiological processes, a database for stress was built.

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2. Sood P, Priyadarshini S, Aich P (2013) Estimation of Psychological Stress in Humans: A Combination of Theory and Practice. PLoS ONE.
3. Priyadarshini S, Aich P.(2012) Effects of psychological stress on innate immunity and metabolism in humans: a systematic analysis. PLoS ONE.

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2. Understanding effects of Psychological Stress on physiology and disease through human stressome - An integral algorithm. [Priyadarshini S, Aich P]
3. A brief overview of stress, stressors and manifestation. [Priyadarshini S, Aich P]

c. In preparation:

1. Modulation of the metabolic and immune balance in adipose tissue by stress hormones: An in vitro simulation.
2. StressdB: A webserver for mining and analysis of stress data.

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Signature of Student:

Date: 14/07/2015

Doctoral Committee

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4.	Dr. Manjusha Dixit	Member	Manjusha	14/07/15

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AA	-	Arachidonic Acid
acetyl CoA	-	Acetyl Coenzyme A
ACTH	-	Adrenocorticotrophic Hormone
ANGPTL4	-	Angiopoietin Like Protein 4
AP1	-	Activator Protein 1
AP2	-	Adipocyte Protein 2
APC	-	Adenomatous Polyposis Coli
ATF4	-	Activating Transcription Factor 4
BCL2	-	B Cell CLL/Lymphoma 2
BDNF	-	Brain Derived Neurotrophic Factor
CCL	-	Chemokine (C-C motif) Ligand
CCL2	-	Chemokine (C-C motif) Ligand 2
CD	-	Cluster of Differentiation
CDKN1a	-	Cyclin-Dependent Kinase Inhibitor 1A
COPD	-	Chronic Obstructive Pulmonary Disorder
COX	-	Cycloxygenase
CREB1	-	cAMP Responsive Element Binding Protein 1
CREBBP	-	CREB Binding Protein
CREBp	-	Cyclic AMP Response Element Binding Protein
CRF	-	Corticotrophin Releasing Hormone
CRP	-	C-Reactive Protein
CXCR3	-	Chemokine (C-X-C motif) Receptor 3
DAG	-	Diacylglycerol
DAPI	-	4',6-Diamidino-2-Phenylindole
DECR1	-	2,4 Dienoyl-CoA Reductase 1
DHA	-	Docosahexaenoic Acid
DMEM	-	Dulbecco's Modified Eagle's Medium
DO	-	Disease Ontology
EC	-	Enterochromaffin Cells
EDTA	-	Ethylene Diamine Tetraacetic Acid
EGR1	-	Early Growth Response Protein 1
ELISA	-	Enzyme Linked Immunosorbent Assay
EPA	-	Eicosapentaenoic Acid
EPHB2	-	EPH Receptor B2
FABP3	-	Fatty Acid Binding Protein 3
FASN	-	Fatty Acid Synthase

FBS - Fetal Bovine Serum
FDFT1 - Farnesyl-Diphosphate Farnesyl Transferase 1
FFAs - Free Fatty Acids
GAPDH - Glyceraldehyde 3-Phosphate Dehydrogenase
GAS - General Adaptation Syndrome
GC - Glucocorticoids
GLUT - Glucose Transporter
GMCSF - Granulocyte Macrophage Stimulating Factor
GO - Gene Ontology
GR - Glucocorticoid Receptor
GRE - Glucocorticoid Responsive Element
GSH - Glutathione
HIF1α - Hypoxia Inducible Factor 1 alpha
HTRs - 5-HT Receptors
IBD - Inflammatory Bowel Disease
IBMX - Methyl IsoButyl Xanthine
ICAMs - Intercellular Adhesion Molecules
IFN - Interferon
IKKBb - I kappa B Kinase Subunit beta
IL - Interleukin
iNOS - Inducible NOS
IRF1 - Interferon Regulatory Factor 1
JAK - Janus Activated Kinase
JAK2 - Janus Kinase 2
LIPE - Hormone Sensitive Lipase
LPL - Lipoprotein Lipase
MAP3K7 - Mitogen Activated Protein Kinase Kinase Kinase 7
MAPK14 - Mitogen-Activated Protein Kinase 14
MCP - Monocyte Chemotactic Protein
MIF - Migration Inhibitory Factor
MIP - Macrophage Inflammatory Protein
MMP9 - Matrix Metallopeptidase 9
MUFAs - Monounsaturated Fatty Acids
MVK - Mevalonate Kinase
NADH - Nicotinamide Adenine Dinucleotide
NCCS - National Center for Cell Science
NCOR2 - Nuclear Receptor Co-Repressor 2
NFκB - Nuclear Factor kappaB
NOD2 - Nucleotide Binding Oligomerization DomainContaining Protein 2
NOS - Nitric Oxide Synthase
OLR1 - Oxidized Low Density lipoprotein receptor 1

oxLDL	-	Oxidised Low Density Lipid
OXTR	-	Oxytocin Receptor
PBS	-	Phosphate Buffered Saline
PDGH	-	Phosphoglycerate Dehydrogenase
PDX1	-	Pyridoxal Phosphate Synthase Protein
PGE2	-	Prostaglandin E2
PI3K	-	Phosphatidylinositide 3-Kinase
PKC	-	Protein Kinase C
POMC	-	Proopiomelanocortin
PPAR	-	Peroxisome Proliferator Activated Receptor
PRKAA1	-	5'-AMP-Activated Protein Kinase Catalytic Subunit alpha-1
PRKAG2	-	5'-AMP-Activated Protein Kinase Subunit Gamma-2
PS	-	Psychological Stress
PSTD	-	Post Stress Traumatic Disorder
PTGS2	-	Prostaglandin Endoperoxide Synthase 2
PTGS2	-	Prostaglandin Endoperoxide Synthase 2
PUFA	-	Polyunsaturated Fatty Acid
RHOA	-	Ras Homolog Family Member A
ROS	-	Reactive Oxygen Species
SDF	-	Stromal Cell-Derived Factor
SERPINE1	-	Serpin Peptidase Inhibitor
SERT	-	Serotonin Transporter
SFAs	-	Saturated Fatty Acids
SIRT1	-	Sirtuin 1
SREBP	-	Sterol Regulatory Element Binding Protein
SSRI	-	Serotonin Reuptake Inhibitors
STAT3	-	Signal Transducers and Activators of Transcription 3
SYK	-	Spleen Tyrosine Kinase
TCA	-	Tricarboxylic Acid
TGFβ1	-	Tumor Growth Factor beta 1
TLR	-	Toll Like Receptor
TNF	-	Tumor Necrosis Factor
TRH	-	Thyrotrophin Releasing Hormone
UCP3	-	Uncoupling Protein 3
UGTs	-	Uridine 5’-Diphospho Glucuronosyl Transferases
VEGF	-	Vascular Endothelial Growth Factor
ZHX2	-	Zinc Fingers and Homeoboxes 2

Chapter 1

Introduction
&
Review of Literature

1.1 General Introduction

Living systems detect and integrate clues from changes in their external and internal states, and respond appropriately to retain a state of dynamic equilibrium or homeostasis which is essential for their survival [1-3]. Events, whose demands outweigh body resources, as perceived by the body to successfully cope, are known as stressors. Stress response is primarily a normal physiological response to the stressors, which allows the organism to respond to its environment. The stress response probably evolved as a survival mechanism, enabling an organism to react quickly to life-threatening situations [4, 5]. In the line of evolution, complex organisms like humans, brain controls coping with aversive situations by modulating the activity of the adaptive systems [6-8]. Psychological stressors are events that are cognitively identified (based on genetic make-up and priming of the individual) as a threat by an organism and can trigger the stress response system. It is important to note that stressful events do not automatically lead to psychopathology, indicating that not the stressor itself, but the individual sensitivity to stress is crucial to determine the extent of vulnerability to stress induced pathology. Genetic predisposition (that determines individual characteristics such as resiliency to stress) plays a critical role in susceptibility of an individual to stress in addition to psychosocial and biological factors[9]. With changes in life-style of humans over the past few centuries, dependence of physical activity for survival has declined, and at the same time, there has been increase in performance-driven perceived survival pressure on humans, based on social hierarchy of socio-economic as well as community, and interpersonal level. This perceived survival pressure or modern-life stress, on one hand helps a person adapt to the demands needed to ensure his survival, however on the other hand may pose a threat when the individual perceives a discrepancy between the demands of a situation and the individual's biological, psychological

or social resources. In such a situation, the stressor may have become too severe for the person to cope with. Effective coping of stress implies that the stress response is activated rapidly when it is needed and is efficiently terminated afterwards, thus enhancing the probability of survival of the concerned individual. However, over time, repeated or prolonged activation of the stress response takes a toll on the body[10]. Psychological stress was identified a potential health risk quite recently and investigations over the past few decades were largely in the domain of sociology, epidemiology and social psychology, which contributed very little to the understanding of its biological implications. Clinical data implicated stress in the etiology of many psychiatric disorders as well as dysautonomia resulting in organ dysfunction and/or disorders such as chronic-adrenal fatigue, insomnia, high blood pressure, circulation disorders, heart disease, gastrointestinal disorders, obesity, dyslipidemia, diabetes, autoimmune disorders, anxiety disorders, depression, addiction, panic attacks, etc; with most of these observations based on physiological parameters and very few studies providing insights to underlying signaling events. The neuro-endocrine signaling conveying the status quo at gene and molecular level during stress response remained largely unexplored and demanded a closer examination of the mechanisms by which the stress gets under the skin and affects body and health[10].

The stress response system spans body's neuro-endocrinal circuitry and affects all the major physiological process including over-all metabolism, circulation, respiration, digestion and in due course immunity, reproduction, ageing [8, 11-26]. Understanding the relationship between stress-response system and each of these processes as well as how stress can disturb the homeostatic balance between these processes requires aggregation of all the investigations relating to stress, followed by downstream data refinement and analysis. In order to explore such complex issues with statistical confidence, one of the methods generally used is meta-analysis,

where comparable physiological parameters across various studies are taken into account to establish the significance of causative molecular or signaling events. Studies reported so far, on psychological stress, have only been able to establish a few signaling events at neuro-endocrine level to understand psychological stress response and associated changes in human physiology [27, 28]. However, meta-analysis of these reports could not be attempted as effects of stress are not reported to be associated with stress physiology and stress-associated disorders in the same studies making identification of comparable parameters affected by stress or consequence of stress difficult. Another major approach is gene-networking which allows segregation of multiple parameters across various dispersed studies[29].Gene networking is an emerging technology that is used in systematic analysis that can integrate data from scattered studies and reduce multiple parameters to gene associations, thus providing critical tools for unifying physiological parameters with novel gene-associations and identifying new gene-signaling clues. The advantage of gene-networking analysis is that large dimension data across various studies can be compressed into a single or a few reducible networks. Such data compression simplifies the daunting dimensions and makes comparative analysis feasible at molecular (gene) level with the potential to makedata-association driven predictions in areas where studies are limited in numbers, diverse or unrelated and scattered. We report here effects of psychological stress on various physiological functions and diseases using analysis of text-mining based gene-association networks with the goal to understand and predict role of psychological stress on human physiology and diseases. We have done hypothesis testing and validation of prediction-driven models based for based on the gene-association networks through *in-vitro* models of psychological stress simulation.

1.2. Review of Literature

1.2.1. Conceptualization of stress and impacts on life

The concept of stress as a "nonspecific response of the body to any demand placed upon it" was first introduced by Hans Selye. Selye proposed a stress model in the year 1973, dividing stress into eustress and distress. Stress when has enhancing effect on the efficiency of an individual (physical or mental) may be considered positive or eustress. Eustress forces us to adapt and helps to increase the strength of our adaptation mechanisms, warns us that we are not coping well and that a lifestyle change is warranted if we are to maintain optimal health. This action-enhancing stress gives the athlete the competitive edge and the public speaker the enthusiasm to perform optimally. Persistent stress that is not resolved through coping or adaptation is deemed negative or distress. Stress is harmful when it exceeds our ability to cope, fatigues body systems and causes behavioral or physical usually results in sub-par performance[30].Although the stresses of modern urban life rarely demand strenuous or even moderate physical activity, social and psychological circumstances can turn on the stress response and divert energy and resources away from many physiological processes. The body's autonomic nervous system does not clearly distinguish between daily stressors and life-threatening events e.g. our bodies can still react to a normal argument with a colleague, traffic jam during commutation to work, or a long money bill, as if facing a life-or-death situation. While a short fight-fight reaction might appear to actually help the body deal with the situation, the repeated of stress response in daily life, can raise blood pressure, suppress the immune system, increase the risk of heart attack and stroke, speed up the aging process and make us vulnerable to a host of mental and physical health problems. Cognitive Activation Theory of Stress, based studies have shown that coping, helplessness, worrying, rumination and

hopelessness are important contributors to health [31].Psychosocial risks such as continuing anxiety, insecurity, low self-esteem, social isolation and lack of control over work and home life, accumulate during life and increase the chances of poor health and premature death.

1.2.2. Symptoms of body under psychological stress

The following have been identified as symptoms of stress overload in general:

- **Cognitive Symptoms:** memory problems, disorganization, lack of concentration, confusion, pessimism, anxious or racing thoughts, constant worrying, difficulty in making decisions
- **Emotional Symptoms:** moodiness, irritability or short temper, agitation, inability to relax, feeling overwhelmed, sense of loneliness and isolation, depression or general unhappiness, frustration, agitation
- **Behavioral Symptoms:** eating too much or too little - appetite changes, sleeping too much or too little-sleep disorder, social isolation, procrastination or neglecting responsibilities, increased abuse of alcohol, cigarettes, or drugs to relax - addiction, nervous habits (e.g. nail biting, pacing), edginess, excessive gambling, impulse buying, hostility
- **Physical Symptoms:** aches and pains, muscle spasms, diarrhea or constipation, excess belching, flatulence, nausea, dizziness, chest pain, rapid heartbeat, loss of libido, frequent colds and infection, insomnia, cold or sweaty hands and feet, dry mouth, low energy

Onset of psychological stress triggers systemic adaptation response (termed as GAS - General Adaptation Syndrome by Hans Selye [32]) in the body. GAS involves activation or suppression of one or more physiological functions to cope up with the demands brought in by stressors through production of adaptive stress hormones[33]. In emergencies, our hormones and nervous system prepare us to deal with an immediate threat by triggering the fight or flight response: raising the heart rate, mobilizing stored energy, diverting blood to muscles and increasing

alertness. Cascades of physiological events, such as elevated blood pressure and impaired heart rate affecting blood circulation, and increased blood sugar level, take place when challenged with a threat or stress. This series of events are designed to give targeted focus along with very fast supply of energy to withstand the emergency situation [34].The adaptive responses to stress are energetically expensive [35], since biological events invoked by PS are energy consuming and are aimed at channelization of body's energy stores towards meeting immediate demands of stress. This prioritized energy mobilization creates an energy crisis in the body, as a result of which non-vital energy-demanding physiological functions like reproduction, digestion, immunity and growth undergo a state of transient adaptive suppression [36]. Chronic stress results in sustained glucocorticoid production rendering these physiological functions in a state of prolonged altered activity. High sympathetic neurological activity leads to overstimulation of these neurotransmitters which eventually leads to their exhaustion [37]. Hyperactivity of sympathetic neurological responses may also lead to higher blood insulin level [38], disruption of hormonal regulation [39] and impairment of detoxification process. For example, adrenalin, as a sympathetic response mediating hormone, triggers liver to dump its glucose reserve into blood. High levels of glucose in the blood alert pancreas to release high amounts of insulin [40] which may lead to various deleterious health conditions [41, 42] like insulin resistance [43], obesity [44], type-2 diabetes [45], heart diseases [46, 47] and heightened inflammatory state [48, 49]. Prolonged sympathetic hyperactivity [50] increases the allostatic load [51, 52] and it becomes increasingly difficult for the body to repair actions of GAS [53], making it susceptible to various disorders of deranged metabolic homeostasis.

The body naturally prefers to be in the parasympathetic or regenerative state, where most of the physiological processes function with an optimal activity. It is thus natural for the body to

have robust feed-back signaling mechanisms to allow its transition from sympathetic to parasympathetic mode after a state of heightened sympathetic SRS activity. Although these mechanisms are not well understood, there are bits and pieces of evidence that support their existence. One such mechanism is via production of cortisol during stress response. Stress induced sympathetic activity increases blood glucose content through catecholamine action [54]. High glucose content in blood stream stimulates production of pro-inflammatory cytokines [55]. IL1b – Interleukin1b has been shown to activate the Hypothalamic Pituitary Axis (HPA) and cause release of Corticotropin Releasing Hormone (CRH) from Para Ventricular Nucleus (PVN) in brain, leading to increase in circulating glucocorticoids (GCs) [56, 57]. GCs play important roles in glucose management and immune function such as suppression of pro-inflammatory response. This mechanism leads a feedback signaling from hyperactive sympathetic nervous system to activation of parasympathetic signaling and restoration of homeostasis. Again sustained cortisol release too can inflict harm to body. A very high level of cortisol is required under intense influence of stress, which results in disruption of the hormonal system e.g.high and persistent cortisol demands lead to adrenal fatigue, a situation where adrenal glands can no longer produce enough cortisol [58]. In order to synthesize more cortisol, physiological system uses up precursors needed for synthesizing other hormones like aldosterone, estrogen, progesterone, testosterone and Dehydroepiandrosterone (DHEA). This phenomenon, known as "cortisol steal", creates deficiency of these hormones and increases the allostatic load on physiology by disturbing various systems such as nervous, endocrinal, digestive, immune and cardiovascular, reproductive systems. These systems are under autonomic nervous control. As a result, stress response system negatively affects Autonomic Nervous System (ANS) that might lead to a plethora of disorders such as chronic fatigue, high blood pressure, circulation disorders,

gastrointestinal disorders, heart diseases, ulcers, autoimmune disorders, anxiety disorders, depression, addiction, and panic attacks. Thus, psychological stress (PS) accompanied by metabolic energy crisis leads to disturbances in physiological homeostasis [59] and increased disease susceptibility [41, 50, 53].

1.2.3. Molecular markers of psychological stress

1.2.3.1. Glucocorticoids

Overshoot of HPA axis activity is kept within borders by feedback mechanisms responding to variations of glucocorticoid hormone levels. Glucocorticoids suppress the transcription of genes coding for CRH and ACTH via binding to glucocorticoid receptors (GR) at various stages of the HPA axis. Recent understanding of role of glucocorticoids in counteracting negative effects of stress and maintaining homeostasis has shed light on the fundamental mechanisms cell signaling in inflammation and how they are able to switch off multiple inflammatory genes (encoding cytokines, chemokines, adhesion molecules, inflammatory enzymes, receptors and proteins) that have been activated during the chronic inflammatory process. In chronic inflammatory diseases, increased expression of most of the inflammatory proteins is regulated at transcriptional level through the activation of pro-inflammatory transcription factors, such as nuclear factor-kB (NF-kB) and Activator Protein-1 (AP-1). Pro-inflammatory stimuli (such as IL-1b, TNF-a or endotoxin) that switch on inflammatory genes do so by changing the chromatin structure of the gene, whereas switching on of inflammatory genes requires the engagement of co-activator molecules (such as cyclic AMP response element binding (CREB) binding protein (CBP), p300 and p300-CBP associated factor (pCAF)) [60]. These co-activators have intrinsic Histone Acetyl Transferase (HAT) activity and their activation causes acetylation of core histones, thereby allowing the

transformation of resting closed conformation of chromatin DNA to an activated unwound or open form. This allows binding of TATA box binding protein (TBP), TBP-associated factors and RNA polymerase II, which interact with pro-inflammatory transcription factors, such as NF-kB, that bind to specific recognition sequences in the promoter region of inflammatory genes and initiate transcription of inflammatory genes.

The histone acetylation associated with increased expression of inflammatory genes is counteracted by Histone Deactylases (HDACs) activity of corticosteroids that can reverse this process. Inactive glucocorticoid receptors (GRs) are phosphoproteins that remain bound to molecular chaperones (such as heat shock protein-90, hsp90 and FK-binding protein) in the cytoplasm. These proteins protect the GR and prevent its nuclear localization by covering the sites on the receptor that are needed for nuclear translocation [61]. Corticosteroids diffuse readily across cell membranes and bind to glucocorticoid receptors in the cytoplasm and change the receptor structure resulting in dissociation of molecular chaperone proteins and exposing nuclear localization signals on GR. This causes rapid transport of the activated GR-corticosteroid complexes into the nucleus, where they homodimerize. Activated GR homodimers may bind to specific sequences (GREs) in promoter region corticosteroid-responsive genes, causing acetylation of lysines on histone H4, which leads to transcription of anti-inflammatory genes (trans-activation); or they can also interact with negative GREs to suppress pro-inflammatory genes (cis-repression). Another way in which corticosteroids may regulate gene expression is by interacting with co-activators, where binding to GRE is not required. In these cases, activated GR binds to CBP or other co-activators directly to inhibit their HAT activity thus reversing the unwinding of DNA around core histones and thereby repressing inflammatory genes (non-DNA binding trans-repression). Glucocorticoids cause trans-repression of inflammation not only by

acetylation of histones, but also by methylation [62], phosphorylation [63] and ubiquitination [64]. GRs are also subject to modifications like phosphorylation [65, 66], which may alter the response to corticosteroids by affecting ligand binding, translocation to the nucleus [66], trans-activating efficacy, protein-protein interactions or recruitment of cofactors [67].

Neurotransmitters (like endorphins, serotonin and dopamine) are also needed to modulate the autonomic nervous system and restore parasympathetic state or the regenerative phase.

1.2.3.2. Serotonin

95% of the body's serotonin (5-HT) is produced from the precursor molecule Tryptophan and housed in the gut, where it acts as a neurotransmitter and endocrine hormone. In the brain serotonin is unable to pass the blood brain barrier, and has to be synthesized locally from tryptophan and its availability the rate limiting step in 5-HT synthesis. After synthesis, 5-HT is stored into small vesicles in the axon terminal and its release activates both pre and post-synaptic receptors. Extracellular serotonin is taken up into the cells via the serotonin transporter (SERT) and either reused or degraded by the monoamine oxidase (MAOa) into 5-hydroxy indoleacetic acid (5-HIAA). Until now, 14 structurally and pharmacologically distinct mammalian 5-HT receptor subtypes have been identified, which are now assigned to one of seven families 5-HT. Normal 5-HT function is important in the control of sleep, wakefulness, feeding behavior, the control of sensory transmission, mood and a wide range of behavior. Abnormal cerebral serotonin function has been associated with depression, anxiety, aggression and poor impulse control. Studies have shown that 5-HT manipulations also are associated with increased impulsivity and aggression. Acute tryptophan depletion has also been shown to increase cortisol levels in patients with seasonal affective disorder and attenuates cortisol levels after a stressful task in subjects with a family history of depression, indicating

multiple and complex interactions between 5-HT and the HPA axis. Serotonin is also implicated in sending signals from the gut to the head by increasing in gut-serotonin levels. There can be various causes of serotonin release in the gut including psychological stress. The digestive process begins when a specialized cell, an enterochromaffin, releases serotonin in gut lumen which communicates with nerve cells to start intestinal peristalsis and secretion of digestive juices. After initiation of intestinal reflex, 5-HT is whisked out of the bowel by the serotonin transporter, or SERT, found in the cells that line the gut wall. Apart from regulating mood, anxiety and depression, serotonin also helps in digestion and is implicated in gut disorders like Irritable Bowel Syndrome (IBD) and ulcers. People with irritable bowel syndrome do not have enough SERT, so they wind up with excess gut serotonin that might cause diarrhea. Excess serotonin can also cause constipation, by overwhelming its receptors in the gut, shutting down their signaling.

1.2.3.3. Histamine

Another mechanism that lends credence to physiology as the source of intestinal dysfunctions is the system of mast cells in the gut. that have an important role in immune response. During stress, trauma or 'fight or flight' reactions, the barrier between the lumen, the interior of the gut where food is digested, and the rest of the bowel could be broken, and bad stuff could get across. So the big brain calls in more immune surveillance at the gut wall by activating mast cells. These mast cells release histamines mobilizing the enteric nervous system to expel the perceived intruders, and causing diarrhea. A gut under stress, with chronic mast cell production and consequent inflammation, may become tender. Enteric neurons are known to express histamine receptors. Both histamine H1 and H2 receptors have been localized in the enteric plexus. Inflammation makes the sensory neurons in the gut fire more often, causing a kind of sensory

hyperactivity. Mast cell induced histamines may turn out to be crucial in understanding and treating GI disorders.

1.2.3.4. Dopamine

Dopamine (DA) is an important endogenous catecholamine, which exerts widespread effects on both neuronal (as a neurotransmitter) and non-neuronal tissues (as an autocrine or paracrine agent). Dopaminergic system is also known to play a regulatory role in gastric ulcers under various stressful conditions. Patients with Parkinson's disease have higher rate of ulcer, where DA becomes deficient. But in patients with schizophrenia, DA level usually becomes high, and the rate of gastric ulcer becomes very less. This indicates that DA levels must have a link with gastric pathology. Studies also suggested that modulation of dopaminergic transmission induced by DA drugs facilitates the gastric cytomodulatory effects.

1.2.4. Mechanism of Stress Response

The stress response begins in the brain's neocortex. It controls imagination, logic, decision making, memory, problem solving, planning, and apprehension, thereby interprets sensory signals and accordingly modulates motor (musculoskeletal) responses[68]. The limbic system, composed of hypothalamus, hippocampus, septum, cingulate gyrus and amygdala, represents the second major component of brain w.r.t stress response because of its role as the emotional control center[69]. The pituitary gland plays a major functional role in this system as a major effector endocrine gland. The spinal cord represents the central pathway for neurons as they conduct signals to and from the brain. Together they form the CNS (Central Nervous System). The PNS (Peripheral Nervous System) consists of the Somatic Nervous System (SNS) and the Autonomic Nervous Systems (ANS). SNS innervates sensory organs as well as the skeletal

musculature and carries sensory and motor signals to and from the CNS. ANS innervates the heart, smooth muscles and glands and regulates the body's internal environment and maintains homeostasis. The hypothalamus (command center) communicates with the rest of the body through the ANS. The ANS is sub-divided into two branches, the sympathetic and the parasympathetic. The sympathetic nerves descend from the anterior hypothalamus through the cranial and sacral spinal cord regions; the parasympathetic nerves then follow and innervate the end organs. The sympathetic branch creates the stress or survival response, and has a generalized arousal effect and provides the body with bursts of energy so that it can respond to perceived dangers[70]. The parasympathetic branch creates a relaxation response, whose effects are those of slowing, restoration, and relaxation of the body after the danger has passed[71]. The body prefers to stay naturally under parasympathetic or regenerative phase. Table 1.1 summarizes the difference in the nature of responses triggered by the activation of these two branches of the ANS.

Table1.1: Difference in effector functions of Sympathetic and Parasympathetic Nervous Stimulation

Physiological Function	**Sympathetic**	**Para- Sympathetic**
Stimulation	Prevertebral Ganglia	Vagus nerve
Heart rate	Increases	Slows down
Breathing rate	Increases	Slows down
Blood pressure	Increases	Decreases
Digestion	Decreases	Increases
Appetite	Inhibited	Increases
Pupil	Dilates	Constricts
Saliva	Watery	More mucous
Pancreatic activity	Decreases	Increases
Urinary Bladder	Inhibits contraction	Stimulates contraction
Acid reflux	Increases	Decreases
Intestinal motility	Decreases	Increases

Both the sympathetic and parasympathetic nervous systems interact with another, less well-known component of the autonomic nervous system — the enteric nervous system[72]. The enteric nervous system is sometimes referred to as a "second brain" because it relies on the same

types of neurons and neurotransmitters that are found in the CNS system (brain and spinal cord). It consists of sheaths of neurons embedded in the walls of gut or alimentary canal. It has a sophisticated, nearly self-contained network of neural circuitry (approx. 100 million neurons) and more than 30 neurotransmitters and proteins (such as Serotonin, Dopamine, Glutamate, Nor-epinephrine, Acetylcholine, Substance P, and Vasoactive Intestinal Polypeptide (VIP))[73]. The command neurons and gut interneurons are spread throughout two layers of gut tissue called - myenteric plexus and the submuscosal plexus (figure1.1).

1.2.4.1. The Gut-Brain Axis

The enteric brain, when under parasympathetic (vagal control), processes food and enhances metabolism and overall growth. The gut is under reciprocal control of both cholinergic and adrenergic innervation, and numerous data have established the involvement of peripheral CRF signaling in the modulation of gut secretory functions under stress conditions via activation of both CRF1 and CRF2 receptors[74, 75]. Peripheral CRF/CRF1 signaling pathways are locally present in the gut and contribute to increased GI motility and secretion through CRF1, delayed gastric emptying through CRF2[76]. Peripheral signaling through both CRF2 and CRF1 is also known to increase intestinal permeability[77].Parallel alteration of the brain-gut interaction is mediated by Corticotropin-Releasing Hormone (CRH), a hormone that stimulates release of cortisol from adrenal glands during stress. When the central brain encounters a threat situation, it stimulates the vagus nerve that innervates the gut walls and releases neurotransmitters such as serotonin, dopamine, etc. that are now known to have endocrine functions as well[78]. CRH (released during stress) is capable of stimulating serotonin release from Enterochromaffin cells (ECs) [79] into the viscera by increasing intestinal permeability [80]. This shows that vulnerability of gut to stress hormones is very high. SERT (Serotonin Transporter) expressed in

the inner lining of gut is responsible for reuptake of serotonin into the gut and thus terminate its function, while anti-depressants (SSRIs- Selective Serotonin Reuptake Inhibitors) recycle serotonin to keep it in circulation. Clinical observations established strong positive correlation between body mass index (BMI) and antidepressant SSRIs [81, 82], thus implying that more serotonin in circulation correlates with higher BMI/obesity There are observations that also indicate that under regulated feeding conditions, excess gut-serotonin might contribute to obesity. It has been reported that serotonin is capable of modulating adipocyte function in an autocrine and paracrine manner [83, 84]. Growing number of evidence associate stress and gut-derived serotonin with central/visceral obesity, adipose tissue inflammation and atherosclerosis [85-88], pointing to peripheral mode of action of serotonin. The stress hormone cortisol is also known to induce gastric glands to secrete acid and pepsin that can further deteriorate gastric mucosal integrity[89]. In cases of extreme stress, where there are chances of luminal antigen infiltration into the gut due to compromised intestinal epithelial integrity, the brain signals the mast cells in the plexus to secrete serotonin, histamine, prostaglandin and other agents that help produce inflammation [90, 91]. Stress has also been shown to induce mast cell degranulation in colon[92], which can lead to exceeding huge accumulation of serotonin, histamine, tryptase, prostaglandin E2[93]. By inflaming the gut, the brain primes the gut for surveillance in the face of an impending immune attack. In excess concentration, these hormones alter the gut normal functioning and if these effects happen over a period of months or years as in chronic stress, the gut physiology goes out of balance (homeostasis is lost). This arrested state of gut physiology can have disastrous effects on digestion, inflammation, mood, behavior and overall physical health. Environmental events/stimuli activate the sensory perception centers of the brain and

trigger mobilization of the stress responses. Individual interpretation of the environmental event creates most stressors and subsequent stress responses[94].

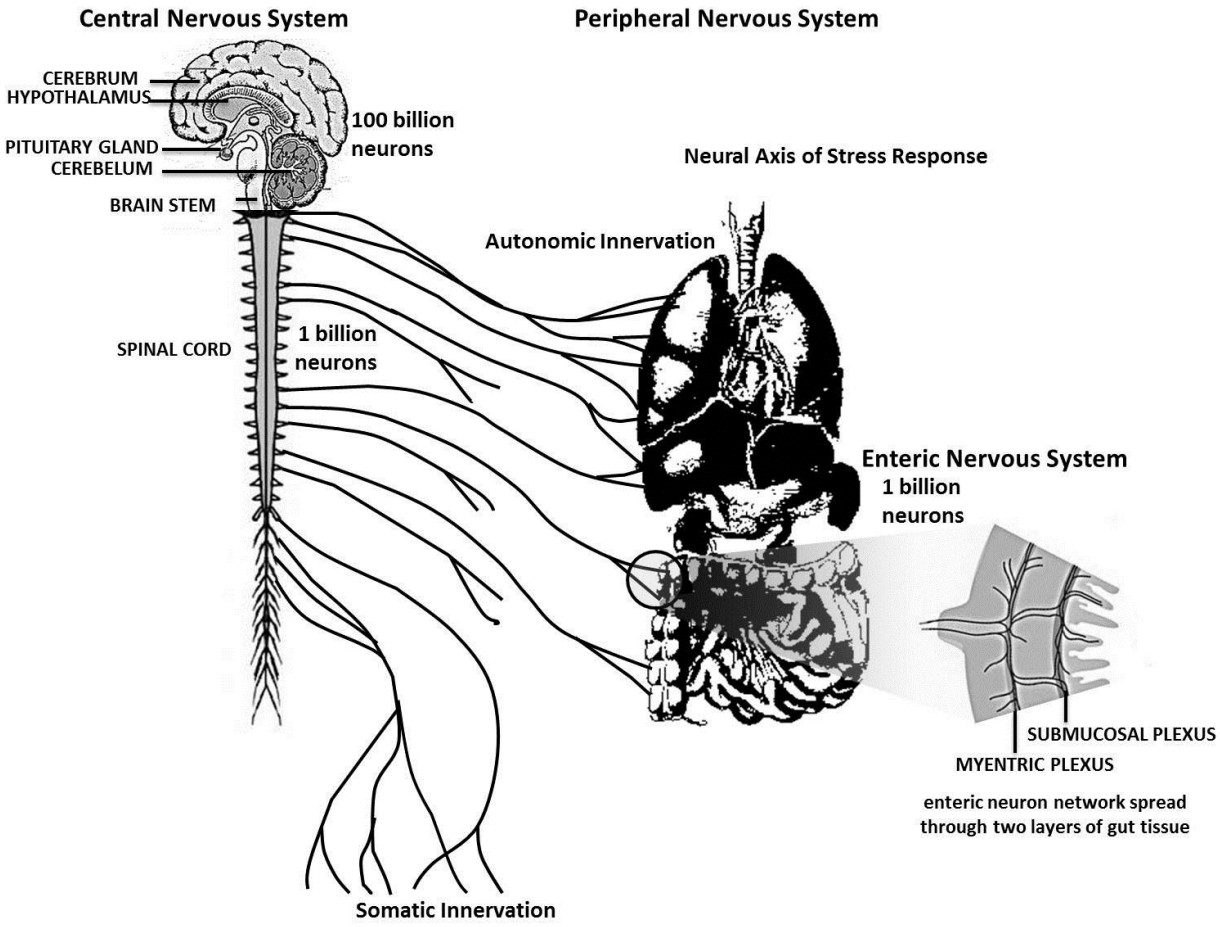


Figure1.1: Neural circuitry of Stress Response

The limbic complex and hypothalamic nuclei trigger efferent neurological, neuroendocrine, and endocrine reactions in response to higher cognitive stress perception [95]. After the amygdala sends a distress signal, the hypothalamus activates the sympathetic nervous system by sending signals through the autonomic nerves to the adrenal glands. These glands respond by pumping the hormone epinephrine (also known as adrenaline) into the bloodstream. As epinephrine circulates through the body, it brings on a number of physiological changes. The heart beats faster than normal, pushing blood to the muscles, heart, and other vital organs. Pulse rate and

blood pressure go up. The person undergoing these changes also starts to breathe more rapidly. Small airways in the lungs open wide. This way, the lungs can take in as much oxygen as possible with each breath. Extra oxygen is sent to the brain, increasing alertness. Sight, hearing, and other senses become sharper. It also triggers the release of blood sugar (glucose) and fats from temporary storage sites in the body. These nutrients flood into the bloodstream, supplying energy to all parts of the body. As the initial surge of epinephrine subsides, the hypothalamus activates the second component of the stress response system known as the Hypothalamic-Pituitary Axis (HPA)[96]. The HPA relies on a series of hormonal signals to dampen the sympathetic nervous system. If the brain continues to perceive something as dangerous, the hypothalamus releases Corticotropin-Releasing Hormone (CRH), which travels to the pituitary gland, triggering the release of adrenocorticotropic hormone (ACTH). This hormone travels to the adrenal glands, prompting them to release cortisol. The body thus stays revved up and on high alert. When the threat passes, cortisol levels fall. The parasympathetic nervous system then becomes activated and dampens the stress response. Figure 1.2 is a representation of the multi-axial and temporal sequenced stress response model. As a result of heavy mobilization of the stress response axes (Box 1) target organ are activated. There is a potential for the activational overlap of each of these axes.The most common axes to be simultaneously active are the neuroendocrine and endocrine axes—both of which have potential for chronic responsiveness. If the adaptive responses generated by these axes are excessive in either intensity or chronicity, target organ dysfunction and/or pathology occur. These axes together form the General Adaptation Syndrome (GAS) and play a major role in manifestation of chronic diseases and dysfunction [97, 98].

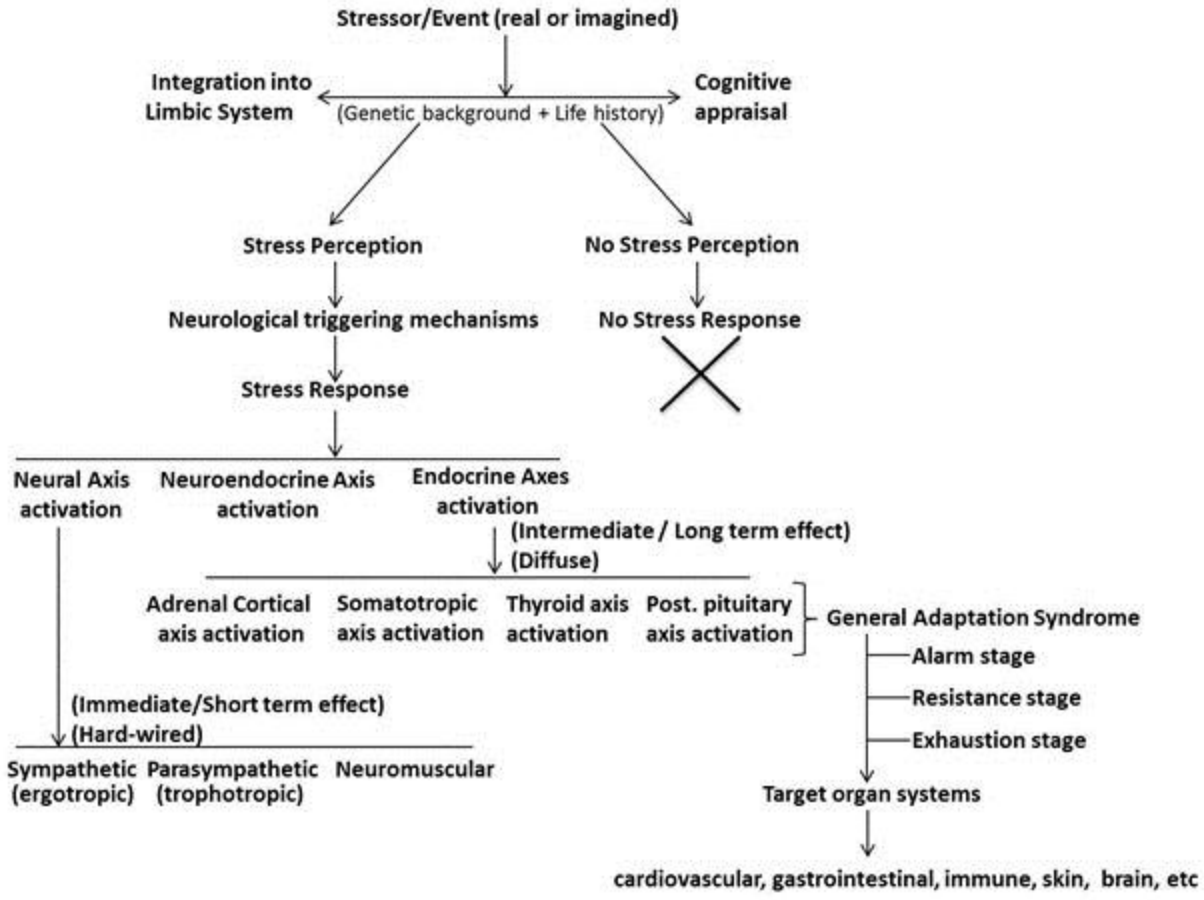


Figure1.2: Schema of multi-axial stress response

The GAS manifests in three stages. The first stage is called the Alarm Stage. This is characterized by stimulation of adrenal medulla dominated by epinephrine activity, followed by the activation of the endocrine axes. This stage is basically aimed at preparing the body for action by mobilizing energy reserves. If the stressor still exists, the body enters the second stage called the Resistance stage. In this stage, the body tries to regain its homeostasis by reducing the SAM and adrenal cortical activity. Here the stress response remains activated, but at less intensity than during the alarm stage. If the stressor is still present, the body enters the Exhaustion stage where its organ systems can no longer function normally. It is usually

characterized by enlargement of lymphatic structures and psychological exhaustion. In this stage, body's vulnerability to opportunistic disease and disorders of mal-adaptation increases[99].

The stress response follows three major efferent axes—neurological, neuroendocrine, and endocrine. The neurological axis is the most rapid stress axis causing immediate response to a stressful stimulus via direct neural innervations of end organs. **The neural axis** (shown in figure1.1) is the most direct of all stress pathways and follows either of the two descending circuitry- the posterior hypothalamus (in the case of a sympathetic activation) and the anterior hypothalamus (in the case of a parasympathetic activation)[100]. The neurological responses consist of rapid mobilization of the sympathetic, parasympathetic, and neuromuscular nervous systems. The brain also sends signals to the gut through command neurons, which in turn send signals to gut interneurons that carry messages into the gut viscera. Following neural stress response, the target organs are triggered for body functions that involve quick, brief actions, such as movement, sensation, and cognition.

The major effects of neural activation on target organs are short term and cease very quickly once the signaling ends, due to the limited ability of the sympathetic telodendria to continue to constantly release neurotransmitters during prolonged high stimulation. Therefore, in order to maintain high and sustained levels of stress response for chronic stressors, an additional activation of stress response axis is needed. The **endocrine system** represents another method of communication, where signals are sent by the endocrine organs that secrete **hormones** to be transported primarily via the bloodstream throughout the body, where they can bind to receptors on target cells and induce the desired characteristic response. As a result, endocrine signaling requires more time than neural signaling to trigger a response in target cells, though the effects last much longer.

The neuroendocrine axis (figure 1.3), also called as the Sympatho-Adreno-Medullary system (SAM, also known as the flight-or-fight response axis) is next to be mobilized[101]. Its activation leads to the excess release of catecholamines (80% epinephrine and 20% nor-epinephrine) from the adrenal medulla into the circulatory system. The effects of this axis are tenfold higher but functionally identical to direct sympathetic innervation except a lag of 20 to 30 second delay of onset for measurable effects. The reaction time of this axis is increased by utilizing the systemic circulation in addition to neuronal signaling as a transport mechanism. However, its effects range from intermediate to chronic in duration and may overlap with the last stress-response system - the endocrine axes.

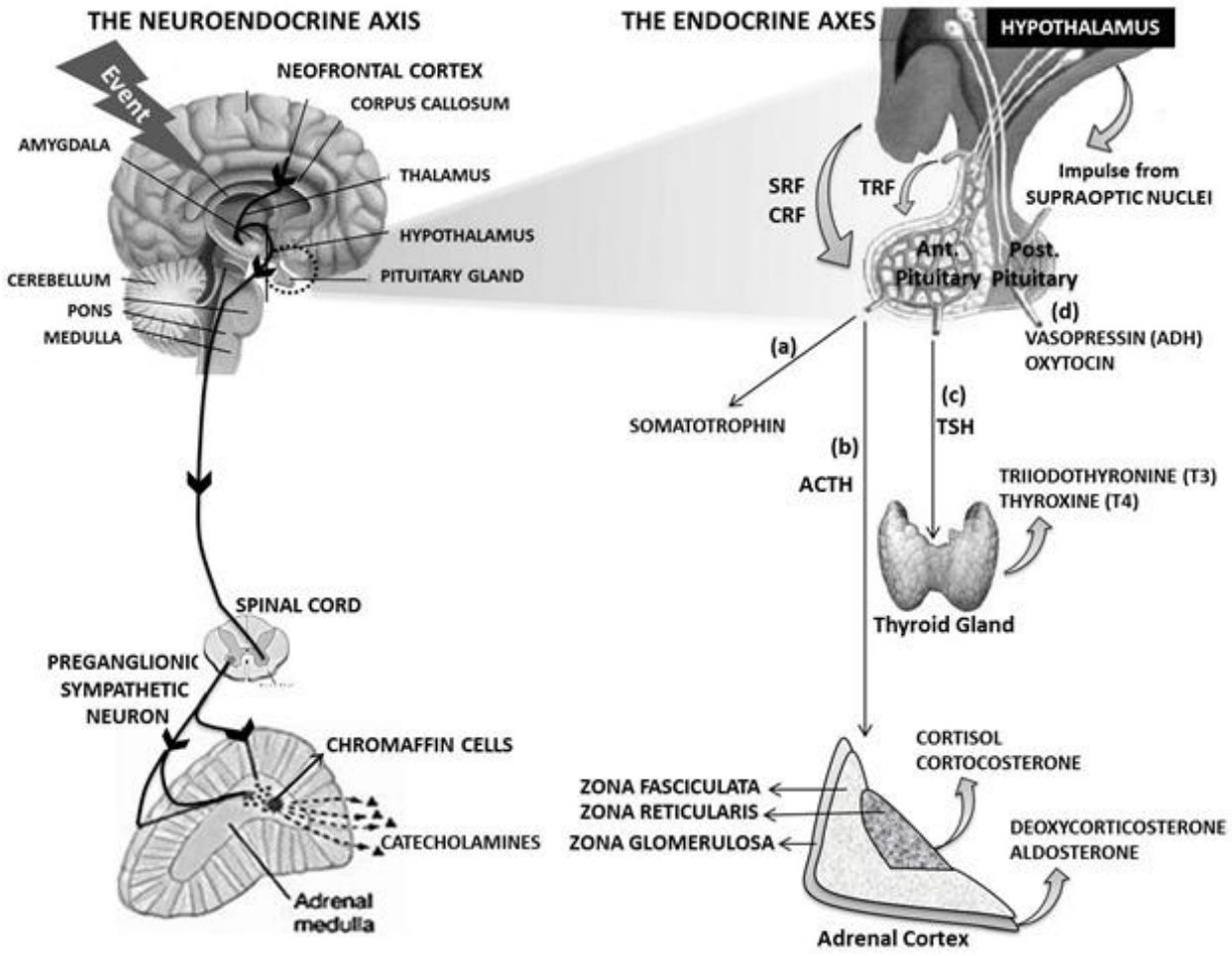


Figure 1.3: The Neuroendocrine and Endocrine axes of stress response

The endocrine axes (figure 1.3) are the final pathways to react to stressful stimuli, relying almost completely on the circulatory system for transportation[102]. A high intensity stimulus is needed to activate these axes consisting of somatotropic axis, hypothalamic-adreno-cortical axis, thyroid axis and posterior pituitary axis.

(a) **The somatotropic axis**

The anterior pituitary responds to the Somatotrophin Releasing Factor (SRF) by releasing growth hormone (somatotropin) into the systemic circulation[103]. Somatotrophin stimulates the release of the mineralocorticoids, increases mobilization of stored fats, free fatty acids and glucose, thereby producing a diabetic-like insulin-resistant effect [104-106].

(b) **The hypothalamic-adreno-cortical axis**

CRF signals the anterior pituitary to secrete ACTH, which causes release of mineralocorticoids and glucocorticoids from the adrenal gland. It is also called the passive coping system and becomes activate when active coping mechanisms fail. Aldosterone (prepotent mineralocorticoid) release results in increase in the absorption of Na^+ and Cl^- by the renal tubules[107] and a decrease in their excretion by salivary glands, sweat glands, and gastrointestinal tract, followed by subsequent fluid retention. Aldosterone also

increases glycogen deposits in the liver [108]and decreases circulating eosinophils[109]. The adrenal medullary and cortical activities can be highly separate, even inversely related.

(c)**The thyroid axis**
Thyroid Stimulating Hormone (TSH) released from anterior pituitary under influence of Thyrotrophin Releasing Factor (TRF) stimulates the thyroid gland to release two thyroid hormones: triiodothyronine (T3) and thyroxine (T4) which mostly remain in bound form in circulation. At the level of target cell tissue, only free hormone is metabolically active. The T3 and T4 hormones serve to participate in a negative feedback loop, thus suppressing their own subsequent secretion. Active thyroid hormone (T3) has been shown to increase general metabolism, heart rate, heart contractility, peripheral vascular resistance and sensitivity of some tissues to catecholamines [110, 111]. Hypothyroidism has been linked to depressive episodes. High thyroid hormone activity is reported to be involved in increasing oxidative stress[112].

(d) **The posterior pituitary axis.**
The posterior pituitary (neurohypophysis) receives neural impulses from the supraoptic nuclei of the hypothalamus. Stimulation from these nuclei results in the release of the hormones vasopressin (antidiuretic hormone, or ADH) and oxytocin into the systemic circulation [113, 114]. ADH affects the human organism by increasing the permeability of the collecting ducts that lie subsequent to the distal ascending tubules within the glomerular structures of the kidneys resulting in water retention. Oxytocin, the other major hormone found in the posterior pituitary axis role in the human stress response is currently unclear.

Box1: The Neuro-endocrine and endocrine axis of stress manifestation

1.2.5. Disorders of Energy Maladaptation

Allostatic load is defined as the cumulative toll of adaptive coping stress responses (various biological processes of the body are re-adjusted to cope with the demands of stress, and in long term accumulation of these abnormally functioning processes cost the overall well-being) on the body. A high allostatic load might result from chronic over-activation or inadequate activation of the stress response system. When a stressor arises, metabolic needs of most tissues increase, thus spiking the energy demands of the body. The combined catabolic activity of stress hormones allows the release of energy substrates and aid in maintaining fuel homeostasis. Stress hormones act to supply this energy, as they liberate energy by oxidative energy metabolism of substrates such as glucose, amino acids, glycerol, and FFA (free fatty acids).Glucose is the most readily available substrate in the body, and triggers the activation of metabolism regulator thyroid hormone (T3)[115].T3 induces increased substrate flux into the mitochondria for oxidation and

energy release. The increased energy demand of the body is met by increased oxygen consumption through increased rate of oxidation [116]. T3 also uncouples oxidation of substrates from ATP production, resulting in reduced ATP production, increased thermogenesis and basal metabolic rate[117]. To accommodate the ATP deficit thus created, more substrates are oxidized, resulting in weight loss. T3 promotes catecholamines to activate HSL, leading to increased lipolysis or fat mobilization[118].

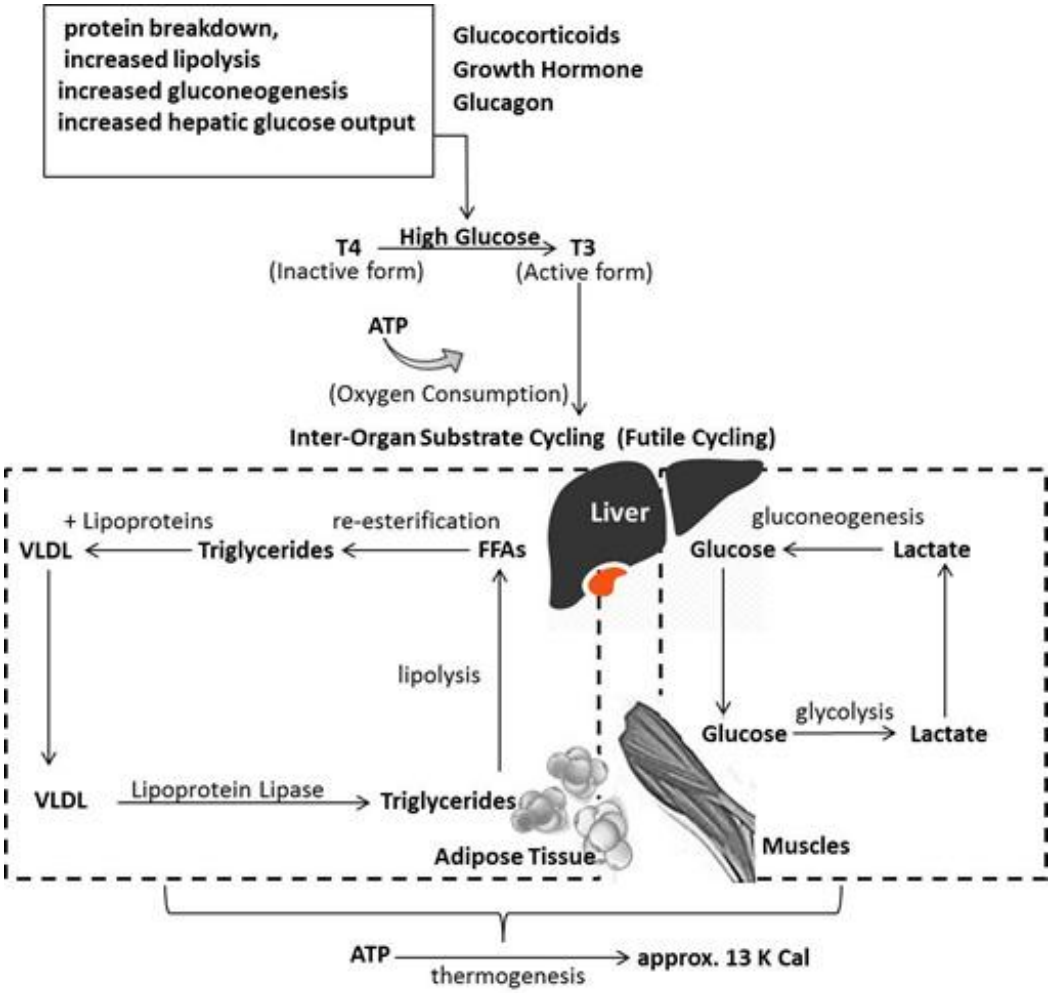


Figure 1.4: Inter-organ futile cycle triggered by body's energy sensing endocrine barometer, Thyroid.

It also increases beta 2 receptor density in adipose tissue and human skeletal muscle[112]. Fat deposits and muscle glycogen is hence more rapidly depleted, and less efficiently stored during hyperthyroidism, which may create muscle atrophy or cachexia (wasting syndrome)[119].

Studies have shown that glucose overload in stressed individuals activates inter-organ futile cycling [120] that utilizes the excess energy produced by the oxidative metabolic reactions by thyroid hormone (figure 1.4), which might further deteriorate the body's energy deficit. High blood glucose is already established to heighten the production of pro-inflammatory cytokines (IL6, TNFα, IL1β, IFNγ) [55, 121, 122], which are also potential activators of the energy-wasting futile cycles [123]. Thus, there is an oxidative metabolism overload mounted by endocrinal activity during stress response due to dysregulated energy metabolism[124].

Elevated levels of oxidative stress and inflammation have been implicated as contributing factors that link acute mental stress to endothelial dysfunction. Apart from several other environmental or genetic factors, oxidative stress (OS) leading to free radical attack on neural cells contributes calamitous role to neuro-degeneration. Though, oxygen is imperative for life, imbalanced metabolism and excess reactive oxygen species (ROS) generation end into a range of disorders such as Alzheimer's disease, Parkinson's disease, Epilepsy, Huntington's disease, and Multiple sclerosis and many other neural disorders. Prolonged or frequently occurring elevations of the catecholamines can also result in vasoconstriction in most systemic arteries and veins leading to allostatic alterations in cardiovascular responses. These alterations provide conditions favorable for the development of hypertension, endothelial dysfunction (impairment of endothelium-dependent vasodilation, also known as endothelial dysfunction), and may contribute to the development of arteriosclerosis. Oxidation of LDL in the vascular endothelium is a precursor to arterial plaque formation. Oxidative stress also plays a role in the ischemic cascade due to oxygen reperfusion injury following hypoxia. Micro vascular reactivity is affected by chronic stress through induction of transient endothelial dysfunction.Figure 1.5

summarizes the disorders that are triggered by deregulation of the balance between free radicals and anti-oxidants leading to accumulation of oxidative stress.

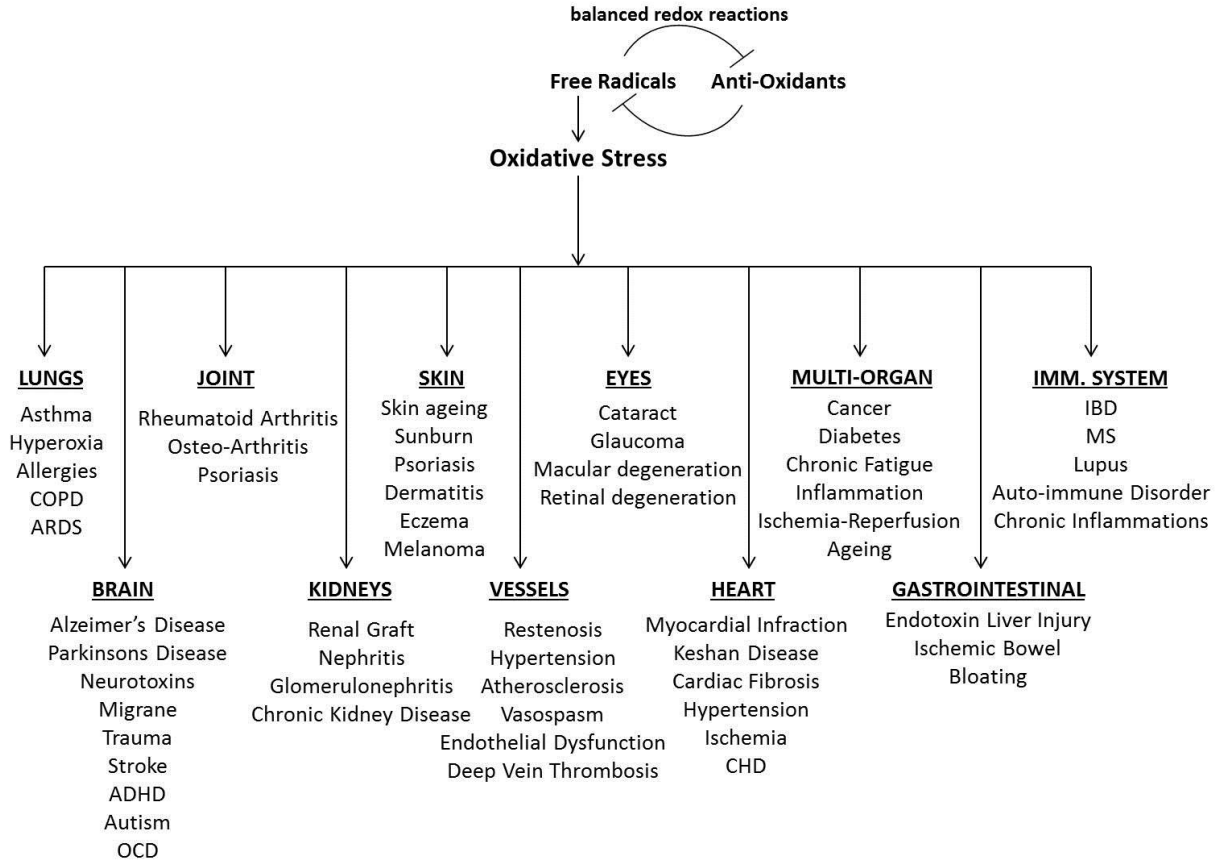


Figure 1.5: Disorders of mal-adaptation due to oxidative stress. (**Nb:**Refer the studies [125-145])

1.2.6. Quantification of Stress

Understanding the physiology of stress and its associated pathways in associated diseases is very important and requires quantification of stress as an important parameter in these studies. Since stress in an individual depends on his/her perception of the event as well as previous limbic priming, hence assessment of stress status of an individual based on stressor categorization is improbable. Although adaptive stress response hormones have been used to quantify stress of an individual, the robustness of these hormones as quantitative stress bio-markers is highly questionable. This is owing to the fact that some of these hormones have diurnal rhythmicity

(such as cortisol), while others may vary greatly due to the temporal nature of stress response which again depends on the stressor and individual perception. In addition to this, search for stable robust biomarkers for stress is also difficult in human, due to limitations on the study invasiveness. Hence, subjective assessment of stress status of humans has been done using the following tests:

1. Trier Social Stress Test (TSST) [146-148].
2. Perceived Stress Scale (PSS) is the most widely used psychological instrument for measuring the perception of stress. [149, 150].
3. Dexamethasone (Dex)-human corticotropin-releasing hormone (hCRH) test [151-153].
4. Visual analog scales: Five scales for visual analogues are established (viz. feelings of fatigue, relaxation, arousal, pressure, and tension), mood questionnaire and mood diaries [37, 154].
5. Profile of Mood States (POMS) : Six identifiable mood or affective states can be measured- Tension, Anxiety, Depression-Dejection, Anger-Hostility, Vigor-Activity, Fatigue-Inertia, Confusion, Bewilderment [155-157].

However, subjective assessment is always prone to bias and cannot be sometimes directly related to physiology.

1.2.7. Pilot study to identify unique molecular and behavioral markers of stress in human

Owing to the inaccuracy of individualistic methods solely by subjective assessment through questionnaire alone or with molecular profiling alone, we attempted to determine the combination of multiple strategies for identifying stress levels in normal random population of humans. A pilot study was undertaken in a cohort of random local population for identification

of probable molecular and/or behavioral symptoms of psychological stress. A questionnaire available online at http://oa.niser.ac.in/SMS/login.htm was used for preliminary subjective quantification of stress in these individuals and their blood samples were collected for characterizing their metabolite and proteomic profiles. We aimed to evaluate the natural stress of individuals as a result of dealing with regular chores, rather than evaluating stress status by constituting experimental and artificial stress situation for a group of individuals; and to see if methodologies we employed are sensitive enough to identify and evaluate stress status. Based on questionnaire scoring (Q-Scores), the cohort could be divided to 3 major groups which we named stressed(S), non-stressed (NS) and borderline (BL) based on the cumulative Q-score of each individual. Similarly, metabolite scoring (M-score) was determined by taking sum of the areas under the normalized peaks which were assigned to specific metabolites by annotating using Human Metabolome Database (HMDB) available at http://www.hmdb.ca/. The individuals were grouped independently based on their Q-Scores into three groups A, B, C. It was found that the pattern of Q and M scores for each individual had positive correlation for 82 out of 124 individuals, where positive association indicates that a high Q-score corresponds to high M-score for an individual.

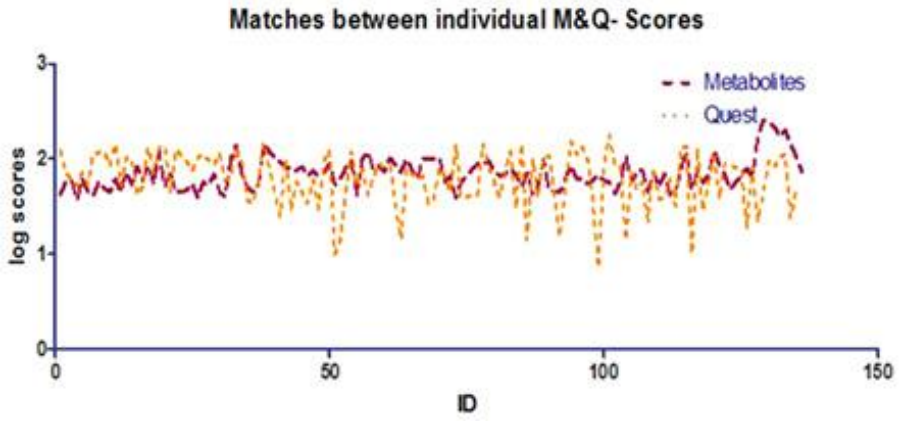


Figure1.6: Variation in normalized scores between questionnaire and metabolite scores for each individual

A double blind study performed for questionnaire and metabolite profiles with grouping based on distribution plots for these 82 individuals showed that the groups becomes tighter with increased contribution of each function to the distinctiveness of the groups, hinting at correlation of metabolite scores with stress levels.

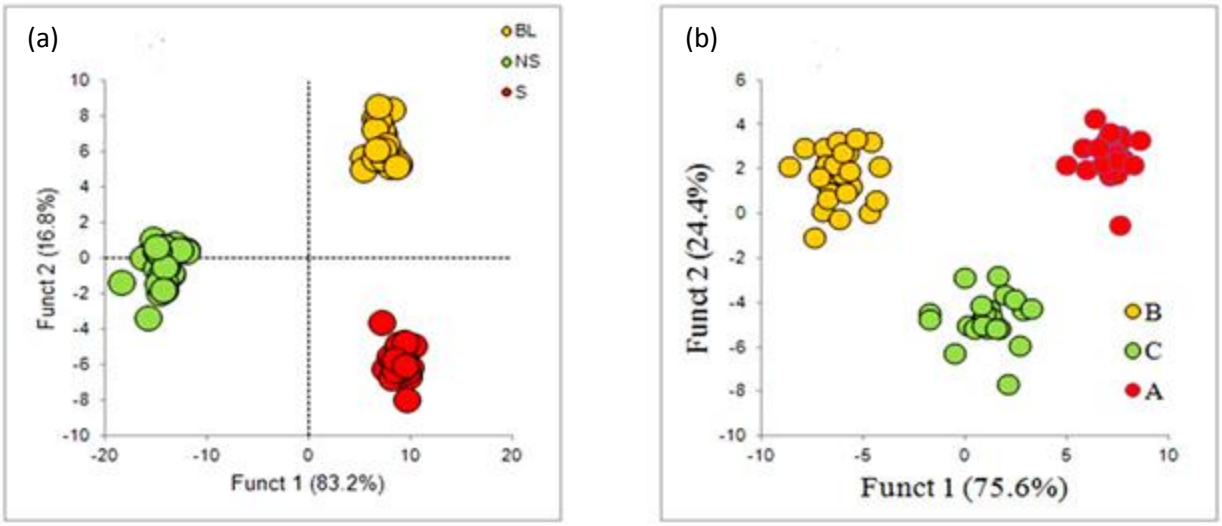


Figure 1.7: Comparison of questionnaire and metabolite data. Discriminant analysis of a) questionnaire and b) metabolite based profiles where individuals were assigned to S, NS, BL groups and A, B and C groups on the basis of questionnaire score and metabolite scores respectively.

With the background that stressed individuals show a distinct molecular and questionnaire profile pattern, an analysis of significant metabolite peaks showed that 41 potential metabolites were could contribute to the differences between these groups. 18 out of a total of 41 potential metabolites were found to be associated either directly with PS or with PS associated disease. Interestingly, 16 of these 18 metabolites were reported to be up-regulated in diseased states [65, 89, 158-176]. This was indicative of an increase in overall abundance of metabolites associated with stress. Ten out of 13 metabolites(viz. 6-Phosphogluconic acid, Aminoadipic acid, D-Arabitol, Cysteine, Sorbitol, D-Fructose, Threonic acid, 2-Methylglutaric acid, Chenodeoxycholic acid, L-dihydroorotic acid), which were upregulated in stress (S) condition compared to BL and NS, were already reported in the literature in association to stress [73, 177-

185]. In questionnaire profiles, the question markers, which were key to the differences between groups correspond to both physiological and psychological aspects, include anxiety, feeling of depression, disinterested or disinclined to do things, moody, jealous based on psychological perspective. From physiological perspective examples include excessive sweating of palms and hands, increased or decreased appetite, struggle to overcome minor sickness, dry mouth. All these factors have been correlated with PS and hence justify the assessment by the questionnaire as is also justified by the consistency in the patterns of up-regulation or down-regulation of molecular profiles that makes stressed group distinctive of NS and BL groups. This study thus clarified and laid down preliminary evidences to the fact that stress initiates certain changes at the molecular level as well as neurological level in terms of perception of a situation and established the effectiveness using combination of subjective and quantitative strategies (such as questionnaire and metabolite profiling in this case) to assess of stress levels in humans.

1.2.8. Nature of studies involving psychological stress

A number of clinical trials have also been conducted for accessing effects of psychological stress on various aspects of physiology including behavioral, psychological parameters as well as on various stress-associated diseases. Associations between psychological stress and disease have been established, particularly for depression and other behavioral and mental disorders, neurological disorders, and general symptoms and pathologies associated with stress (figure 1.8).Recently due to identification of stress and a major modulator of body's energy homeostasis, these clinical trials have also shifted focus to other diseases like metabolic, endocrine and circulatory disorders. Other areas in which evidence for the role of stress is beginning to emerge include upper respiratory tract infections, asthma, viral infections, autoimmune diseases, cancers and reproductive disorders (see Annexure 1 for details). Evidence derived from these studies

provides support for stress as an important factor in certain diseases but cannot establish a causal relationship. However, the these studies conclusively support experiments carried out in laboratory conditions on various models systems (*in vitro* and *in vivo*) regarding the effects of stress on disease susceptibility; with results showing that stress is capable of regulating various disease-relevant physiological processes. This consistency of research findings strongly supports the hypothesis of a causal link of stress with these physiological processes and disease susceptibility.

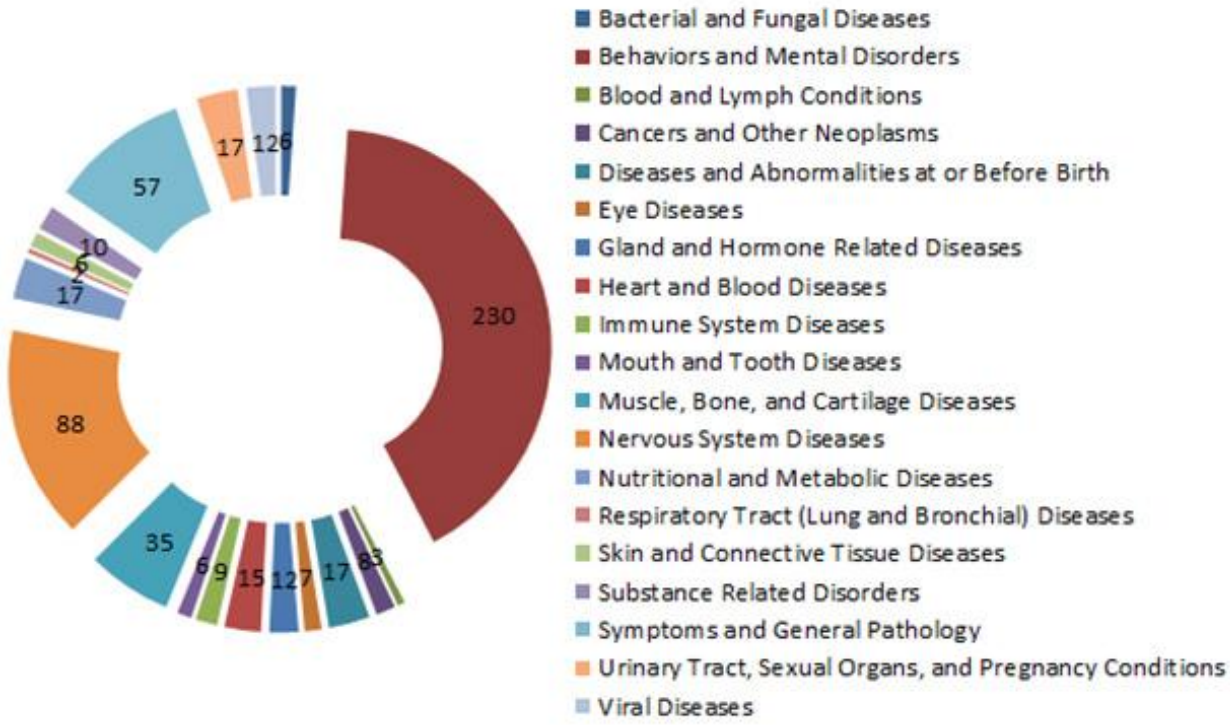


Figure 1.8: Distribution of clinical trials aimed at studying association of psychological stress with various diseases/disorders and stress pathology. Each fragments represents the number of clinical trials done with psychological stress as a condition done in each category as obtained from NIH (National Inst. of Health) resources.

1.2.9. Need for data-integration

The paucity of our understanding even in the face of recently growing research advances in psychological stress owing to its pathogenic consequences on health, when accounting for causal genes and their causative functional relationship with stress reflects a lack of integrative analysis.

With the amount of biomedical literature regarding the identification of genes related to stress-etiology and associated disorders on the rise, one of the major bottle necks in this domain face is that huge amount of relevant information remains hidden in the unstructured text of published literature. Given that these experiments have been performed in different animal model systems, and experimental conditions, there is a clear and present need for mining and assembling the information for creation of comprehensive data that clearly represent the associative links among these genes or between diseases/phenotypes or physiological functions and these genes. Classically identification of new genes related to a particular disease/phenotype or physiology required laboratory experiments, which required huge resources in terms money, time and manpower. These data, which we have created in this work and represented in the form of gene-association networks, provide critical leverage to the process of identification and discovery of new gene functions related to stress and associated pathological biological processes by providing a platform for making predictions, generating hypothesis and targeted experimentation; thereby cutting on the resources that would have been required otherwise if the traditional methods would have been used. In fact, to maximize the impact on the predictive power of the model, the experimental focus and type can be decided based on current experimental model drawbacks. The implications of this type of studies is transformative and spans discovery of gene functions and their emerging functional mappings at physiological level, generating and testing predictable hypotheses in well-defined simulated environments, guided experimentation, and accelerates the in-depth understanding of complex interconnected and interdependent of biological processes. Concomitantly, research advances in the computational front have reached the level of maturity needed for the analysis and integration of these datasets.

Chapter2

ESTABLISHMENT OF SYSTEMATIC-ANALYSIS METHODOLOGY THROUGH GENE-NETWORKING

2.1. Effect of psychological stress on innate immunity and metabolism: A systematic analysis

We developed strategy to establish the much required connectivity among the scattered information through an unique systematic-analysis approach which required three major steps: 1) Screening of existing data in the published literature relevant to the biological contexts of interest (namely innate immunity and metabolism), 2) Building of an association between the elements of screened data, and 3) Analysis of the screened data based on the relationship between innate immunity and metabolism in the context of psychological stress. The entire report is based on the results of our systematic-analysis of literature in this area to understand the inter-dependence and balance between innate immune and metabolic processes when perturbed by psychological stress. The uniqueness of this method lies in use of association-networks generated by dual-mining techniques (data-mining and text-mining, see materials and methods) at systems level as an approach to explore the functionality and relationship between the genes involved in the present context. Drawing any biologically relevant information from these networks could help us identify certain important patterns hitherto hidden and subsequently help us in designing newer strategies to establish the relationship experimentally. Information in the current report could be used for elucidating any possible predictive mechanisms which would serve a critical role, in part or whole, in answering key biological questions pertaining to human physiology.

2.1.1. Materials and Methods

Efficient screening of data requires extracting data from both structured repositories (databases) as well as from unstructured textual documents. Powerful exploratory techniques like data-mining which apply machine learning, neural networks, genetic algorithms and statistics, etc. is used for automatic discovery of patterns from structured databases. While for handling

unstructured data, text-mining comes in handy since it uses language-based techniques, such as semantic analysis and taxonomies to parse textual data and identify patterns. A number of data-mining and text-mining tools exist that cover different aspects of information extraction from databases and from biomedical literature in a variety of domains but, none of these are customized for an integrated approach that combines both the mining techniques to extract information in relation to specific contexts and also build associations from the extracted terms at the same time. We have taken a unique dual approach in combining data-mining and text-mining techniques which could overcome their individual short-comings and also at the same time build associations among these screened elements, thereby forming association networks.

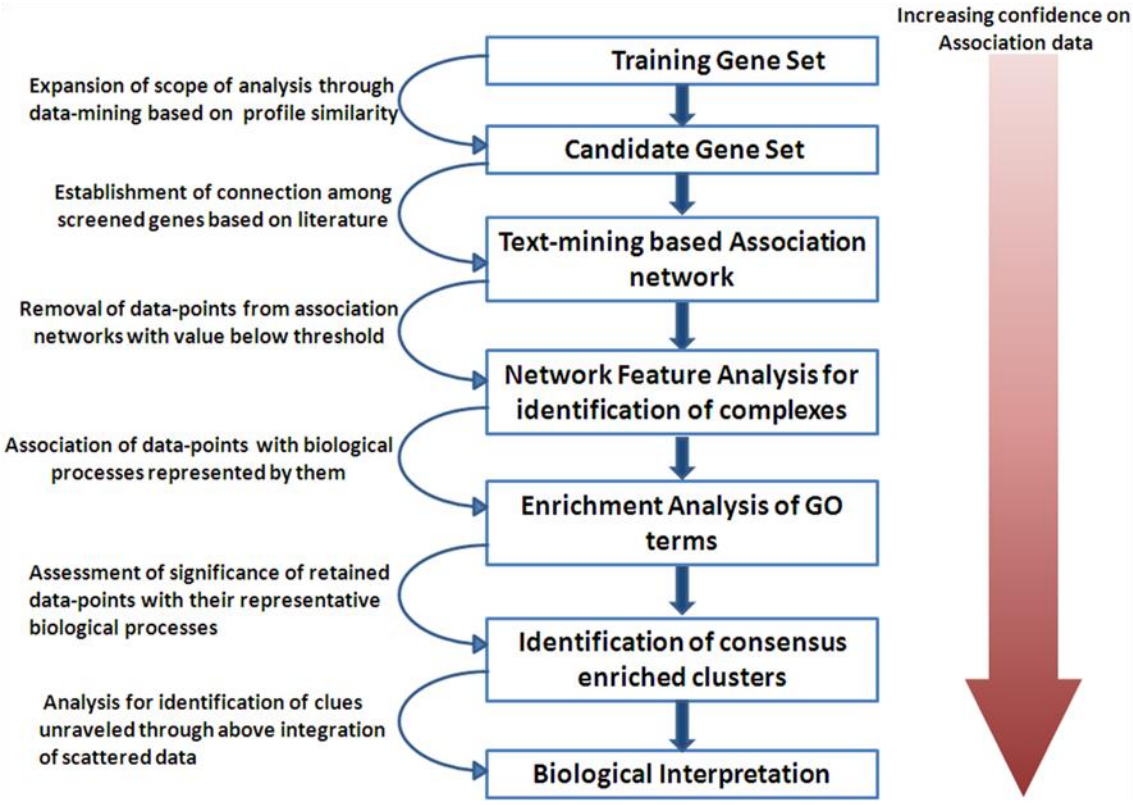


Figure 2.1: The step-by-step methodological schema of systematic analysis.

Drawing any biologically relevant information from these networks requires a detailed analysis which could help us identify hidden patterns in the huge data-sets created by the dual-mining

techniques. We have focused on a bi-facetted analysis of the networks in 1) identifying biological modules through topological distinctions by analyzing the gene association network structure 2) carrying out an enrichment analysis for identification of over-represented Gene Ontology terms. This was then followed by an analysis which identifies any correlation between these modules and the over-represented GO-terms. The overall schematic for the approach developed is presented in figure 2.1.

2.1.1.1. Data-mining

Endeavour freely available at http://homes.esat.kuleuven.be/~bioiuser/endeavour/index.php was used for identifying the candidate genes from whole genome based on their similarity/association with the "training set". The analysis initializes by gathering all information about the training set genes to build a model by consulting the following data sources: A, literature (abstracts in EntrezGene); B, functional annotation (Gene Ontology); C, microarray expression (Atlas gene expression); D, EST expression (EST data from Ensembl); E, protein domains (InterPro); F, protein-protein interactions (Biomolecular Interaction Network Database or BIND); G, pathway membership (Kyoto Encyclopedia of Genes and Genomes or KEGG); H, cis-regulatory modules (TOUCAN); I, transcriptional motifs (TRANSFAC); J, sequence similarity (BLAST); K, additional data sources, which can be added (e.g., disease probabilities)[186]. We used whole human genome as the test-set against which the annotation profile of the training-set genes has to be compared. Vector-based data are scored by the Pearson correlation between a test profile and the training average, whereas attribute-based data are scored by a Fisher's omnibus analysis on statistically overrepresented training attributes. These test genes are then ranked based on their similarity with the training properties, which results in one prioritized list for each data source. The rankings from the separate data sources are then fused into a single ranking which provides

an overall prioritization for each test gene. The top ranking genes were used for text mining and association network generation.

2.1.1.2. Association network generation and network feature analysis

Agilent Literature Search plugin in Cytoscape was used for text-mining literature using structured queries and generating gene-association networks based on the text-mining results. The literature search plugin allows the user to control the search options, and provides for custom-made query construction so that the biological relevance of the context can be tightly regulated. One network was generated each for innate immunity, metabolism, innate-immunity and psychological stress, and metabolism and psychological stress. Four different query structures were used for generation of these four primary gene association networks. Biologically relevant contexts were provided as context terms in the context panel, while the genes from the candidate gene-set were used as search terms. The query structures thus built are as follows:

Innate Immunity:
Gene name AND (("innate immunity" AND (Homo sapien OR human)) OR (inflammation AND (Homo sapien OR human)))

Metabolism:
Gene name AND (("metabolism" AND (Homo sapiens OR human)) OR ("metabolic syndrome" AND (Homo sapiens OR human)))

Innate Immunity and Psychological Stress:
Gene name AND (("innate immunity" AND "psychological stress" AND (Homo sapiens OR human)) OR (inflammation AND "psychological stress" AND (Homo sapiens OR human)))

Metabolism and Psychological Stress:
Gene name AND (("metabolism" AND "psychological stress" AND (Homo sapiens OR human)) OR ("metabolic syndrome" AND "psychological stress" AND (Homo sapiens OR human)))

The query set is submitted to selected search engines, for example PubMed or OMIM. The resulting documents are fetched, parsed into sentences, and analyzed for known interaction terms, like 'binding' or 'activate'. Agilent Literature Search uses a lexicon set for defining gene

names (concepts) and aliases, drawn from Entrez Gene, and interaction terms (verbs) of interest. An association is extracted for every sentence containing at least two concepts and one verb. Associations are then converted into interactions with corresponding sentences and source hyperlinks, and added to a Cytoscape network [187].

For generation of networks as union or intersection derivates of these primary networks, **Advanced Network Merge plugin** was used in Cytoscape. The force-directed layout tends to expose the inherent structure of the network in way that facilitates identification of clusters of tightly connected nodes which suggest functional modules and hub nodes which have many interactions and mainly represent functionally important genes. But, it doesn't eliminate unconnected or sparsely connected nodes. This might lead to many false positive predictions due to false positives in the original network. MCODE (Molecular Complex Detection) plugin of Cytoscape was used for solving this issue and finding regions with significant local density to identify complexes for predictive assignment of function to unknown nodes as a part of the complex. MCODE uses a method which allows retention of an edge in the network only if the given edge is among the highest scoring candidate edges for both genes [188]. This is referred to as the "top overlap" method and it helps in overcoming the issue of false positives. The significant modules i.e. modules with MCODE score more than 2.5 and minimum 4 nodes have been taken into analysis for functional annotation.

Network Analysis plugin was used for measuring the node degree and the clustering co-efficient of the nodes in these networks after applying clustering algorithm to them. It has provisions for visual representation of node degree (the number of connections per gene in a network) and how it relates to function.

2.1.1.3. Enrichment Analysis

Gene Ontology enrichment anaLysis and visualization tool freely available at the website http://cbl-gorilla.cs.technion.ac.il/ was used for this analysis. For the kind of analysis we are doing, GORILLA uses a statistical framework called hyper-geometric model and performs the enrichment analysis on thousands of genes and thousands of GO terms in a few seconds and provides a DAG representation of the GO hierarchy, emphasizing on the enriched nodes [189]. This graphical representation is color coded based on the p-value attained for each GO term. Comparison of the genes associated with metabolism, innate-immunity and psychological stress with a background of whole genome would give a wrong representation of the involvement of these genes in psychological stress in terms of Gene Ontology enrichment. To sort out this issue, innate immunity and metabolism genes associated with psychological stress were compared against a background that consisted of complete set of genes associated with innate immunity and/or metabolism irrespective of their association with psychological stress. GO terms with minimum enrichment p-value 10^{-7} were included in the analysis.

2.1.2. Results

2.1.2.1. Identification of Candidate Genes and Generation of Association Network

Top 100 and 200 genes from the candidate gene set for innate immunity and metabolism were used in the generation of text-mining based association networks II and M respectively. A view to the topologies of the first four primary association networks is shown in figure 2.2and their network statistics are given in table 2.1.The table contains details of the first four primary association networks obtained by text-mining results using Agilent Literature search (ALS) plugin. ‘Total references’ refers to the total number of literature references used by the ALS

plugin to generate the networks, 'Unique references' refer to the number of references that were used only once for the network generation while 'Duplicate references' refer to the number of references used more than once for network generation. 'Network clustering coefficient' is the average of the clustering coefficients for all nodes in the network and 'average number of neighbors' indicates the average connectivity of a node in the network.

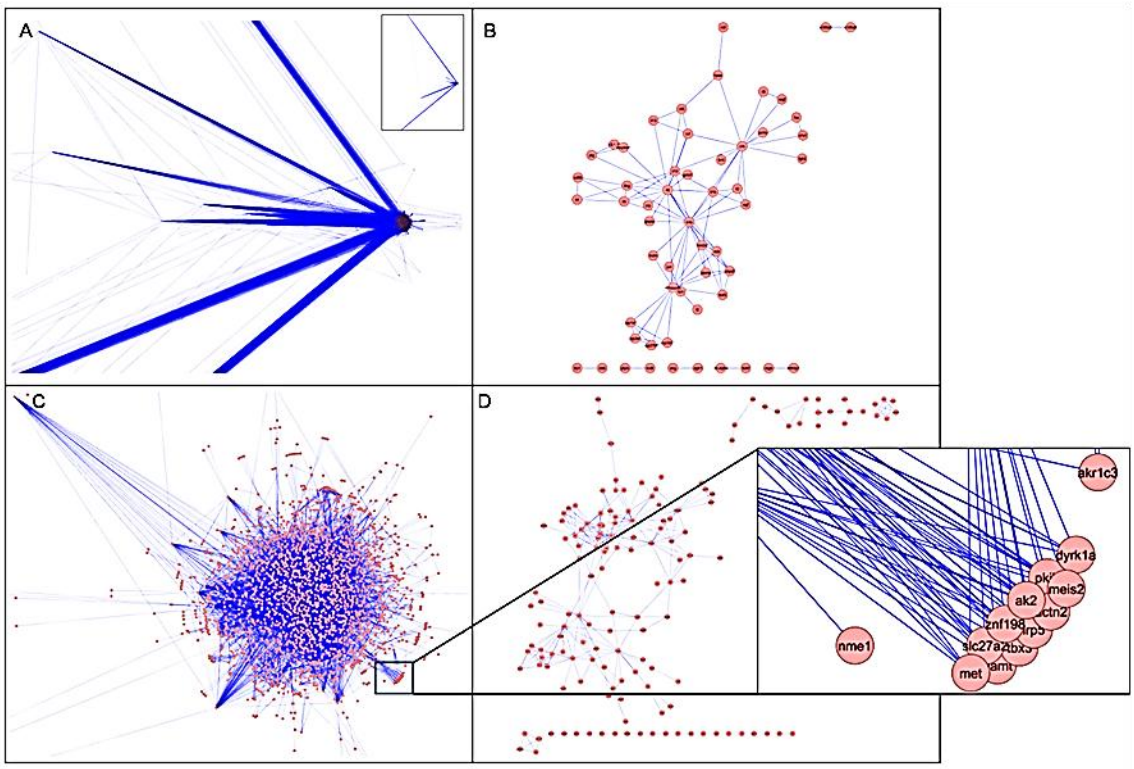


Figure 2.2: Network topologies of the text-mining association networks obtained by using ALS plugin. The networks consist of genes associated with A) Innate Immunity, B) Innate immunity and psychological stress, C) Metabolism and D) Metabolism and psychological stress

Text-mining association networks thus generated, do not contain experimentally determined interactions, rather these have more general association types and offer an alternative network source where interaction data are limited [187].

Table 2.1: Details of reference distribution and network statistics of the four primary association networks

	Reference distributions				Topology feature details				
Network	**Total refs**	**Unique refs**	**Duplicate refs**	**Network Confidence**	**Nodes**	**Edges**	**No. of isolated nodes**	**Avg. num. of neighbors**	**Clustering co-efficient**
Metabolism, M	7765	7052	713	0.7059	2108	9833	0	9.329	0.528
Innate Immunity, II	11411	8153	3258	0.5763	1721	13989	0	16.257	0.581
Metabolism & Stress, M(S)	328	217	111	0.0298	148	314	0	4.243	0.452
Innate Immunity & Stress, II(S)	1155	1038	117	0.0586	55	102	0	3.709	0.476

As can be seen from the reference distribution shown in figure 2.3, the network confidence(C) for **M(S)** [metabolism and psychological stress] and **II(S)** [innate immunity and psychological stress] networks is very less compared to that of **M** [metabolism] and **II** [innate immunity] networks respectively.

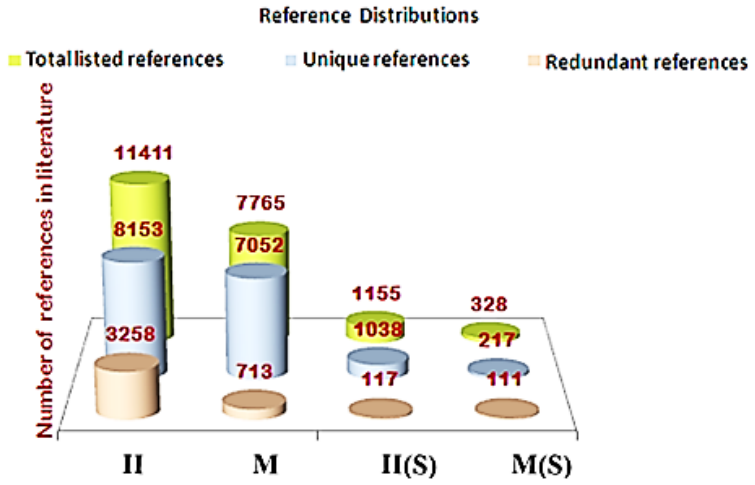


Figure 2.3: References distribution used by the Agilent literature Search plugin for generation of the four primary networks.

This is due to the keyword "psychological stress" that is used as an additional filter which indicates that very fewer studies have been done on psychological stress in relation to innate immunity and metabolism. This, together with the assessment of the screened references revealed that keyword search-based text-mining didn't draw non-contextual data-points and thus this technique adds on to the confidence on the approach adopted by us so far. It was also

interesting to note that even though **II** network contained less number of nodes compared to the **M** network, the number of edges is more for **II** network. This indicates that either the nodes representing genes in **II** network are multifunctional, or literature present in relation to the nodes in **II** network is more; but since the unique reference distribution doesn't reflect significant differences between **II** and **M** network (**II**-8153, **M**-7052), the chance that genes which have been so far associated with innate immune functions of being multi-functional is more likely.

2.1.2.2. Network analysis

Identification of modules provided another level of functional annotation above the guilt-by-association methods used for generation of text-mining based association network [188][190], where, any false positive association pulled out could lead to faulty functional annotation of those genes. Figure 2.4shows the network topologies of the derived networks i.e. **II** $\cap$ **M**, **II(S)** $\cap$ **M(S)**, **II** $\cup$ **M** and **II(S)** $\cup$ **M(S)** after applying clustering algorithms and making normalizations for proper representation of node degree and clustering-coefficient in these networks.It can be seen that data points with lower confidence have been removed; leaving only those nodes and edges that participate in cluster formation (their retention is owing to their high scores) for functional analysis.Table 2.2 represents the number of nodes, edges and number of clusters in each network after clustering and normalization to significant values. The networks derived as union functions have been allowed to retain clusters with minimum MCODE score 1.25, while all the clusters have been retained in the networks derived as intersection functions.

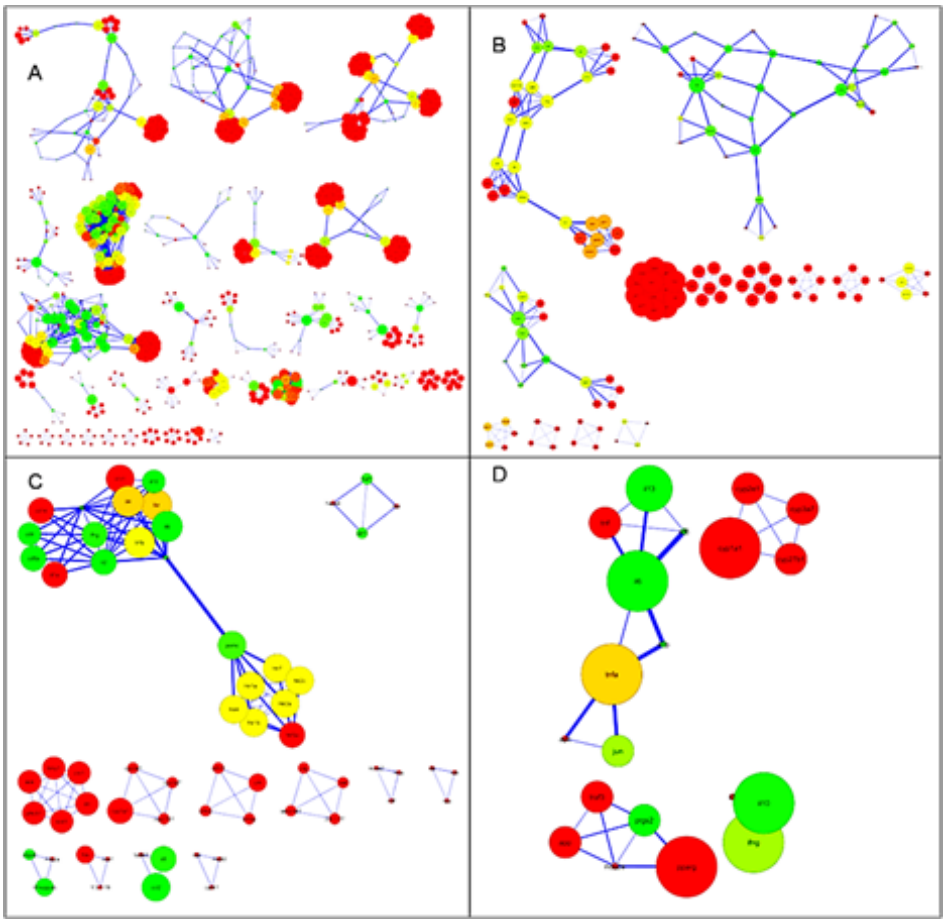


Figure 2.4. Network topologies of stress (immunity and metabolism) networks.

A) II ⋃ M network contains clusters with a minimum MODE score of 1.5,
B) II ⋂ M network contains clusters with a minimum MCODE score of 1.25,
C) II(S) ⋃ M(S) and
D) II(S) ⋂ M(S) networks contains all clusters.

Larger nodes represent higher node degree, while warmer colors represent higher clustering co-efficient, where warmer to cooler colors are represented by a gradient from Red to Green.

Table 2.2: Network statistics of the derived networks reflecting the effect of clustering.

Network	Nodes		Edges		Clusters
	Before clustering	After clustering	Before Clustering	After clustering	
II∪M	2904	639	22581	3413	30
II(S)∪M(S)	158	562	341	143	7
II∩M	925	89	1241	265	8
II(S)∩M(S)	45	20	75	29	4

Enrichment analysis of **II ⋂ M** against **II ⋃ M** background showed the GO terms enriched due to genes that are associated both with innate immunity and metabolism (irrespective of their association with psychological stress), but genes that have extensive reported implications in relation to psychological stress and lack enough evidence for their association with innate

immunity and metabolism wouldn't be presented in the enriched terms. This called for an enrichment analysis of **II(S)⋃M(S)** against **II⋃M** background to identify such genes and it revealed that among all other genes CNR1, HTR1B, HTR2A, CCL2 were consistently present in most of the enriched terms. Enrichment analysis of **II⋂M** (enriched GO biological processes represent genes involved in regulating both innate-immunity and metabolism only) and **II(S)⋃M(S)** (enriched GO biological processes represent genes common to both innate immunity and metabolism and at the same time are associated with psychological stress) against a background of **II⋃M** revealed certain specific consensus clusters that were consistently enriched (shown in figure 2.5).

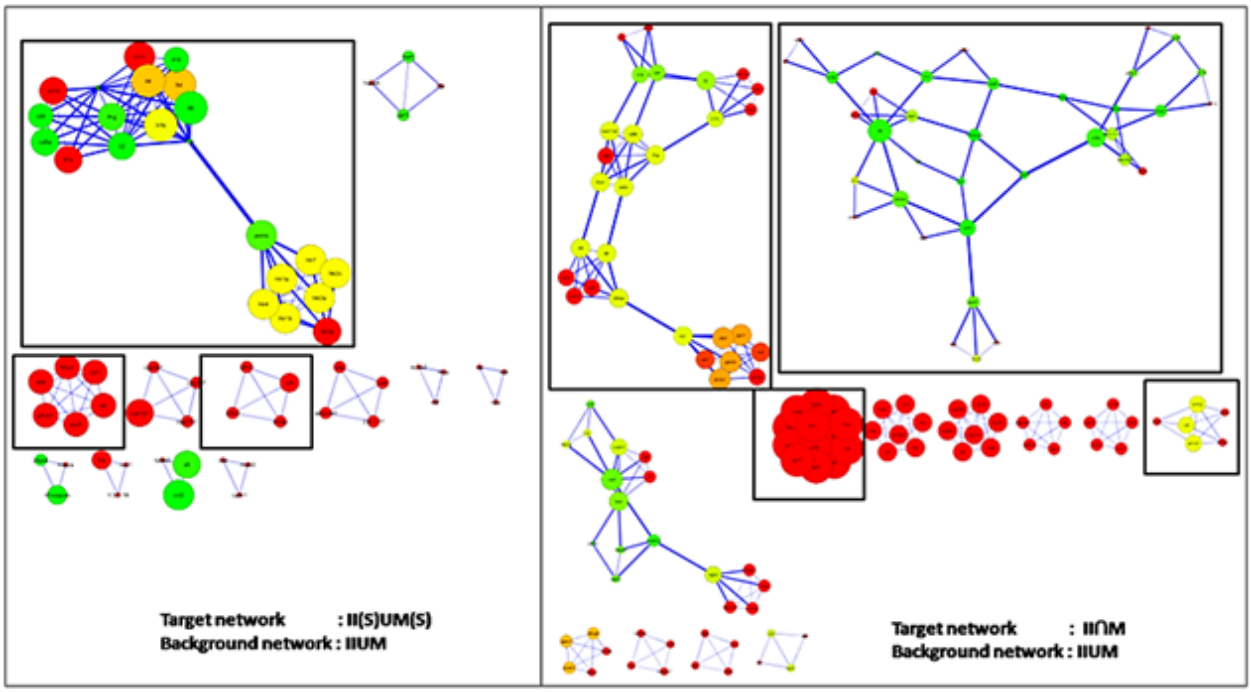
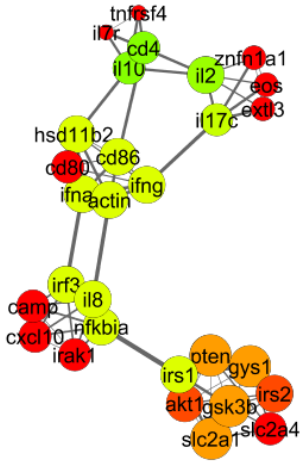
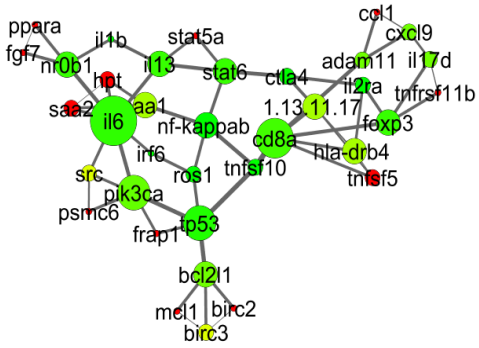


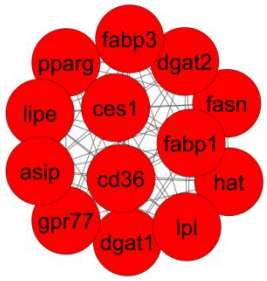
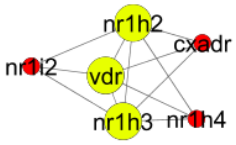
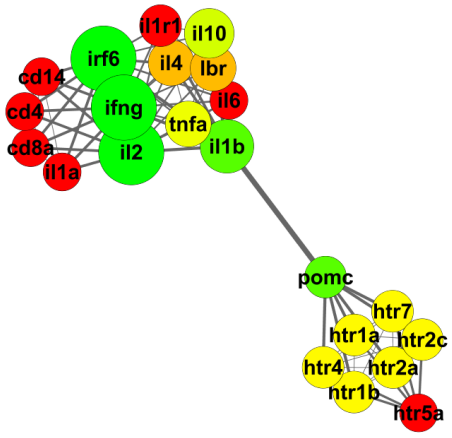
Figure 2.5: Consensus enriched clusters after enrichment analysis shown in boxes in the networks II(S)⋃M(S) network and II⋂M network.

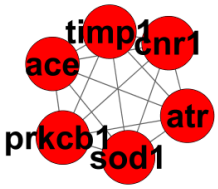
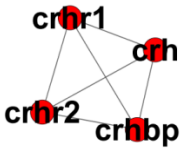
The details of the enrichment terms and genes associated with these clusters are shown in table 2.3.This table contains details of the consensus clusters (consistently populated by majority of the significantly enriched GO terms) in the enrichment analysis of genes populating II∩M and

II(S)∪M(S) networks against a background of II∪M network; where a minimum of 10^{-7} enrichment p-value is set as limit of significance for our analysis. The clusters have been named according to their original ranks in MCODE clustering analysis.

Table 2.3 Enrichment details of the consensus enriched clusters

	Network and Cluster Name	Cluster	Genes Involved	GO terms enriched
1.	**Target:** II∩M **Background:** II∪M Cluster 4		actin, irs2, eos, irak1, cxcl10, nfkbia, slc2a4, cd80, extl3, tnfrsf4, il2, cd4, IFNγ, znfn1a1, gys1, cd86, hsd11b2, irs1, pten, il8, akt1, gsk3b, slc2a1, il17c, il7r, il10, irf3, ifna1, camp	-Regulation of protein secretion - Positive regulation of cell activation - Regulation of Immune response - Positive regulation of leukocyte activation -Positive regulation of lymphocyte activation -Positive regulation of lipid metabolic process - Regulation of fatty-acid B-oxidation
2.	**Target:** II∩M **Background:** II∪M Cluster 10		cxcl9, hpt, src, saa1, adam11, hla-drb4, mcl1, tnfsf5, tnfsf10, 1.13.11.17, irf6, nf-kappab, il6, ros1, tp53, fgf7, ppara, il17d, cd8a, , stat6, saa2, il13, il1b, ctla4, birc3, foxp3, smc6, bcl2l1, frap1, stat5a, pik3ca, tnfrsf11b, ccl1, il2ra, nr0b1, birc2	-Regulation of protein secretion - Positive regulation of cell activation - Regulation of Immune response - Positive regulation of leukocyte activation - Positive regulation of lymphocyte activation - Regulation of lipid storage - Regulation of cholesterol storage - Positive regulation of lipid metabolic process -Regulation of fatty-acid B-oxidation

3.	**Target:** II∩M**Background:** II∪M Cluster1		asip, lipe, lpl, PPARγ, fasn, dgat1, fabp3 gpr77, cd36, ces1,dgat2, hat, fabp1	-Regulation of Immune response - Positive regulation of leukocyte activation - Positive regulation of lymphocyte activation - Regulation of lipid storage - Regulation of cholesterol storage - Positive regulation of lipid metabolic process - Regulation of fatty-acid B-oxidation
4.	**Target:** II∩M **Background:** II∪M Cluster8		nr1h2, nr1i2, vdr, nr1h4, nr1h3, cxadr	-Regulation of protein secretion -Positive regulation of cell activation -Regulation of Immune response -Positive regulation of leukocyte activation - Positive regulation of lymphocyte activation - Regulation of lipid storage -Regulation of cholesterol storage -Positive regulation of lipid metabolic process
5.	**Target:** II(S)∪M(S) **Background:** II∪M Cluster1		htr5a, il1b, il2, htr1b,htr2c, il10, pomc, il6, cd4, il1a, htr4, il1r1, IFNγ, htr7, irf6, htr2a,tnfα, cd14, cd8a, il4, lbr, htr1a	-Regulation of systems process - Regulation of systems neurological process - Negative regulation of multicellular organismal process - Positive regulation of protein secretion - Regulation of immunoglobin - Response to temperature stimulus - Behaviour -G-protein coupled receptor protein signaling pathway

6.	**Target:** II(S)∪M(S) **Background:** II∪M Cluster2		Ace, sod1, atrcnr1, timp1, prkcb1	-Regulation of systems process -Regulation of systems neurological process -Negative regulation of multicellular organismal process -Positive regulation of protein secretion -Regulation of immunoglobin -Response to temperature stimulus -Behaviour -G-protein coupled receptor protein signaling pathway
7.	**Target:** II(S)∪M(S) **Background:** II∪M Cluster4		Crh, crhr1, crhr2, crhbp	-Regulation of systems process - Regulation of systems neurological process - Negative regulation of multicellular organismal process - Positive regulation of protein secretion -Regulation of immunoglobin - Response to temperature stimulus - Behaviour -G-protein coupled receptor protein signaling pathway

Detailed analysis of these consensus enriched clusters highlighted tight sub-clusters populated with high MCODE score-bearing genes within select few clusters as shown in figure 2.6. It was seen that in cluster 1 of **II(S)⋃M(S)** network (refer table 2.3), a tight sub-cluster was formed by the genes coding for inflammatory cytokines (marked as '**a**'), which was connected to another sub-cluster formed by 5-HT receptor family genes (marked as '**b**') via POMC gene. Similarly, GSK3, SLC2A1, AKT1, IRS1, IRS2, PTEN, GYS1 formed a tight sub-cluster (marked as'**d**'),

while HSD11b2, IFNa1, ACTIN, CD80, CD86 and IFNγ formed another tight sub-cluster (marked as '**c**') in cluster 4 of **II** ⋂ **M** network (refer table 2.3).

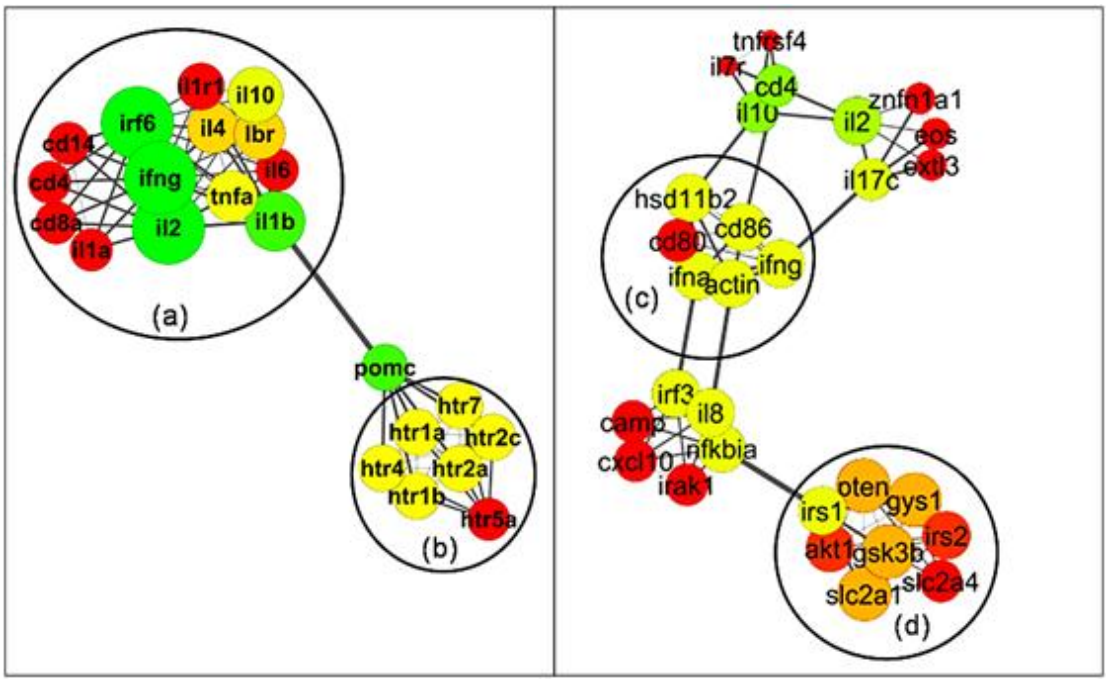


Figure 2.6: Cluster10 from II ⋂ M and Cluster 4 from II(S) ⋃ M(S) networks. Various sub-clusters are shown for (a) inflammatory cytokines, (b) serotonin receptors, (c) genes involved in inflammation and energy metabolism and (d) genes involved in lymphocyte activation as well as positive regulation of catabolic processes.

Details of the significantly enriched terms (only those GO terms with a minimum enrichment p-value 10^{-7} have been included in the functional analysis) obtained in the enrichment analysis of **II(S) ⋂ M(S)** against a background of **II ⋃ M** case have been provided in table 2.4.

Table 2.4: Comparison of the genes of II(S) ⋂ M(S) against II ⋃ M network.

GO Term	Description	P-value	Enrichment (N, B, n, b)	Genes
GO:0002637	regulation of immunoglobulin production	2.47E-07	17.46 (774,14,19,6)	IL13 - interleukin 13 IL6 - interleukin 6 (interferon, beta 2) IL10 - interleukin 10 TNF - tumor necrosis factor IL5 - interleukin 5 (colony-stimulating factor, eosinophil) IFNγ - interferon, gamma

GO:0045428	regulation of nitric oxide biosynthetic process	6.41E-07	15.28 (774,16,19,6)	PTGS2 - prostaglandin-endoperoxide synthase 2 (prostaglandin g/h synthase and cyclooxygenase) IL6 - interleukin 6 (interferon, beta 2) IL10 - interleukin 10 TNF - tumor necrosis factor IL1B - interleukin 1, beta IFNγ - interferon, gamma
GO:0050714	positive regulation of protein secretion	6.41E-07	15.28 (774,16,19,6)	IL13 - interleukin 13 IL6 - interleukin 6 (interferon, beta 2) IL10 - interleukin 10 TNF - tumor necrosis factor IFNγ - interferon, gamma IL5 - interleukin 5 (colony-stimulating factor, eosinophil)
GO:0051043	regulation of membrane protein ectodomain proteolysis	1.29E-06	32.59 (774,5,19,4)	IL10 - interleukin 10 TNF - tumor necrosis factor IL1B - interleukin 1, beta IFNγ - interferon, gamma
GO:0002697	regulation of immune effector process	2.07E-06	6.32 (774,58,19,9)	IL13 - interleukin 13 NOS2 - nitric oxide synthase 2, inducible IL6 - interleukin 6 (interferon, beta 2) IL10 - interleukin 10 TRAF3 - tnf receptor-associated factor 3 TNF - tumor necrosis factor IL1B - interleukin 1, beta IFNγ - interferon, gamma IL5 - interleukin 5 (colony-stimulating factor, eosinophil)
GO:0008285	negative regulation of cell proliferation	3.11E-06	4.48 (774,100,19,11)	PPARγ - peroxisome proliferator-activated receptor gamma APC - adenomatous polyposis coli JUN - jun proto-oncogene CYP27B1 - cytochrome p450, family 27, subfamily b, polypeptide 1 PTGS2 - prostaglandin-endoperoxide synthase 2 (prostaglandin g/h synthase and cyclooxygenase) IL6 - interleukin 6 (interferon, beta 2) IL10 - interleukin 10 TNF - tumor necrosis factor IL1B - interleukin 1, beta IFNγ - interferon, gamma IRF6 - interferon regulatory factor 6
GO:0051222	positive regulation of protein transport	3.72E-06	8.91 (774,32,19,7)	IL13 - interleukin 13 IL6 - interleukin 6 (interferon, beta 2) IL10 - interleukin 10 TNF - tumor necrosis factor IL1B - interleukin 1, beta IFNγ - interferon, gamma IL5-interleukin 5 (colony-stimulating factor, eosinophil)

GO:0060558	regulation of calcidiol 1-monooxygenase activity	3.80E-06	27.16 (774,6,19,4)	CYP27B1 - cytochrome p450, family 27, subfamily b, polypeptide 1 TNF - tumor necrosis factor IL1B - interleukin 1, beta IFNγ - interferon, gamma
GO:0051047	positive regulation of secretion	4.66E-06	8.64 (774,33,19,7)	IL13 - interleukin 13 IL6 - interleukin 6 (interferon, beta 2) IL10 - interleukin 10 TNF - tumor necrosis factor IL1B - interleukin 1, beta IFNγ - interferon, gamma IL5 - interleukin 5 (colony-stimulating factor, eosinophil)
GO:0050708	regulation of protein secretion	7.28E-06	10.63 (774,23,19,6)	IL13 - interleukin 13 IL6 - interleukin 6 (interferon, beta 2) IL10 - interleukin 10 TNF - tumor necrosis factor IL5 - interleukin 5 (colony-stimulating factor, eosinophil) IFNγ - interferon, gamma
GO:0002673	regulation of acute inflammatory response	8.87E-06	14.55 (774,14,19,5)	PPARγ - peroxisome proliferator-activated receptor gamma PTGS2 - prostaglandin-endoperoxide synthase 2 (prostaglandin g/h synthase and cyclooxygenase) IL6 - interleukin 6 (interferon, beta 2) IL1B - interleukin 1, beta TNF - tumor necrosis factor
GO:0045429	positive regulation of nitric oxide biosynthetic process	8.87E-06	14.55 (774,14,19,5)	PTGS2 - prostaglandin-endoperoxide synthase 2 (prostaglandin g/h synthase and cyclooxygenase) IL6 - interleukin 6 (interferon, beta 2) TNF - tumor necrosis factor IL1B - interleukin 1, beta IFNγ - interferon, gamma

2.2. Understanding effects of psychological stress on physiology and disease through human stressome - An integral algorithm

An over-burdened stress response system is capable of negatively affecting physiological homeostasis leading to a plethora of disorders as mentioned earlier. The physiological signaling conveying the status quo at gene and molecular level during stress response thus involves a large number of organ systems, signaling cascades and neuro-endocrinal circuitry which is largely unexplored. So, we took lead from our previous systematic-analysis reports [33, 191], thereby

deriving predictive association of genes with physiology and diseases through analysis of the gene-network topologies, using the power of text-mining. The advantage of gene-networking analysis is that huge dimension data across various studies can be compressed into single or a few networks with the potential to develop critical tools for making predictions in areas where studies are limited in numbers, diverse/unrelated and scattered. We, therefore, report here an integral algorithm to understand and predict role of psychological stress on human physiology and diseases.

2.2.1. Materials and Methods

2.2.1.1. Development of Gene association Network for Psychological Stress (Human Stressome)

Text-mining based gene-association network for psychological stress for humans (human stressome) was generated by scanning literature using genes of entire human genome as gene-based keyword search. Cytoscape plug-in for Agilent Literature Search was used for keyword based text-mining and network generation. For text-mining, this plugin uses each gene as a keyword along with the provided context to search against pubmed and OMIM database, and creates a node (gene) for each match it finds in the relevant context search (the details of the search methods can be found in our earlier work. Once, the text-mining based gene association network for human stressome was created, MCODE plugin was used to identify putative biological complexes/gene clusters followed by in-built network analysis plugin of Cytoscape. This method as already described in our work [191] also removes publication bias. It also provided the node degree and clustering co-efficient for each gene in the network after clustering, which were used in Gene Ontology (GO) enrichment of human stressome using GOriLLA server [189]. To test the robustness of the human stressome gene-network thus

generated, genes of this network were compared with those pulled out by the web-based text-mining server, GeneClip [192]. The nature of distribution of literature evidences for each gene from both the text-mining tools was tested by Pearson correlation analysis

2.2.1.2. Algorithm for association of effects of stress on diseases

Human stress-disease network was further developed for analyzing the association between stress genes (obtained from human stressome network) and disease susceptibility. Disease Ontology (DO) enrichment of genes from human stressome was done using DOSE package in R (Bio Conductor) [193]. The DO enrichment profile of human stressome (table 2.5) showed that all the diseases enriched by these genes could be broadly classified into five major categories, each of which were then comprehensively populated with disease names (table 2.6).

Table 2.5: List of DO terms obtained by DO enrichment of human stressome genes

ID	Description	GeneRatio	p-value
DOLite:246	Hepatitis C	23/257	4.47E-16
DOLite:173	Endometriosis	37/257	4.17E-14
DOLite:322	Lupus erythematosus	33/257	1.00E-11
DOLite:513	Systemic infection	24/257	1.67E-11
DOLite:78	Behcet syndrome	19/257	3.09E-11
DOLite:358	Multiple sclerosis	26/257	6.18E-11
DOLite:411	Pancreatitis	17/257	9.95E-11
DOLite:62	Asthma	33/257	1.11E-10
DOLite:154	Dermatitis	29/257	1.57E-10
DOLite:120	Chronic obstructive airway disease	23/257	2.83E-10
DOLite:477	Rheumatoid arthritis	45/257	4.41E-10
DOLite:175	Enteritis	23/257	6.40E-10
DOLite:507	Stroke	22/257	1.06E-09
DOLite:537	Ulcerative colitis	23/257	9.15E-09
DOLite:353	Mucocutaneous lymph node syndrome	11/257	1.09E-08
DOLite:272	IGA glomerulonephritis	13/257	5.58E-08
DOLite:33	Adenovirus infection	17/257	6.42E-08
DOLite:279	Infectious lung disease	10/257	9.34E-08
DOLite:346	Migraine	12/257	1.02E-07
DOLite:514	Systemic scleroderma	21/257	1.53E-07

DOLite:434	Polyarthritis	19/257	1.65E-07
DOLite:320	Lung cancer	33/257	3.52E-07
DOLite:396	Oral cancer	15/257	6.66E-07
DOLite:306	Leukemia	43/257	6.97E-07
DOLite:68	Autoimmune disease	19/257	8.68E-07
DOLite:44	Alzheimer's disease	31/257	8.69E-07
DOLite:423	Periodontitis	13/257	1.31E-06
DOLite:104	Celiac disease	12/257	1.46E-06
DOLite:156	Diabetes mellitus	46/257	1.99E-06
DOLite:41	Alimentary system disease	9/257	2.17E-06
DOLite:337	Melanoma	25/257	2.54E-06
DOLite:466	Respiratory distress syndrome	7/257	4.71E-06
DOLite:533	Tuberculosis	14/257	4.98E-06
DOLite:298	Kidney failure	17/257	5.81E-06
DOLite:392	Obesity	27/257	5.84E-06
DOLite:264	Hypertension	26/257	6.42E-06
DOLite:441	Pre-Eclampsia	16/257	7.10E-06
DOLite:447	Prostate cancer	45/257	7.54E-06
DOLite:483	Schizophrenia	27/257	8.19E-06
DOLite:495	Skin cancer	8/257	9.65E-06
DOLite:489	Severe acute respiratory syndrome	6/257	1.04E-05
DOLite:95	Bronchopulmonary dysplasia	6/257	2.17E-05
DOLite:96	Brucellosis	6/257	2.17E-05
DOLite:180	Epstein-Barr virus infection	8/257	2.32E-05
DOLite:64	Atherosclerosis	29/257	2.64E-05
DOLite:23	Abortion	14/257	3.26E-05
DOLite:323	Lupus vulgaris	9/257	3.44E-05
DOLite:458	Pulmonary fibrosis	9/257	3.44E-05
DOLite:517	Testicular dysfunction	12/257	3.67E-05
DOLite:165	Embryoma	34/257	3.91E-05
DOLite:100	Cancer	72/257	3.96E-05
DOLite:315	Liver disease	11/257	3.97E-05
DOLite:228	HIV infection	19/257	4.63E-05
DOLite:109	Cervical cancer	15/257	5.22E-05
DOLite:523	Thrombophilia	7/257	6.62E-05
DOLite:46	Amnionitis	6/257	7.23E-05
DOLite:75	Basal cell carcinoma	6/257	7.23E-05
DOLite:277	Infection	19/257	8.70E-05
DOLite:283	Influenza	8/257	9.87E-05
DOLite:152	Dental plaque	13/257	9.99E-05
DOLite:258	Hyperglycemia	10/257	0.000109
DOLite:234	Helicobacter infection	7/257	0.000145
DOLite:417	Parkinson disease	15/257	0.000153

DOLite:60	Arthritis	13/257	0.000166
DOLite:373	Nasopharyngeal cancer	8/257	0.000181
DOLite:160	Drug abuse	18/257	0.000242
DOLite:218	Glomerulonephritis	6/257	0.000287
DOLite:81	Bipolar disorder	14/257	0.000312
DOLite:291	Ischemia	12/257	0.000349
DOLite:280	Infertility	11/257	0.000378
DOLite:232	Heart failure	15/257	0.000391
DOLite:245	Hepatitis B	6/257	0.00042
DOLite:442	Premature birth	6/257	0.00042
DOLite:443	Primary biliary cirrhosis	8/257	0.000516
DOLite:48	Amyotrophic lateral sclerosis	11/257	0.00053
DOLite:271	Hypothyroidism	6/257	0.000597
DOLite:436	Polycystic ovary syndrome	11/257	0.000729
DOLite:145	Cystic fibrosis	10/257	0.000802
DOLite:250	Herpes	10/257	0.000802
DOLite:527	Thyroid gland disease	6/257	0.000826
DOLite:153	Depression	11/257	0.00085
DOLite:205	Gastritis	7/257	0.001423
DOLite:217	Glaucoma	8/257	0.001499
DOLite:314	Liver cancer	21/257	0.001608
DOLite:135	Congenital abnormality	22/257	0.001699
DOLite:123	Chronic simple glaucoma	6/257	0.001933
DOLite:49	Anemia	6/257	0.001933
DOLite:399	Osteoporosis	8/257	0.003055
DOLite:357	Multiple myeloma	10/257	0.00307
DOLite:166	Emphysema	6/257	0.003133
DOLite:53	Anorexia nervosa	6/257	0.003133
DOLite:225	Graves' disease	7/257	0.003252
DOLite:252	Hodgkin's disease	7/257	0.003252
DOLite:191	Fanconi's anemia	6/257	0.003907
DOLite:526	Thyroid cancer	7/257	0.003908
DOLite:89	Breast cancer	41/257	0.005378
DOLite:409	Pancreas cancer	13/257	0.005827
DOLite:226	Growth retardation	6/257	0.005868
DOLite:257	Hypercholesterolemia	6/257	0.005868
DOLite:552	Virus disease	7/257	0.006489
DOLite:47	Amyloidosis	6/257	0.007081
DOLite:505	Stomach cancer	17/257	0.007212
DOLite:159	Down syndrome	11/257	0.008543
DOLite:454	Psychotic disorder	7/257	0.010163
DOLite:179	Epilepsy	8/257	0.012314
DOLite:376	Neoplasm metastasis	17/257	0.014846

DOLite:77	Behavior disease	7/257	0.01516
DOLite:163	Eating disorder	6/257	0.015959
DOLite:103	Cardiovascular disease	7/257	0.017159
DOLite:504	Squamous cell cancer	12/257	0.017216
DOLite:402	Ovarian cancer	12/257	0.021525
DOLite:560	Yersinia infection	9/257	0.033905
DOLite:464	Renal Cell cancer	9/257	0.042711

Table 2.6: List of the disease categories and diseases used to populate each category based on DO enrichment of Human Stressome.

Disease Category (DC_i)	Disease Name (D_{ij})
Autoimmune disorders (# 36)	Encephalomyelitis, Inflammatory Bowel Disease, Monoclonal Gamopathy, Multiple Sclerosis, Cogan's Syndrome, Graft-versus-Host Disease, Primary Biliary Cirrhosis, Antiphospholipid Syndrome, Wegener's Granulomatosis, Systemic Necrotizing Vascolitides, Kawasaki Disease, Chronic Inlammatory Demyelinating Disease,Sarcoidosis, Autoimmune Lymphoproliferative Syndrome, Celiac Disease, Poltradiculoneuropathy, Chronic Active Hepatitis, Primary Sclerosing Cholangitis, Paraneoplastic Neurological Disorders, Cryoglobulinemia, Meniere's Disease, CNS Vasculitis, Stiff Man Syndrome, Raynaud's Phenomenon, Myasthenia Gravis, Polymyalgia Rhematica, Temporal Arteritis, Lambert-Eaton Myasthenic Syndrome, Pernicious Anemia, Throboangitisobliterans, Neuromyotonia /Issacs' Syndrome, Takayasu's Arteritis, Opsoclonus-Myoclonus Syndrome, Autoimmune Inner Ear Disease, Acute Inflammatory Demyelinating Polyneuropathy Polyarteritisnodosa, Hypersensitivity Vasculitis
Infectious diseases (# 70)	Anthrax, Avian Influenza, Babesiosis, Boils and Skin Infection,Pertusis, Brucellosis, Camplylobacteriosis, Chrancroid, Chickenpox, Smallpox, Chikungunya, Chlamydia, Cholera, Creutzfeldt-Jacob Disease,Shingles, Cryptosporidiosis, Diphtheria, Lyme Disease, Epidemic Keratoconjunctivitis, Fifth Disease, Gastroenteritis, Giardiasis, Gonorrhoea, HaemolyticUraemic Syndrome, Haemophilus Influenza Type B, Hand Foot and Mouth Disease, Hepatitis A, Hepatitis B, Hepatitis D, Hepatitis E, AIDS, Tetanus, Typhoid, German Measles, Infectious Mononucleosis, Influenza, Japanese Encephalitis, Listeriosis, Keratoconjunctivitis, Kunjin Virus Disease, Measles, Legionnaires Disease, Leprosy, Leptospirosis, Syphilis, Plague, LymphogranulomaVenereum, Malaria,Dengue, Botulism, Murray Valley Encephalitis, Tularemia, Meningococcal Disease Pandemic Influenza, Pneumococcal Disease, Poliomylitis, Psittacosis, Q Fever, SARS, Rickettsia, Rotavirus Infection, Rubella, Yellow Fever, Salmonellosis, Hepatitis C, Shigellosis, Tuberculosis, Whooping Cough
Metabolic Syndrome (# 15)	Metabolic Syndrome, Obesity, Hyperthyroidism, Hypolipidemia, Tay-Sachs Disease, Hypothyroidism, Dyslipidemia, Galactosemia, G-6-PD Deficiency, Diabetes, Phenylketonuria, Inborn Errors of Metabolism, Metabolic Acidosis, Addison's Disease, Gangliosidosis
Neuro-degenerative	Alzheimer's Disease, Amyotrophic Lateral Disease, Parkinsons Disease, Primary Progressive Aphasia, Amylotrophic Lateral Sclerosis, Progressive

disdorders (# 8)	Supranuclear Palsy, Creutzfeldt_Jacob Disease, Huntington's Disease
Neurophysiological disorders (# 11)	Attention Deficit Hyperactivity Disorder, Autism, Delayed Onset of Speech, Anxiety, Bipolar Disorder, Depression, Dyslexia, Epilepsy Obsessive-Compulsive Disorder, Schizophrenia, Social Phobia

Gene-association networks were generated for each of these diseases using Agilent Literature Search plug-in. The keyword used for text-mining was the disease name for each disease network. Effects of psychological stress on disease susceptibility was probed by studying gene associations among each of these disease networks as well as with human stressome by integrating association data of all these networks into a single network that retains the biological significance. Gene-associations of each disease category were used to create disease-gene associations and the strength of such associations was calculated as described below. Algorithm, schematically represented in figure 2.7, was hence designed for network condensation leading to a single network (Human Stress-Disease Network) that could easily be analyzed for association with stress:

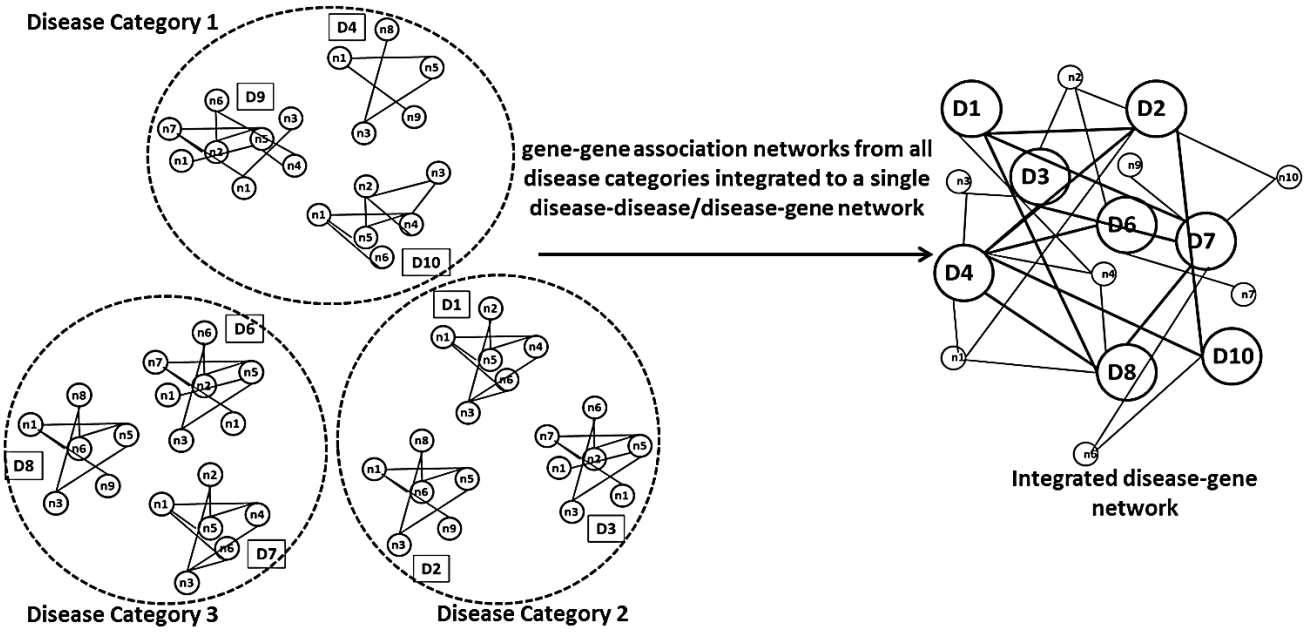


Figure 2.7: Schema of disease-gene network condensation algorithm. Associations from individual gene networks of diseases from various disease categories were used to build final disease-gene network.

Mathematical formulation for the above schema is as follows:

DC_i= ($Di_1, Di_2, Di_3,....,Di_n$) or $D_{ij} \in DC_i$ (example of select diseases are shown in table 2.6)

where, DC_i denotes disease category of *i*-th type consisting of *j*-number of D_{ij} elements.

Similarly,

D_{ij}= ($g_{ij1}, g_{ij2}, g_{ij3}, ...,g_{ijn}$) or $g_{ijk} \in D_{ij}$, where genes g_{ijk}(s) are elements of a disease D_{ij}. Diseases D_{i1} to D_{in} are in unions as $D_{i1} \bigcup D_{i2} \bigcup D_{i3}.....\bigcup D_{in}$ leading to disease category DC_i.

We also define that if n_i is the number of nodes in D_{ij} where $t_i^n = \sum_i^n n_i$ is the total number of nodes in D_{ij} and $d_i^{DC} = \sum_i^n t_i^n$ representing total number of nodes in DC_i.

Genes were associated with each other as nodes for each disease, but for comprehensive representation of human disease network, the association data was integrated into a single network, where each disease was represented as a mega node and edges (connections) were formed between any two diseases $\boldsymbol{D_i}$ and $\boldsymbol{D_j}$, following the rules described below:

Connectivity (*C*) between diseases D_i andD_j (D_i<->D_j) in a disease category is true**,**

if $d_i^{DC} \bigcap d_j^{DC} = C$ for $C \neq 0$,

Strength (*Sij*) of edge or connectivity between two diseases **($\boldsymbol{D_i}$<->$\boldsymbol{D_j}$)**, can be calculated by

$$S_{ij}^D = \left[\left(d_i^{DC} + d_j^{DC}\right) \times C\right] / t_i^n$$

Strength (S_i^D) of nodes (genes) in a disease D_{ij} can be denoted as ratio between number of edges ($d_i^{g_i \leftrightarrow D_{ij}}$) connecting its nearest neighbors (diseases) and number of edges connecting its nearest neighbors (d_i^{DC}), therefore, $S_i^D = \frac{d_i^{g_i \leftrightarrow DC_{ij}}}{d_i^{DC}}$

In the composite disease network, genes were represented as offshoot nodes of disease nodes, and edges were formed between any gene $\boldsymbol{g_i}$ and disease $\boldsymbol{D_i}$, based on the following rules:

Edges between g_i and D_{ij} will form, if, $g_i \in D_{ij} \subseteq DC_i$

Strength (S_i^{DC}) of offshoot gene node is given by $S_i^{DC} = \sum_{j=1}^{n} S_{ij}^{D}$

In order to study effects of stress on physiological homeostasis, similar text-mining based gene-association networks were generated for select stress-affected physiological processes viz., ageing, digestion, growth and development, immunity, metabolism respiration, reproduction. Genes which are already reported to affect each of the physiological process were manually identified, and expanded to candidate-set genes using gene prioritization algorithm (through Endeavour). Top 2% of the prioritized candidate genes were used as keywords to construct query for literature search using Agilent Literature Search plugin for each gene-association network. Significant gene-association clusters were then identified using MCODE plug-in as described by the current group previously [33, 191]. Using the formulations described above, various associations between genes and diseases as well as genes and these physiological functions were generated. Similar mathematical basis was also used to build the composite disease, gene and physiology network. Genes from Human Stress-Disease Network were extended to second order neighbors as background set for GO enrichment. Genes conserved for Human Stress-Disease Network and Human Stressome were used for Gene Ontology (GO) Enrichment Analysis using GOriLLA server.

2.2.2. Results

Gene association network for psychological stress (figure 2.8a) was created using the entire human genome, (hence forth referred as “human stressome”) as keyword by ALS plugin. Out of

all the known human genes, 477 genes were found to be associated with psychological stress. GeneClip, software which also employs algorithm for text-mining based keyword search on pubmed abstracts, yielded 177 genes, out of which 100 genes were shared by both methodologies. A statistical comparison by t-test showed that both the text-mining methods pulled out similarly distributed gene sets and a clustering analysis, based on frequency of occurrence values actually revealed they formed tight clusters, within which the correlation was high.

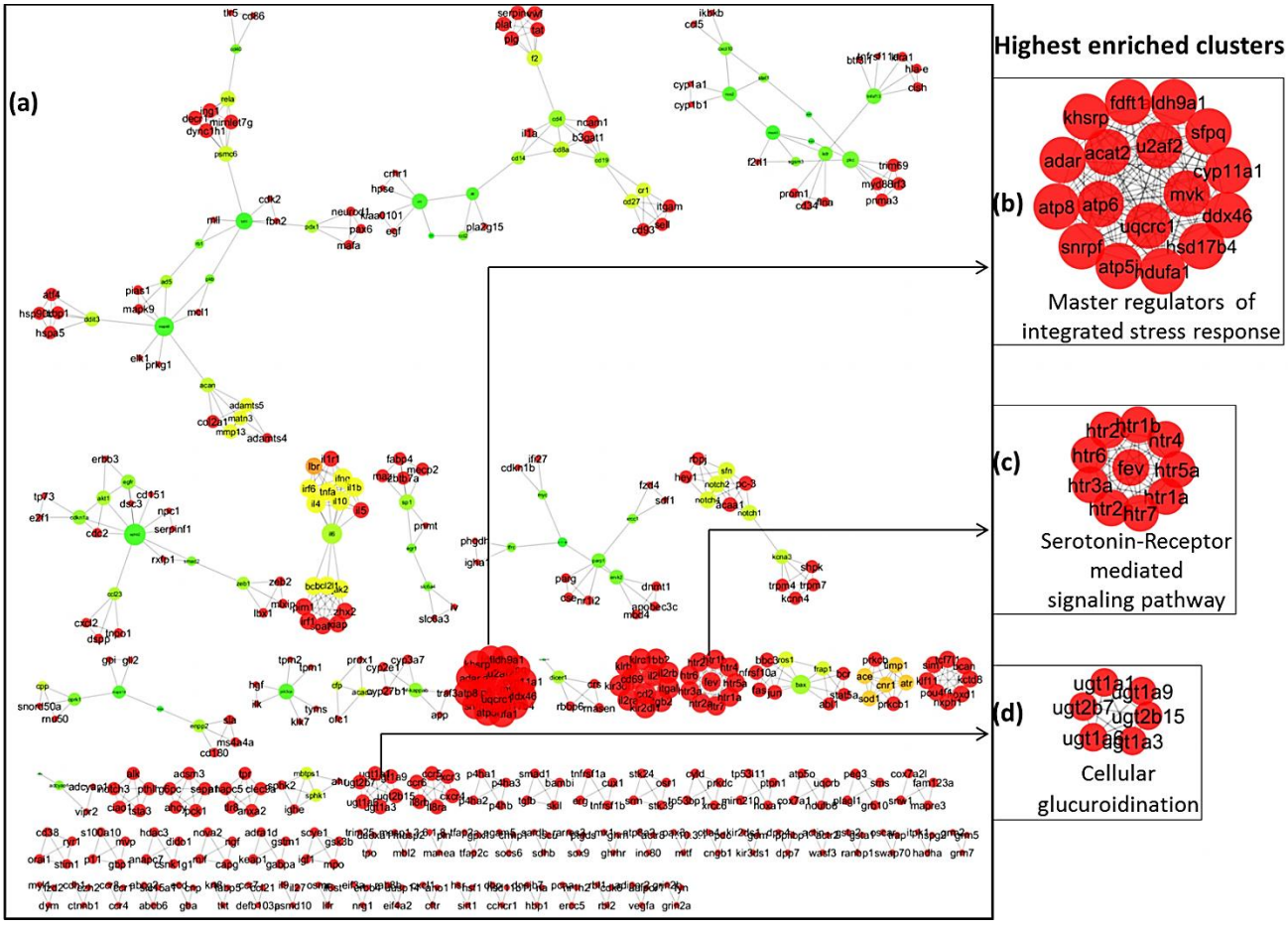


Figure 2.8: Human Stressome - the gene association network of psychological stress for entire human genome. Nodes sizes are directly proportional to their degree, and color is directly proportional to clustering coefficient (green for lower and red for higher clustering coefficient).

2.2.2.1. Functional Enrichment of Stress genes in human stress-network

Clustering-coefficient (degree involvement of a node in the participating cluster) based GO enrichment showed enrichment of G-protein coupled receptor signaling pathway and cellular glucuronidation to be most significantly enriched. It was seen that genes from these enrichment terms actually formed two sub-clusters: serotonin receptors sub-cluster [seen as highly enriched HTRs, 5-hydroxytryptamine receptors - HTR4, HTR3A, HTR2C, HTR7, HTR6, HTR5A, HTR1B, HTR1A, HTR2A in figure 2.8c] and glucuronidation enzymes sub-cluster [seen as enriched UGTs - UGT1A3, UGT2B7, UGT1A1, UGT2B15, UGT1A6, UGT1A9 in figure 2.8d] in human stressome. Genes from these two sub-clusters contributed to all of the GO-terms enriched by clustering-coefficient based GO enrichment. The HTRs were implicated in synaptic transmission, control of appetite, blood vessel size regulation, as well as catecholamine and dopamine secretion. Node-degree (the number of connections of a node) based GO enrichment, however, showed that along with genes responsible for serotonin receptor signaling and glucuronidation, other genes involved in oxidation-reduction processes, mRNA splicing, isoprenoid biosynthesis (FDFT1 and MVK) as well as steroid biosynthesis were also significantly enriched and clustered together [figure 2.8b]. There was also significant enrichment of genes responsible for regulation of nitric oxide biosynthesis process such as TNF, IL10, IL4, JAK2, IL1B, IL6 and IFNΓ along with other cytokines and apoptotic genes such as BCL2.

2.2.2.2. Integrated Human Stress-Disease network

The Human Stress-Disease network contained all the association information among the disease and stress genes condensed in a single network which represented individual contribution of each gene to the diseases they are associated with at multiple dimensions. The same network also showed how many of these disease associated genes were from human stressome network and how the diseases were related among themselves based on conserved genes between them (figure

2.9). Since all genes in this network were associated with one or the other disease, 3 types of GO enrichment was done to understand how (1) disease-genes associated with stress contributed to overall disease profile (2) disease-genes not associated with stress contributed to overall disease profile (3) disease genes, independent of their nature with respect to stress, contributed to normal physiology. It was seen that out of 612 disease-genes in human stress-disease network, 168 genes were associated with stress and 444 genes were associated with diseases but not with stress. We found that stress associated diseases genes showed an enrichment of positive regulation of icosanoids, prostaglandins secretion and calidiol 1-monooxygenase activity. Positive regulation of peptidyl-serine phosphorylation and negative regulation of catabolic process was seen which was absent in human stressome GO enrichment profile. It was also observed that there was positive regulation of B-type cell activation, isotype switching and activation induced cell death of T-cells. However, GO enrichment of disease genes not associated with stress, showed that amino-acid transport (L-glutamate) was positively regulated, along with positive regulation of glutametargic synaptic transmission, which involved PTGS2 and OXTR genes among others. IFNβ and TNFα production were positively regulated, along with production of cytokines. Network topology analysis revealed that among other diseases, Hepatitis C was maximum affected by stress and contribution of PDGH (stress associated gene) to the human stress-disease network was the largest. A positive correlation ($r = 0.82$ with $p \leq 0.003$) between genes of stress-disease network (obtained through text-mining) and genes obtained through disease ontology enrichment analysis of human stressome for the same disease terms was also obtained, reflecting the robustness of the method used for generation of stress-disease network.

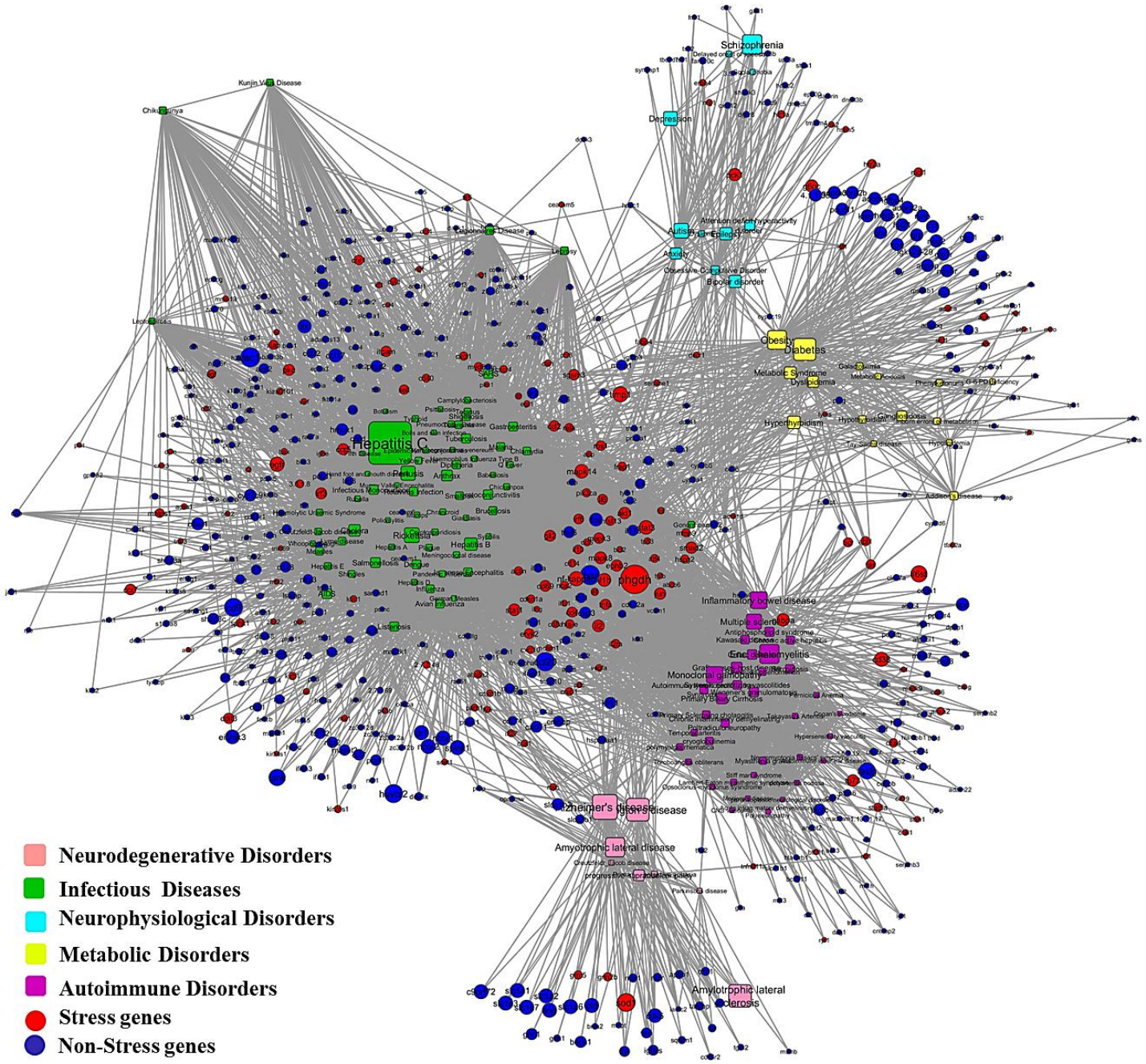


Figure 2.9: Comprehensive network of genes associated with psychological stress and diseases (stress-disease network). Relative contribution of each gene (circular nodes) to its associated diseases is represented by node size. Size of each disease (rectangular node) represents its relative abundance of contributing genes.

GO enrichment profile of stressome network revealed that stress is associated with steroid hormone biosynthesis along with increased redox reactions, isoprenoid biosynthesis and nitric oxide biosynthesis process. Enrichment of stress as well as disease genes from the physiological processes network too showed similar enrichment profile with more specific child terms such as enrichment of response to corticosteroids, hypoxia and serine tyrosine kinase signaling. Nitric oxide synthesis was also enriched by stress genes in most of the physiological processes. These profiles clearly show that stress response system works in a manner that creates mobilization of energy by increasing catabolic oxidative metabolism. This goes with the earlier established notion of high energy demand of stress, which is adequately met by stress response mechanism of the physiology by mobilizing energy produced through oxidative metabolic processes. It is also known that chronic stress increases susceptibility to a wide range of metabolic and inflammatory disorders. Enrichment profile of stress-disease network showed enrichment of calidiol 1-monooxygenase activity and icosanoids, prostaglandins secretion. It was seen that negative regulation of catabolism too was enriched. This observation hints that stress mediated diseases might result due to accumulation of ICs and PGs in the physiological system

2.2.2.3. Involvement of stress genes at physiological regulatory levels

Increasing amounts of these endotoxic oxidation end-products might trigger suspension of catabolism through negative feed-back signaling. High levels of cortisol have been reported to increase PGs level in vitro [194]. Stress has been shown to increase lipid peroxidation and protein oxidation in liver, brain and plasma [195]. It is also known that organs/sites of high oxygen consumption, and/or high lipid content are highly susceptible to redox reactions. PTGS2 which is highly represented among the enriched terms oxidizes arachidonic acid derived from lipids to produce PGs, ECs, prostacyclins, isotrenes, etc. These oxidation derived metabolites

during stress response accumulate and exert deleterious effects on the concerned organs. In order to study the effects of psychological stress on physiological homeostasis and hence disease susceptibility, we generated gene-association networks for physiological processes well known to be affected by stress viz. ageing, digestion, reproduction, respiration, growth and development, immunity, and metabolism (figure 2.10). Each of these physiological processes was analyzed in the light of disease genes and stress genes in these networks. Stress genes were colored red and disease genes were colored cyan and the node size shows its contribution to the stress-disease network. The nodes colored blue represented the genes associated with the physiological process, but no associations with stress or disease have been reported in literature so far.

Ageing: GO-biological processes enrichment profile of ageing network showed that response to steroid hormones, and stress induced premature senescence were among the highly enriched terms; thereby implying that genes responsible for ageing are also responsive to stress hormones and stress might be capable of affecting genes responsible for acceleration of senescence or ageing. It was also seen that the stress genes TP53, MAPK14 and CDKN1a contributed to other significantly enriched terms like positive regulation of ROS (Reactive Oxygen Species) metabolic process, cellular response to hypoxia, stress-induced premature and cell-cycle check-point regulation. Regulation of lipid homeostasis and fat cell differentiation were also enriched implying the involvement of lipid synthesis and metabolism in stress associated ageing process. Among the genes from disease network contributing to ageing, the most abundantly present genes were PTGS2, SRC and APC- adenomatous polyposis coli. ATM serine/threonine kinase gene, known to contribute to cell cycle control, was also among the significantly enriched genes

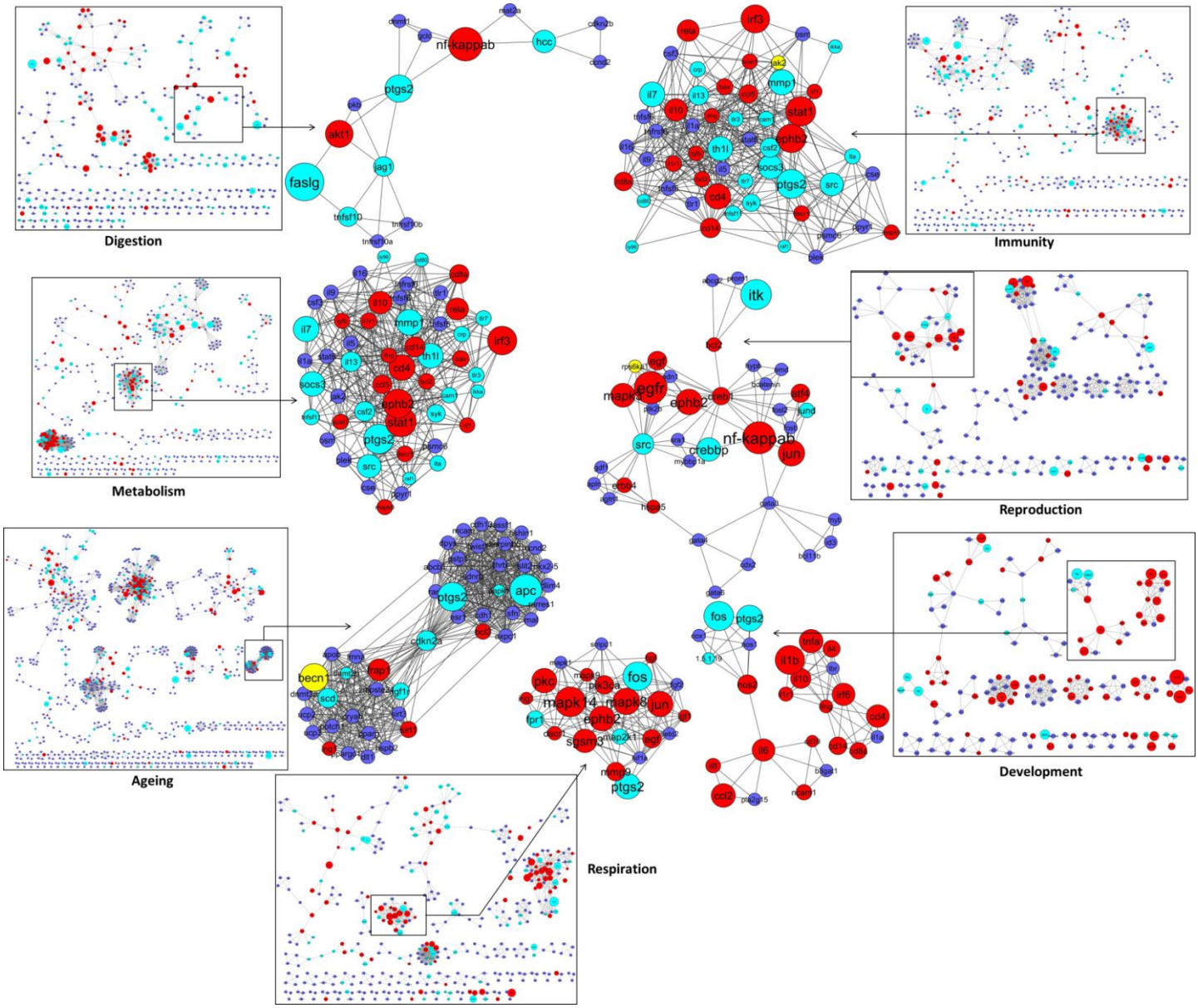


Figure 2.10: Gene association network of physiological processes. Genes associated with diseases (from stress-disease network) are represented with cyan color and genes associated with stress are in red color. Nodes sizes are directly proportional to their relative contribution to stress-disease network.

and was associated with negative regulation of serine/threonine kinase (a highly enriched GO term).

Development: GO enrichment of the genes from development network showed overall negative regulation of lymphocyte as well as leukocyte proliferation and activation. While serotonin receptor signaling was enriched the most, regulation of insulin secretion also was enriched in this

network. Response to growth factor binding was also enriched and was contributed to by IL2Ra, IL2Rb, and ERBB2. However, when genes contributing to both stress and diseases were enriched against entire development network genes, activation of immune system, particularly T-cell activation was seen. It was also seen that negative regulation of developmental process was enriched and all the contributing genes viz. CCL2, IRF1, IFNγ, TLR4, ZHX2, Coagulation factor II, EPHB2, TP53, SERPINE1, IL6, SIRT1, BDNF, BCL2, IL4 belonged to stress and disease category implying that these stress associated genes might impair developmental process.

Digestion: Enrichment of hexose and glucose metabolism, t-cell differentiation, granulocyte chemotaxis, inflammatory and myeloid cell apoptotic process were seen as a result of GO biological process enrichment of digestion network. Positive regulation of JAK-STAT cascade was enriched along with positive regulation of innate immune responses by TLR signaling through TRIF- dependent signaling (Myd88-independent signaling), and vitamin metabolism. Enrichment of stress and disease attributed genes against digestion gene background however didn't show very different enrichment scores except for glucose and hexose metabolism. The enrichment also showed that PDX1 and TRH contributed to the enriched corticosteroid response term.

Immunity: Enrichment of immunity network showed that immunity-associated genes enriched fatty-acid metabolism (PRKAG2, PRKAA1, SYK, FABP3, UCP3, STAT5A, LPL, LIPE, DECR1, PTGS2, CYP1A1, CYP1A2, CYP1B1, FASN and PPARγ) suggesting a possible role of these fatty-acid metabolism genes in immune regulation. Two of the immune genes (STAT1 and IFNγ) were seen to be associated with negative regulation of epithelial cell differentiation involved in kidney development. Results further revealed that TLR3, TLR6 TLR7 and NOD2 significantly enriched PAMP-dependent induction by symbiotic relation of host innate immune

response (implying that symbiotic bacteria could have profound effect on innate immunity). Along the line, TGFβ1, CCL5 and CD4 were similarly enriched. These genes are known to be involved in modification of physiology of organisms involved in symbiotic interaction with host. These genes are also known to have profound activating effect on brain microglial cells [196]. However, when genes associated with stress and disease from the network were enriched against immunity network, it was observed that trans-membrane receptor protein serine/threonine kinase signaling pathway (comprising of TP53, SP1, NCOR2, RHOA, TGFB1, SERPINE1, MYC and MAP3K7) was highly enriched showing that serine threonine kinase phosphorylation events are necessary for mediating stress response/effects.

Metabolism: Enrichment map of metabolism network showed that positive regulation of neutrophil migration and chemotaxis, positive regulation of IL23 production, positive regulation of IL12 production, positive regulation of acute inflammatory process, and negative regulation of IL17 were significantly enriched. It also showed enrichment of COX activity and vitamin metabolic process enrichment along with positive regulation of monoxygenase activity and negative regulation of glucose transport. Enrichment map obtained by comparison of genes contributing to stress and diseases against metabolism showed enrichment of positive regulation of IL8 production, regulation of fibroblast growth factor signaling, regulation of fatty acid transport, response to hypoxia, TGFβ signaling and regulation of nitric oxide biosynthetic process.

Reproduction: The enrichment map of reproduction network showed that gene involved in regulation of stress response, response to cytokine signaling and enhancement/activation of immune system were enriched (esp. TLR signaling through LPS). This result hints at regulations of psycho–neuro immunity on reproduction process. The genes ATF4 and ATM serine/threonine

kinase were associated with MAPK, NFκB, JUN, CREB1, CREBBP, HSPA4 and HSPA5. It was also seen than IL6 was the highest contributing node in reproduction network. Type 2 immunity cytokines viz. IL6, IL2, IL4, IL27, TNF and STAT3 were also among the significant contributors.

Respiration: GO enrichment map of respiration network showed enrichment of nitric oxide biosynthesis, stress activated MAP kinases, and positive regulation of phosphatidyl serine and tyrosine residues. However, when stress and disease genes were enriched against respiration background, positive regulation of lipid synthesis was enriched, along with glucose and hexose metabolism.

2.2.2.4. Physiological processes and stress-mediated disease susceptibility network

Finally, to do an integrated analysis of how stress affects physiology to increase disease susceptibility, a combined network of the genes affecting several physiological processes along with genes associated with diseases and stress was generated (figure 2.11). As can be seen from the integrated gene network of physiological processes, there are genes that are not directly associated with these physiological processes, however, they are associated with their first order (nearest) neighbours. This observation implies that such genes might even have associations (direct/indirect) with physiological processes that are yet to be reported.

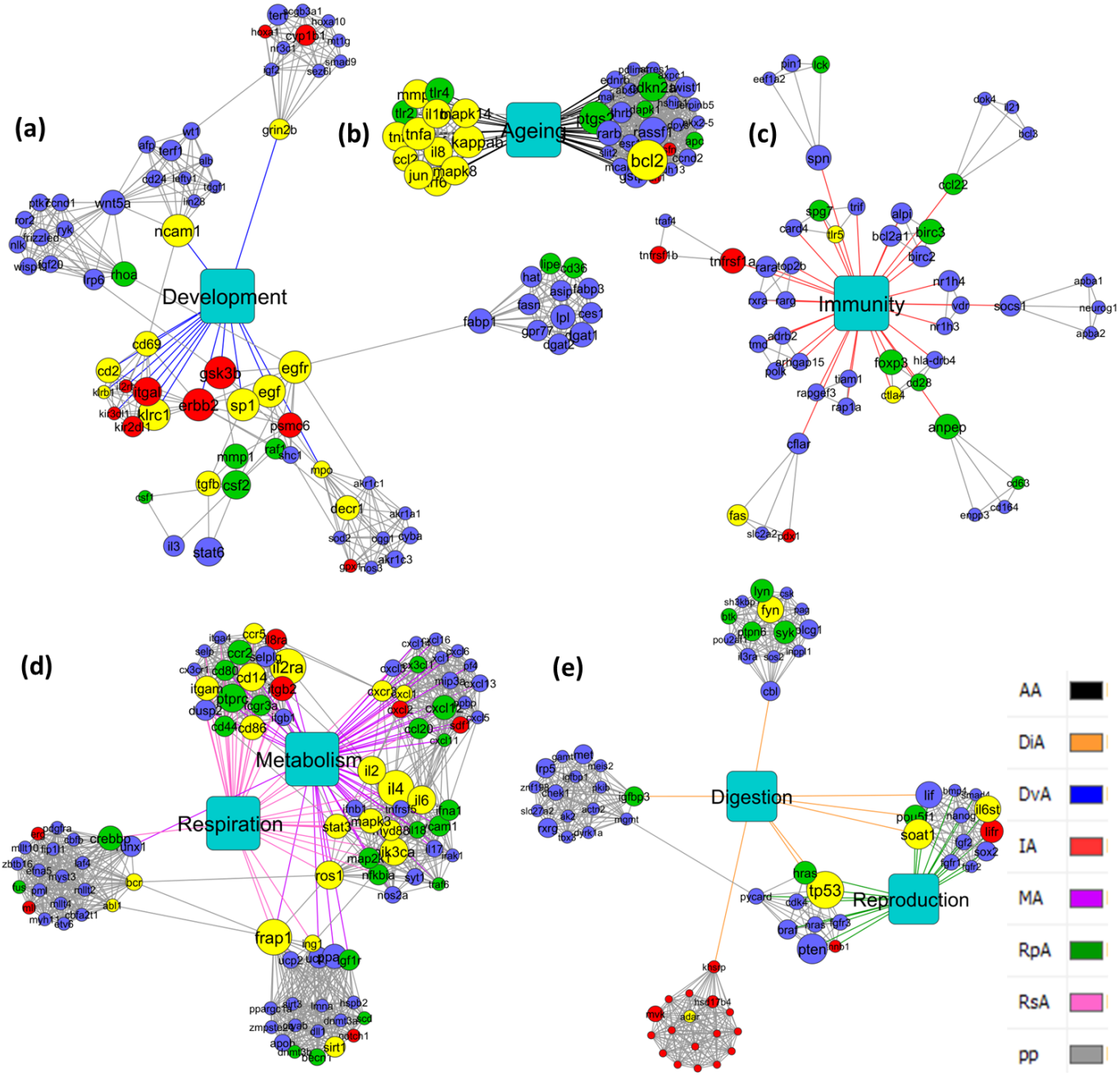


Figure 2.11: Combined network of genes affecting the physiological processes, stress and diseases. Genes, associated with stress, diseases and both stress and diseases are colored in red, green and yellow, respectively. Edges, showing associations between physiological processes [a) Development, b) Ageing. c) Immunity, d) Metabolism and e) Digestion] and genes, are colored as described in the figure sidebar with acronyms where AA- ageing associations, DiA- Digestion associations, DvA- Development associations, IA- Immunity associations, MA- Metabolism associations, RpA- Reproduction associations, RsA- Respiration associations, pp- not directly associated with any physiological process.

2.3. Discussion

The initial systematic analysis methodology developed to understand the inter-dependence between innate immune and metabolic processes when perturbed by psychological stress established networks that helped identify important patterns hitherto hidden and subsequently helped us in designing strategies to explore the predicted relationship experimentally.

2.3.1. Involvement of Serotonin receptor in energy homeostasis

HTR1B and HTR2A genes were consistently present in most of the enriched terms in **II(S)∪ M(S)** network, thereby hinting to the association of 5-HT receptors (5-Hydroxytryptamine receptors) to psychological stress. 5-HT receptors are implicated in the regulation of feeding behavior, mood and even temperature regulation. Interestingly, in the cluster 4 of **II(S)∪M(S)** network (table 2.3 and figure 2.6), the sub-cluster formed by (5-HT) receptor family genes (marked as '**b**') is linked to the sub-cluster of inflammatory cytokines (marked as '**a**') via POMC gene. It is already clear from previous discussions that psychological stress seems to induce a systemic inflammatory state in the body as is also justified by the enriched clusters of inflammatory cytokines. Inflammation is an energy intensive process [197] and might induce sickness syndrome (which also includes elevation of body temperature) in the body to provide for this energy demand, since it one of the energy conservation strategies of the body. On the other hand, digestion is also a high energy demanding process and so, it is quite expected that feeding behavior and digestion would be inhibited during systemic inflammation. The serotonin receptor HTR1A is known to induce hyperphagia[198] while, HTR1B and HTR2C are known to reduce food intake [199][200]. This kind of enrichment again looks contradictory, but here it should be noticed that this is an association study and should not be analyzed in the same manner as that of co-expression study. The enrichment of HTR1A, HTR1B and HTR2C in the same

cluster indicates their association with POMC and with each other in different studies (i.e. quite possibly in different physiological conditions), and does not necessarily need them to be co-expressed under the same situation. While HTR2C and HR1B might contribute to the anorexigenic effect of the body during the inflammatory stage, HTR1A might work in conjunction with CNR1 to increase appetite during body's adaptive response in a compensatory manner. Another effect of HTR1A is induction of various hormones including cortisol, corticosterone, ACTH, oxytocin, B-endorphin etc. Cortisol and corticosterone are known to suppress the pro-inflammatory cytokines (thereby causing immune suppression)[58], while B-endorphin contributes to anti-depressant effect, thereby controlling inflammation and enabling the body to revert back to basal levels of energy metabolism. There are different reported mechanisms by which HTR1A causes activation of CRH, followed by activation of ACTH, finally induces secretion of cortisol [201]. As has already been pointed out, many other proteins which might be acting during the compensatory response viz. CNR1 might also contribute to the activation of CRH and thereby lead to cortical/corticosterone production. Now, if the compensatory phase stretches beyond certain physiological limits, which might be the case in chronic stress, there could be a continuous over-activation of the ECS, serotonin receptor signaling and many more process which could have a possible role in the post-stress recovery system [202]. This could be possible causes of obesity, dyslipidemia, insulin resistance and many more metabolic syndromes and also a state of reduced immune resistance [197].

2.3.2. Role of the Endo-Cannabinoid System (ECS)

As mentioned earlier, CNR1 gene is consistently present in most of the enriched terms in **II(S)∪ M(S)** network, which already hints towards direct or indirect association of this gene with psychological stress. This gene is one of the two components of the Endo-Cannabinoid System,

the other component being CNR2 (though not present in the enriched gene ontology terms). Literature review interestingly revealed the fact that CNR1 gene is associated with systemic homeostasis and can be considered as a potential candidate that can bridge stress response with energy balance, as it reportedly gets activated post stress as one of the recovery mechanisms [203]. Interestingly, the enrichment data shows clear absence of any association of these genes in **II**$\cup$**M** network since, when only "innate immunity" or "metabolism" is used as the keyword, there would not be many reports, which relate the CNR1 or CNR2 genes with them indicating that ECS would be inactive in repose physiological conditions. While, when "psychological stress" is used as a key-word the genes of this system represent the enriched fraction of the total genes pulled out indicating that ECS is active only in physiologic conditions associated with stress. Cannabinoid receptors are expressed mainly in brain (central receptors), but some of them are also present in organs involved in energy homeostasis like adipose tissue, liver, gastro-intestinal tract, pancreas, skeletal muscle, etc (peripheral receptors). The endo-cannabinoid system has dual mode of action on energy metabolism; activation of central cannabinoid receptors is believed to increase satiety which may lead to obesity, thereby promoting food energy efficiency ,while activation of peripheral cannabinoid receptors (adipose tissue, liver,etc) can regulate metabolism and lipid storage without increasing food intake by some unknown mechanism[204, 205]. Reported evidences also hint that long-term effects of endo-cannabiod system on metabolism is basically due to the peripheral cannabinoid receptors[204]. This implies that if ECS is activated for longer time frames, it might cause food-intake independent weight gain (i.e. increased energy efficiency), which fits in ideally in a situation of chronic psychological stress. Reported observations thus hint towards the hypothesis that during stress, when the body requires instantaneous energy, this system might be dormant, but post-trauma the

body tries to compensate for the energy expenditure during stress, by increasing the activity of ECS and thereby promoting storage of fats causing unnecessary energy conservation. An intriguing question to address here would be to find out whether the production of endo-cannabinoids increases more or the endo-cannabinoid receptor expression is increased more post trauma. Acute stress activates systems which require using up of energy, while chronic stress might lead to a constant over-activation of the ECS, leading to the metabolic syndromes like excessive weight gain, insulin resistance and dyslipidemia[206].

2.3.3. Detoxification systems during stress response and associated disorders

UGTs are major phase 2 drug metabolizing enzymes and are highly expressed in liver and GI-tract (also present in kidney, brain skin, etc in lesser extents). It has been shown by Krishnaswamyet. al[207-209] that serotonin is a highly specific substrate for human intestinal UGTs, and serotonin UGT activity is induced by oxidative stress. This observation suggests that apart from detoxification, intestinal UGTs might contribute to intestinal homeostasis of 5-HT. Liver is one of the most important organs in terms of detoxifying or getting rid of foreign substances or toxins (including micro-organisms), especially from the gut, with major percentage of blood being filtered is from the portal vein, which carries blood from intestines. Liver is also the major site for breakdown of fats. It has been reported that UGTs are responsible for cholesterol homeostasis in liver. During sustained stress response, when there is continuous release of esterified fatty acids from fat deposits of body, liver is under high pressure and might eventually malfunction due to over-activity. A functional analysis of the GO enrichment profile of human stressome thus aptly prioritizes liver as one of the major organs to be affected during stress response. Moreover, efferent sympathetic system has been implicated in stress-induced exacerbation of liver diseases while, the efferent parasympathetic nervous system elicits an

inhibitory effect on the development of hepatic inflammation [210]. The stress-disease network also consequently showed that liver associated disease (Hepatitis C) to be the most over-represented disorder.

Signaling through HTR1c and HTR2a have also been reported to increase mean arterial blood pressure and renin activity [211] as is also reflected by the stressome enrichment profile. Nitric Oxide (NO) is known to control blood pressure. NO is produced by iNOS and eNOS from L-arginine and known to counteract angiotensin II and inhibit glomerulosclerosis, interstitial fibrosis, microvascular lesions and podocyte stress [212] in hyper-cholesterolomic rodents. Adrenergic innervations have been identified in the renal vasculature and renal sympathetic activity tends to be increased during depleted circulating volume [213-215], thereby implying renal-controlled blood pressure as a stress responsive measure. This shows that kidneys on the other hand seem could be another major organ highly affected by stress.

As was seen from the stress-disease network, PTGS2 was also associated with L-glutamate transport and glutaminergic nerve transmission. It is already known that glucocorticoids mediate stress-induced accumulation of extracellular glutamate. This creates exitotoxicity in the nervous system and might be one of the leading causes of neuro-degenerative disorders [216]. PGDH (Prostaglandin dehydrogenase) is a stress gene which is highest represented in stress-diseases network. This again emphasizes the relevance of lipid metabolism/turnover and resulting accumulation of oxidized products as one of the major causes of stress-associated diseases.

2.3.4. Hints of compensatory mechanisms in psychological stress manifestation

Enrichment analysis of $\mathbf{II(S)} \cap \mathbf{M(S)}$ with $\mathbf{II} \cup \mathbf{M}$ as background revealed that most of the enriched terms were populated by the following genes: IL6, IL1B, IL10, IL13, TNF and IFNγ. These are the genes which are involved in the inflammatory response. Owing to the very high

enrichment of the inflammatory cytokines, the data thus hints at the association of inflammatory response with psychological stress manifestation in the body, as has already been observed and reported in literature. Critical to this observation is the fact that while IL6, IL1B, TNF, IFNΓ etc are pro-inflammatory cytokines and are generally involved in acute inflammatory response, IL10 and IL13 are believed to be anti-inflammatory cytokines. This would suggest that both pro-inflammatory and anti-inflammatory cytokines are associated with manifestation of stress (as is also justified by the enrichment of GSK3 in cluster 4 of II(S)⋃M(S) network [217]), which looks contradictory. But, it looks quite relevant in the context of psychological stress since, pro-inflammatory cytokines are produced in response to a psychological stressor, but if they continue to be produced, they can have a damaging effect on the body which includes septic shock. So the body tries to counter-balance its effects by inducing an anti-inflammatory response. The enriched GO terms also show the up-regulation of protein secretion and transport in addition to regulation of acute inflammatory response as would be quite expected in body's inflammatory responses. It is already known, CARS (Compensatory Anti-inflammatory Syndrome) develops in response to SIRS (Systemic Inflammatory Response Syndrome) to protect the body from the harmful effects of pro-inflammatory cytokines and acute phase proteins to restore the basal homeostasis of the body. In case of psychological stress also, it seems quite logical that the body's adaptive response follows the same rule as can also be observed from the pattern of enriched GO terms in the enrichment analysis.

2.3.5. The Feed-back loop in chronic stress

Cortisol/Glucocorticoid suppresses production of inflammatory cytokines and mobilizes glucose out of the cells, while high levels of glucose is known to induce production of IL6 (a pro-inflammatory cytokine) from monocytes. Glucose level in plasma is elevated during

psychological stress as an effect of the HPA activity by corticosteroids, most probably when the plasma glucose level shoots beyond a certain limit; an inflammatory condition is created in the body. Such a situation is typical of chronic stress or high corticosteroids in body. This inflammatory condition might as well cause activation of a compensatory phase response which would now stretch beyond its normal range due to frequent induction (priming).Based on the above observations and discussions, a probabilistic model has been suggested for explaining the interplay between various biological processes going on inside the body when a body is subjected to psychological stress and its manifestations on the body in long run (figure 2.12).

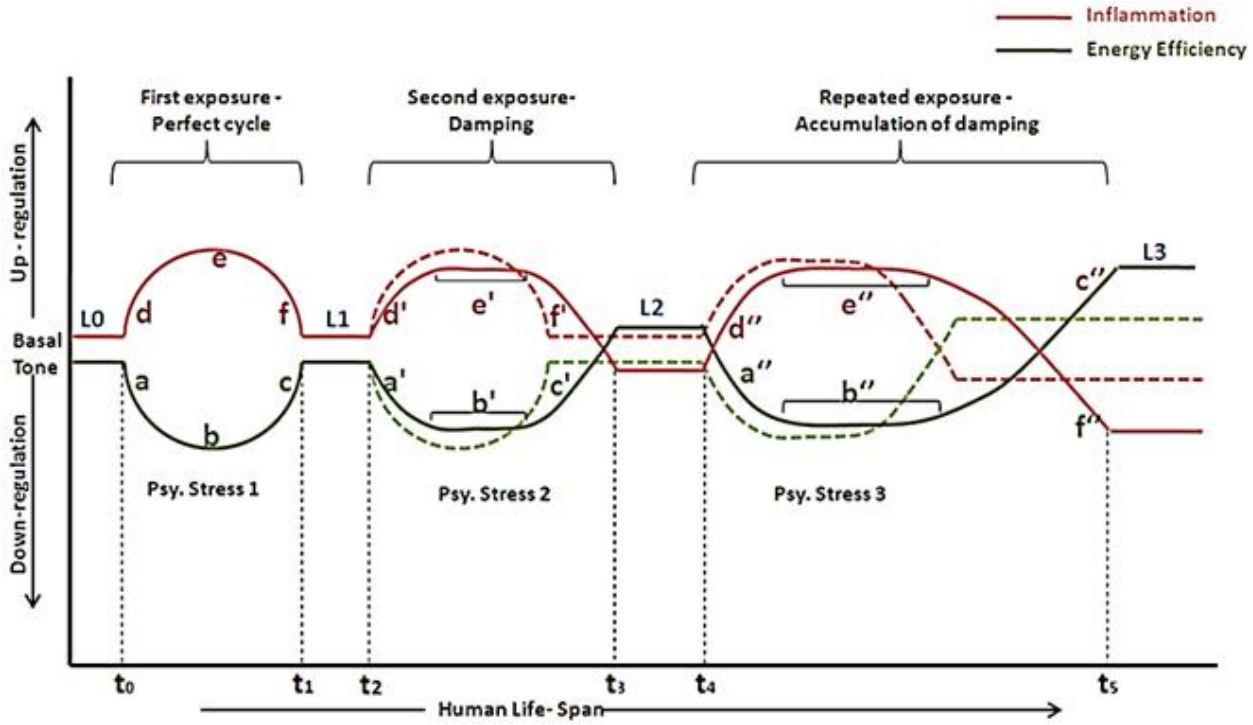


Figure 2.12: Systematic-analysis based prediction model of psychological stress and associated homeostatic imbalance. A probabilistic model is developed to establish coping and adaptive responses to understand psychological stress as a challenge to normal physiology. Colored solid lines indicate the actual deviations from the basal tone, colored dotted lines represent expected, non-deviated basal tone after each cycle, while colored dashed lines show actual deviations from basal tone in case of a psychological stress challenge extending over a long time-period.

Body's basal tone of inflammatory state and energy efficiency in terms of body mass index are represented by L0. Whenever there is psychological stress, an inflammatory response is evoked

(shown as ‘d’) by activation of innate defense responses and increase in production of pro-inflammatory cytokines. While at the same time, a sickness behavior [218] is induced in the body which cuts down all energy expensive processes including food intake and digestion, raises the body temperature and makes the body socially withdrawn to conserve energy. The initial phase of is dominated by production of flight-fright hormones (catecholamines) which trigger lipolysis, glycogenolysis, etc[219]. All these processes are aimed to provide for the energy required for maintenance of the high inflammatory state thereby reduce the energy efficiency in terms of body mass (shown by ‘a’). Flight- fright response is followed by an increase in the production of cortisol which tends to raise the glucose level in blood by stimulating catabolic processes, but at the same time it has an inhibitory effect on the production of pro-inflammatory cytokines, thus acting as a brake on the increasing slope of inflammatory state (only after attaining threshold concentrations in blood) which plateaus (‘e’) and starts declining (‘f’) under its action. The ECS and other stress recovery systems get activated by some unknown mechanism/s, but they increase appetite, reduce the sickness behavior and increase energy efficiency by inducing weight gain (‘c’). Both these processes bring the body back to its basal tone (L1).What remains unclear here is what signals the transition from ‘b’ to ‘c’ and activates ECS and other stress recovery systems. It looks quite justified from the model that extremely high levels of cortisol in blood during ‘b’ might trigger the activation of stress recovery systems and thereby transition from ‘b’ to ‘c’. This should also be the point, where cortisol levels start declining and reach basal levels (L1) by time t_1. An interesting point to take note of is, excess of glucose levels are known to increase production of pro-inflammatory cytokines. So, blood glucose levels might be one of the mechanisms which might be involved in controlling cortisol levels (since excess of cortisol can create a hyperglycemic state). This looks like a quite

simplistic cycle working towards restoring homeostasis in the face of a psychological stress, but the scenario becomes a bit complicated if the body is repeatedly faced with such challenges or if the challenge itself persistently lasts extending over a considerable period. As can be seen from the figure 7, when the body encounters the challenge second time, the slope of increasing inflammatory state (d') and decreasing energy efficiency (a') decreases implying that the response is slower. The plateau stage also probably remains for a longer time (b'), which implies that cortisol acts for a longer period and hence suppresses the inflammatory cytokines below the basal levels (f'). In this situation body glucose levels would not be able to regulate cortisol levels by feed-back mechanisms, since most probably the stress recovery systems which act longer now (c') use up the excess blood glucose for anabolic activities for increasing energy efficiency. Thus, by time t_4 both these process reset the body's basal tone at L2, which denotes a higher energy efficiency in terms of body mass, and a lower than normal inflammatory state. Again when the body encounters the challenge a third time, the basal tone becomes reset at a much deviated level, L3 by time t_6. Thus with each cycle, a damping effect on these systems is most likely to be exhibited if they are frequently stimulated. This dampening effect causes the compensatory mechanisms to deviate from the basal tone and become reset such that the body weight is increased and the innate immune responses are lowered down. This mechanism might increase susceptibility to infection and might lead to obesity and other metabolic syndromes. An important point to note here is that body's adaptive response might also depend on the interval between two challenges viz. t_1-t_2 and t_4-t_5, which might also decide where the body's basal tones are being reset after each cycle. However, in the case of a challenge which extends over a long time-period t_1-t_3 (shown by dashed lines), where cortisol acts longer and thereby suppresses the inflammatory state below the basal tone, while the compensatory stress recovery system works

longer in response to it and the body's basal tone becomes rest at L1' instead of L1 at time t_3. This suggests a justified explanation for infectious disease susceptibility [220], [221], obesity [222] and other metabolic syndromes associated with chronically stressed people. This also hints to the aberrant weight loss, withdrawn behavior and flu-like symptoms (due to acute phase response) seen in people have short-term acute stress shown by the initial fluctuations from basal tone when body encounters psychological stress. Based on this model, physiological homeostasis of our body can assumed as an elastic system, which rebounds to its original shape after the stress stimuli is withdrawn. But when the body tries to push beyond the elastic limits of its adaptive scope, a hysteresis loss (biologically exemplified by mal-adaptation/ dysfunction) is expected, which is well supported by the analysis based on observations so far in literature related to psychological stress.

2.3.6. Glucocorticoid induced candidate chronic compensatory mechanism during stress response

ANS, through epinephrine, initiates stress response system (coping mechanism) by targeting physiological energy reservoirs and mobilizing energy. Low energy state of physiology is detected and relayed across the system to activate various compensatory mechanisms to replenish energy storage. Adipose tissue is one of the most important endocrine organs, which also acts as a reservoir of energy and might be one the immediate targets of sympathetic nervous stimulation. Studies show that epinephrine causes a significant rise in adipose tissue blood flow and net efflux of non-esterified fatty acids (NEFA) under the action of both HSL (Hormone sensitive lipase) and LPL (Lipoprotein Lipase) [223]. It is also hinted by GO enrichment of phospholipase C-activation term in human stressome.

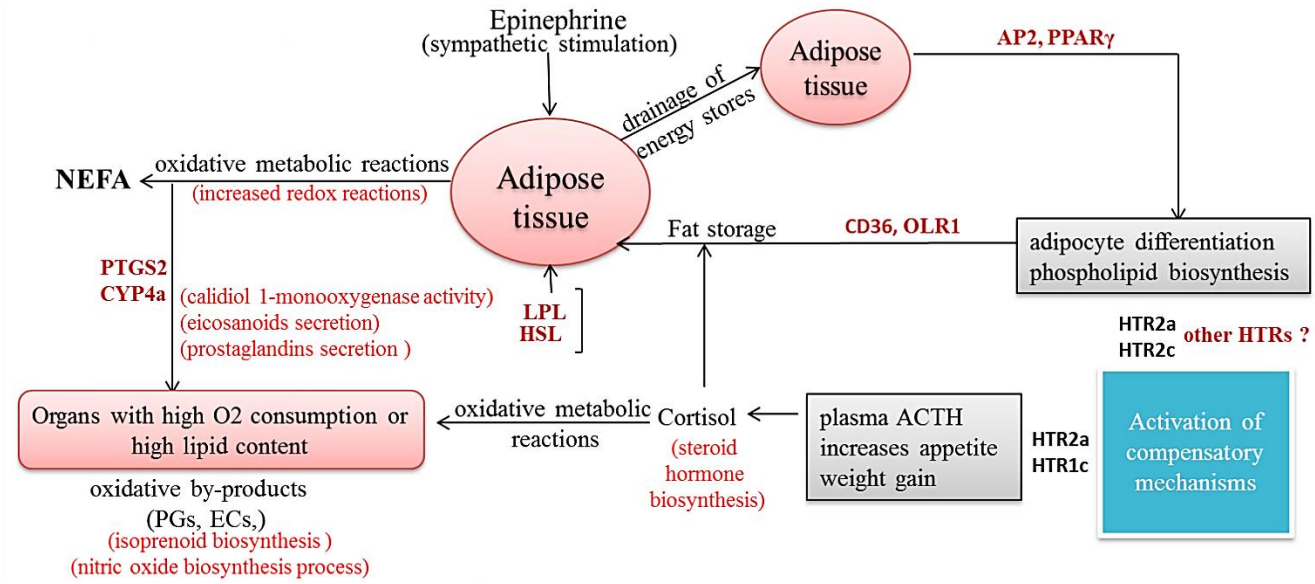


Figure 2.13: Model for cortisol induced chronic compensatory mechanism during stress response

This analysis suggests involvement of adipose tissue in providing instantaneous energy during sympathetic response to stress; a response that can drain vital reserves of the system so the physiology would try to replenish the energy storage by activating compensatory mechanisms during an extended stress exposure. It has long been demonstrated that cortisol negates epinephrine-induced limbic arousal during acute stress response. Various isotypes of HTRs are implicated in diverse mechanisms of stress response. Neuronal signaling through HTR1c and HTR2a is known to increase appetite and body weight. In rodent models of hypertension, neuronal signaling through HTR1c and HTR2a has been shown to have stimulatory effect on plasma levels of ACTH and epinephrine (not norepinephrine). Enriched HTRs (esp. HTR2c and HTR2a) are implicated in phospholipid biosynthesis, suggesting signaling through these receptors might contribute to lipid accumulation during stress. HTR2c and HTR2a are already established in promoting adipocyte differentiation and fat cell development, thereby contributing to obesity [224]. This adipogenic signaling through HTRs in adipose tissue is boosted by corticosteroids [225], implying that cortisol aids in fat storage to compensate action of

epinephrine. High levels of cortisol have also been reported to increase PGs level in vitro [194]. Stress has been shown to increase lipid peroxidation and protein oxidation in liver, brain and plasma [195]. It is also known that organs/sites of high oxygen consumption, and/or high lipid content are highly susceptible to redox reactions. PTGS2 which is highly represented among the enriched terms oxidizes arachidonic acid derived from lipids to produce PGs, ECs, prostacyclins, isotrenes, etc. These oxidation derived metabolites during stress response accumulate and exert deleterious effects on the concerned organs. Hence, we synthesized the following model to validate the action of stress hormone cortisol and serotonin as a candidate hormone that could be acting in a compensatory manner during stress response (figure2.13).

CHAPTER 3

VALIDATION OF SELECT GENE-ASSOCIATION PREDICTIONS THROUGH GENE-ASSOCIATION NETWORK SYSTEMATIC-ANALYSIS

Adipose tissue, as a potent endocrine organ as well as the body's primary energy storage reserve, plays key roles in systemic metabolism by modulating metabolic and immune homeostasis. Psychological stress is a well-established contributor to inflammatory metabolic disorders like obesity, dyslipidemia, etc. often marked by local inflammation[226-228], aberrant hormonal signaling and adipokine secretion, and altered lipid storage dynamics [229] of adipose tissue. Based on the predicted model of compensatory stress response described in previous, we speculated that serotonin might be involved in the inflammatory and/or metabolic stress response system, via HTRs receptors. A detailed back-ground search on the endocrine role of serotonin revealed that, gut is the major site of serotonin production. Serotonin content in inflamed adipose tissue is also very high [92], which also correlates well with increased visceral obesity [87, 229, 230] observed in subjects of chronic stress. Macrophages and immune cells migrate into adipose tissue during adipose tissue inflammation (stress being one of the contributing factors of adipose tissue inflammation). So, we designed an in vitro simulation of adipose tissue to understand the effects psychological stress on the balance between immunity and metabolism. This was achieved by doing an indirect co-culture of 3T3L1 adipocytes cells and RAW 264.7 macrophage cells in a stress micro-environment (figure 3.1), where they were treated with cortisol (the established stress hormone) [34] and serotonin as a candidate regulatory hormone [191, 231] in adaptive stress response.

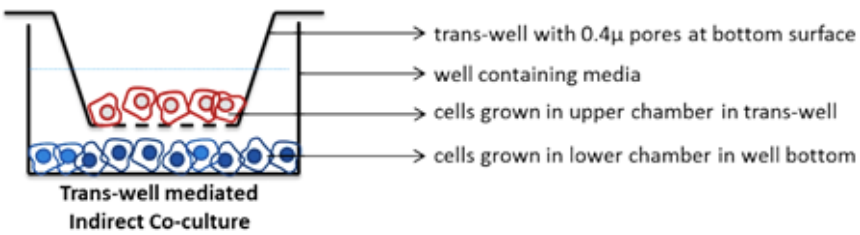


Figure 3.1: Experimental set-up for in-vitro simulation through indirect adipocyte and macrophage co-culture.

3.1. Role of HTR5a and HTR2c in cortisol-induced peripheral serotonergic signaling in adipocytes during stress

Psychological stress causes release of Corticotrophin Releasing Factor (CRF), a hormone that stimulates the release of cortisol from adrenal glands and is responsible for sustained glucocorticoid production during chronic stress. CRF is also capable of altering brain-gut interaction by stimulating serotonin release from Enterochromaffin cells (ECs) [79] showing that vulnerability of gut to stress hormones is very high. Growing number of evidence has started pointing towards association of stress and gut-derived serotonin with central/visceral obesity, adipose tissue inflammation and atherosclerosis [85-88]. Systematic-analysis studies discussed earlier have further indicated that serotonergic pathways could be one of many possible adaptive regulatory mechanisms of stress[232]. In the present study, our goal is to understand the adipogenic signaling events in adipose tissue during chronic stress. We attempted to achieve the goal by mimicking adipose tissue (which is a combination of adipocytes and immune cells) *in vitro* by subjecting adipocyte-macrophage co-culture systems to glucocorticoids (a stress adaptation hormone) and serotonin (a glucocorticoid-induced candidate regulatory hormone). Our results established the role of cortisol induced peripheral serotonin signaling in adipocytes via., two serotonin receptors HTR5a and HTR2c (which were not reported earlier) in enhancing adipogenesis.

3.1.1. Materials and Methods

3.1.1.1. Cell culture

Pre-adipocyte cells, 3T3-L1 and murine macrophage cells RAW 264.7, were purchased from national cell repository at National Center for Cell Science (NCCS) at Pune, India. 3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM, HiMedia, India)

supplemented with 10% (v/v) heat inactivated fetal calf serum (HiMedia, India), 4 mM L-Glutamine (Sigma, USA) and Amphotericin-B (Sigma, USA) and Gentamycin (Sigma, USA) at $37^{o}C$ with 95% air and 5 % CO2. Medium was renewed every 2-3 days and were harvested for sub-culturing by 0.25% trypsin with 1 mM EDTA (Sigma, USA) for 3 min at 37°C when the confluence reached 70%. RAW 264.7 murine macrophage cells were cultured in DMEM supplemented with 10% (v/v) heat inactivated fetal bovine serum (HiMedia, India), 4 mM L-Glutamine and Amphotericin-B and Gentamycin under similar atmospheric conditions. The cells were harvested for sub-culturing at 80% confluence. Differentiation was induced in 3T3 cells maintained at confluence for 2 days. After two days, the media was changed and supplemented with 10% fetal bovine serum, 0.5 mM methyl isobutyl xanthine (IBMX), 1 µM dexamethasone (Sigma, USA), and 1µg/ml insulin (Sigma, USA) [This media is termed as differentiation media for future reference] and maintained for 2 more days. Subsequent media changes containing 10% fetal bovine serum and 1 µg/ml insulin [maintenance media for future reference] was done until the cells were harvested.

Indirect (trans-well) co-culture was performed in two different ways as follows. In one set-up, macrophages were seeded (1×10^6 cells) on 0.4-µm-pore-size cell-culture inserts (BD Biosciences, India) and then placed in 6-well plates containing 3T3-L1 adipocytes differentiated for 2 days (1×10^6 cells). In another set-up, 3T3-L1 were seeded on 0.4-µm-pore-size cell culture inserts, differentiated for 2 days and then placed in 6-well plates containing RAW 264.7 macrophage cells. Both co-culture set-ups were treated with 100 µM cortisol(C), 100 µM serotonin(S) and mixture of cortisol and serotonin (CS) containing 100 µM of each of cortisol and serotonin for 48h. Cells were harvested from the lower well for RNA extraction in each set-up.

3.1.1.2. Oil-Red-O Triglyceride assay

3T3-L1 adipocytes (36,000/well) were seeded in 96-well plates and differentiation induction was done as described in preceding sections. After 48h of differentiation induction, media was replaced by maintenance media containing either cortisol or serotonin or mixture of cortisol and serotonin as various treatment conditions. After 48h of treatment, cells were fixed with 4% Paraformaldehyde (Sigma, USA) solution and stained with Oil-Red-O (HiMedia, India) stain. Elution of stained fat droplets was done with 100% isopropanol (HiMedia, India) and assayed by taking absorbance at 590 nm.

3.1.1.3. Microscopy

Either 3T3-L1 or RAW 264.7 cells were grown on coverslips and placed in the lower chamber of indirect (trans-well) co-culture systems as described in preceding section. The macrophages were fixed and stained with DAPI (Sigma, USA) and Oil-Red-O after 72 h of treatment and scanned using confocal microscopy. Adipocytes were stained with Oil-Red-O after 72h treatment. Oil-Red-O stained cells were imaged by light microscopy using inverted microscope (Olympus, USA). Confocal imaging of DAPI stained cells was done using Laser Scanning Confocal Microscope (LSM 780, Carl Zeiss, Germany).

3.1.1.3.1. Image Analysis: Quantification of lipid content in macrophages was done using droplet counter plugin in ImageJ software. For each treatment condition, confocal images of co-cultured macrophages were at taken five separate regions of coverslip. Lipid content per cell for each treatment was determined by averaging the total droplet volume with the total number of cells (determined by counting DAPI stained nuclei).

3.1.1.4. Real-time PCR

Total RNA from RAW 264.7 or 3T3L1 cells were extracted using RNeasy Mini Kit (QIAGEN, USA). cDNA was synthesized from RNA using Affinity Script One-Step RT-PCR Kit (Agilent, USA) as per the manufacturer's protocol. Briefly, 5 µg of total RNA was mixed with the buffer containing Affinityscript reverse transcriptase and polyT primer. The mixture was kept in the thermo cycler at 45^{o}C for 30 min to synthesize C-DNA. Then the temperature was raised to 92^{o}C for 1 min to deactivate the enzyme. qRT-PCR reaction was set in a 96 well PCR plate. The template, required primer, buffer and SYBR green along with DNA polymerase were added in PCR plate as per the manufacturer's protocol. The plate was then kept in the q-RT-PCR machine (Mx3005P, Stratagene, USA) and the machine was programmed as follows; 2 min at 92^{o}C to activate DNA polymerase for 1 cycle, 15 sec at 92^{o}C for melting and 1 min at 60^{o}C for primer annealing along with extension of the chain and detection of the florescence for 40 cycles, then a program to find out the melting temperature of each product. Cycle threshold (Ct) values were noted and fold changes of the desired genes were calculated with respect to the control after normalizing with internal control gene β-actin. The qRT-PCR reactions were set up as 3 technical replicates along with no template control and no primer control.

The oligonucleotides used as primers are provided in table 3.1.The expression value of each gene was normalized to β-Actin as internal control.

Table 3.1: Primer sequences used for RT-PCR

	Sense strand	**Antisense strand**
Ap2	5'-AAATCACCGCAGACGACAGGAAGG-3'	5'-CACATTCCACCACCAGCTTGTCAC-3'
Cd36	5'-GGCTAAATGAGACTGGGACCATTG-3'	5'-CAAGTAAGGCCATCTCTACCATGC-3'
Htr1a	5'-CTCGGTCTTTGGCCCTAGGATCAG-3'	5'-GTCCTAGTGGGCATGGTAGATGTC-3'
Htr1b	5'-GATCGTGCTGGTGTGGGTCTTCTC-3'	5'-CTTGCCGGTCTTGTTGGGTGTCTG-3'
Htr1d	5'-CTGGAGTACAGCAAGCGTCGAACC-3'	5'-GTGTTCACCAGGCAGTCGGACATC-3'

Htr2a	5’-TGCTGCTGGGTTTCCTTGTCATGC-3’	5’-TCTGGAGTTGAAGCGGCTATGGT-3’
Htr2b	5’-CAATAGGCATCGCCATCCCAGTCC-3’	5’-CAGTCCACCGTGTTAGGCGTTGAG-3’
Htr2c	5’-CTCTCCCTTCCTTCCGTATTCC-3’	5’-GCCACCTGAGATGAACATCAAC-3’
Htr3a	5’-CTGCCCAGTATCTTCCTCATGGTC-3’	5’-GATGGTCTCAGCGAGGCTTATCAC-3’
Htr3b	5’-TACGGTCTTCAGGGTCAACATGTC-3’	5’-AGTCGCCTTGAAGGCACATAAGAG-3’
Htr4	5’-ATGCTCACCTTGTGGAGCCTTGTC-3’	5’-AAGCAGGGCCTGGGCTTACATTTG-3’
Htr5a	5’-GGCTAACAGCAGCCATGAGCTATC-3’	5’-CTGTGGCCGTATAAACACTGTGTC-3’
Htr5b	5’- GCCCTCCTATGCTGTCTTCTCCAC -3’	5’- GTCTCCGCTTGTCTGGAAGGTTAC -3’
Pparγ	5’- GCCCTGGCAAAGCATTTGTATGAC -3’	5’- TGATCTCTTGCACGGCTTCTACGG -3’
Sert	5’- GACCAGTGTGGTGAACTGCATGAC-3’	5’- GGCCTGCGAACGTACTATCCAAAC-3’
Olr1	5’- CTCAGTCTCCTCTACCTCAGTATG -3’	5’- GGCAAAGAGTACTGGCTGTTCTTC -3’
βActin	5’-CTGACGGCCAGGTCATCACTATTG-3’	5’-GACAGCACTGTGTTGGCATAGAGG-3’

3.1.1.5. Statistical analysis

Statistical analyses were performed with GraphPad Prism version 5.01 using two-way ANOVA and Bonferroni post-tests. p-value < 0.05 was considered to be statistically significant.

3.1.2. Results

We aimed at understanding effects of cortisol and serotonin on adipogenesis and accompanying signaling events in adipocytes grown separately and in indirect co-culture with macrophages. Results revealed that adipocytes showed higher lipid content in co-culture compared to cells grown alone. It was observed that in adipocytes, increased lipid content was accompanied by up-regulation of serotonin receptors HTR5a and HTR2c when treated with high doses of cortisol. When serotonin (natural ligand for these receptors) was provided along with cortisol, the lipid content in adipocytes further increased by two folds and this effect further increased significantly when co-cultured with macrophages. Increased adipogenesis and lipid accumulation was also observed in macrophages on administration of cortisol and serotonin and this effect was enhanced when co-cultured with adipocytes. Results further indicated that the adipogenic

responses of macrophages and adipocytes to cortisol and serotonin are regulated by completely different mechanisms.

3.1.2.1. Cortisol and Serotonin act together to increase lipid content in adipocytes

Differentiated adipocytes treated with cortisol for 48h showed increasing lipid content with cortisol dose (figure 3.2a). Treatment of differentiated 3T3L1 adipocytes with cortisol (C), serotonin (S), and mixture of serotonin and cortisol (CS) over a range of 0.01–500μM resulted in overall increased lipid content with dose (figure 1b) in adipocytes and started saturating at 100 μM of cortisol and/or serotonin given alone or together. It was observed that treatment with CS at 100 μM of each yielded more lipid accumulation than any other conditions reported in this study without compromising cell survivability. Therefore 100 μM concentration was selected for each treatment condition viz. C, S, and CS (cortisol 100 μM and serotonin 100 μM given together) as optimal condition for further studies.

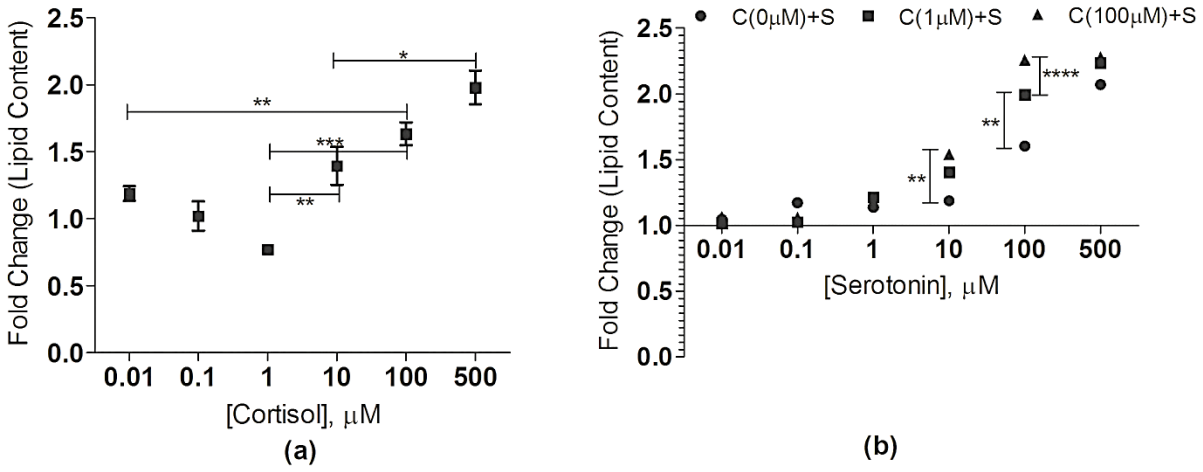


Figure 3.2: Dose titration of cortisol and serotonin in adipocytes. Effect of **(a)** cortisol on adipogenesis presented as relative fold-changes of lipid content in adipocytes measured by Oil-Red-O Assay, represented as mean +/- SEM, **(b)** serotonin (0.01 -500 μM) with no cortisol (C), 1 μM and 100 μM

Adipogenic profile of cortisol treated adipocytes corroborated with earlier reports [224, 233]. Treatment of adipocytes with CS showed 2.4 fold increase in lipid content against C alone (100

μM) at 1.7 and S alone (100 μM) at 1.6 fold change (figure 3.2b). Therefore, transcriptional profile of all HTRs was checked through qRT-PCR in adipocytes treated with CS at 100 μM concentration each (addressed as CS100 hence forth). It was found that serotonin receptors HTR5a, HTR2c and serotonin transporter SERT were up-regulated 4.6, 2.0 and 2.0 folds respectively because of this treatment. To determine whether the increased lipid content was indeed due to increased adipogenesis or adipocyte differentiation, transcriptional expression of adipogenic transcription factor (PPARγ), adipocyte differentiation marker (AP2) were evaluated. It was seen that CS100 treatment also up-regulated PPARγ by 3.1 fold and AP2 by 4.7 folds in adipocytes (figure 3.3). These results showed that the genes HTR5a, HTR2c, SERT might be associated with the increased adipogenicity (as seen by higher transcription of PPARγ and AP2 figure 3.3), and higher lipid content (as seen from figure 3.2b) in differentiated adipocytes treated with CS100.

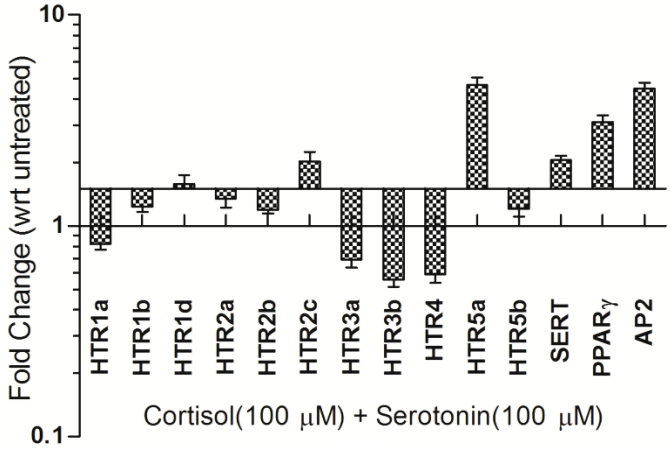


Figure 33: Effect of 100 μM of each of C and S together (CS100) on expression of HTRs, SERT, PPARγ and AP2 at transcriptional level

3.1.2.2. HTR5a and HTR2c antagonists reduce adipogenic transcriptional activity and lipid accumulation adipocytes.

To test the roles of HTR5a and HTR2c as mediators of adipogenic effects of cortisol and serotonin in adipocytes, differentiated 3T3L1 adipocytes were treated with antagonists of HTR5a and HTR2c. Adipocytes were treated with SB699551-A, a selective HTR5a antagonist (pKi =

8.3) and SB242084, a selective HTR2c antagonist (pKi = 9) at various concentrations over a range of 0.1-200 µM for 48h. It was observed that increasing concentration of the receptor antagonists, SB699551-A and SB242084 significantly decreased the lipid content up to 50 µM of antagonist concentration. However, increasing the antagonist concentration beyond 50 µM affected cell survival (figure 3.4a). Hence, 10 µM concentration of each inhibitor was chosen as optimal and effective dose for treatment based on ours and other studies reported elsewhere [224, 234, 235]. It was observed that adipocytes treated with CS as well as only C and only S had higher lipid content than adipocytes treated with each of these antagonists (figure 3.4b).Increased lipid content in adipocytes can be a result of higher transcription of adipogenic genes (PPARγ) and higher adipocyte differentiation. Other factors like increased expression of scavenger receptors (CD36 and OLR1) can also lead to increased lipid content in adipocytes via higher intake of oxidized lipids.

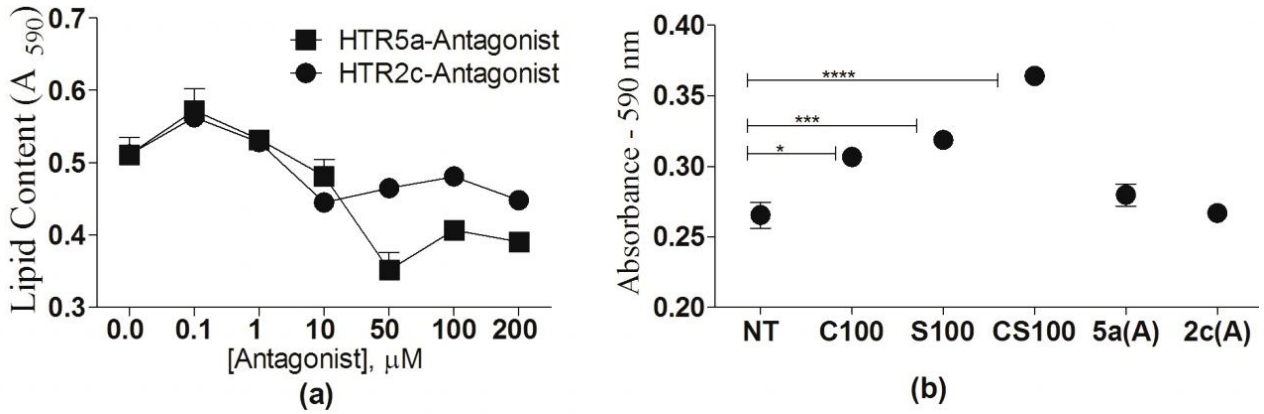


Figure 3.4: Effect of HTR5a and HTR2c antagonist on lipid content in adipocytes**.(a)** Dose-titration of HTR5a and HTR2c antagonist (0-200 µM) in adipocytes measured by Oil Red O assay (in terms of absorbance at 590nm) , **(b)** Lipid accumulation in untreated adipocytes and adipocytes treated with C100, S100, CS100, HTR5a-antagonist (10 µM) and HTR2c-antagonist (10 µM) measured by Oil Red O assay.

Comparison of transcription of these genes viz. PPARγ, AP2, OLR1 and CD36 in adipocytes treated with antagonists of HTR5a, HTR2c as well as natural ligand (serotonin) of HTRs revealed that blocking signaling through HTR2c suppressed the expression of PPARγ (1.2 fold),

OLR1 (no significant change) and CD36 (1.5 fold), while AP2 (3 fold) expression still persisted (figure 3.5a).By blocking signaling through HTR5a expression of AP2 (1.2 fold) was repressed, while PPARγ (2.4 fold) expression still persisted yet dampened. However, HTR5a antagonist did not affect expression of OLR1 (3.2 fold) and CD36 (2.9) (figure 3.5a). Figure 3.5c revealed that treatment with CS100 boosted the expression of PPARγ, AP2, OLR1 and CD36 while treatment with C-5a(A) and C-2c(A) suppressed expression of all the genes except AP2, whose expression still persisted (around approx. 2 fold) although dampened.

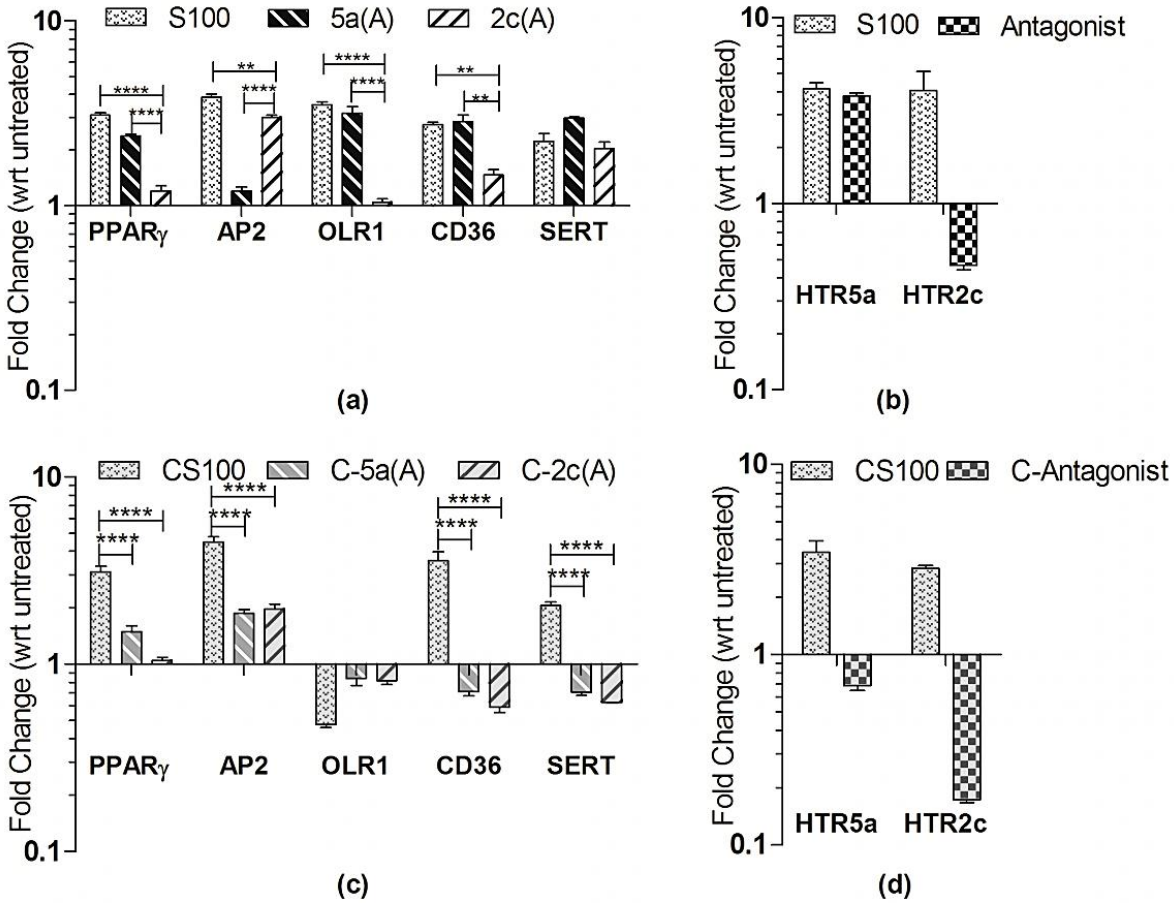


Figure 3.5: Effect of HTR5a antagonist and HTR2c antagonist on expression of PPARγ, AP2, OLR1, CD36 and SERT in adipocytes. Relative transcriptional fold-changes of **(a)** PPARγ, AP2, OLR1, CD36 and SERT in adipocytes treated with S100, HTR5a-Antagonist (10 µM) and HTR2c-antagonist (10 µM) expressed as fold-changes against time-matched untreated controls, **(b)** HTR5a in adipocytes treated with S100 and Antagonists (10 µM), of HTR5a and with respect to time-matched untreated controls, **(c)** PPARγ, AP2, OLR1, CD36 and SERT in adipocytes treated with CS100, antagonists (10 µM) of HTR5a and HTR2c in the presence of C100 [denoted as C-5A or C-2c) against time-matched untreated controls and **(d)** HTR5a and HTR2c in adipocytes treated with CS100 and C-5a or C-2c against time-matched untreated controls.

It was observed that SERT being serotonin transporter did not show any change from serotonin treatment, when HTR5a and HTR2c signaling was blocked, however when cortisol was added along with these treatments, SERT expression was suppressed (figure 3.5a and 3.5c). The results thus showed that signaling through HTR5a and HTR2c is important for enhancing adipogenesis and increasing lipid content in adipocytes.

3.1.2.3. Role of co-culturing in HTR5a and HTR2c mediated adipogenic signaling

Differentiated adipocytes were co-cultured with macrophages (as described in methods earlier) to study whether presence of macrophages influenced the adipogenic effect of cortisol and serotonin on adipocytes. Figures 3.6 and 3.7 show extent of lipid accumulation in adipocytes, grown alone and co-cultured, under various conditions as illustrated. It was observed that lipid droplets, in co-cultured adipocytes, took defined and round shapes only in co-cultured conditions. Number of defined and well-shaped droplets was higher in co-cultured adipocytes treated with CS100 than any other treatment condition.

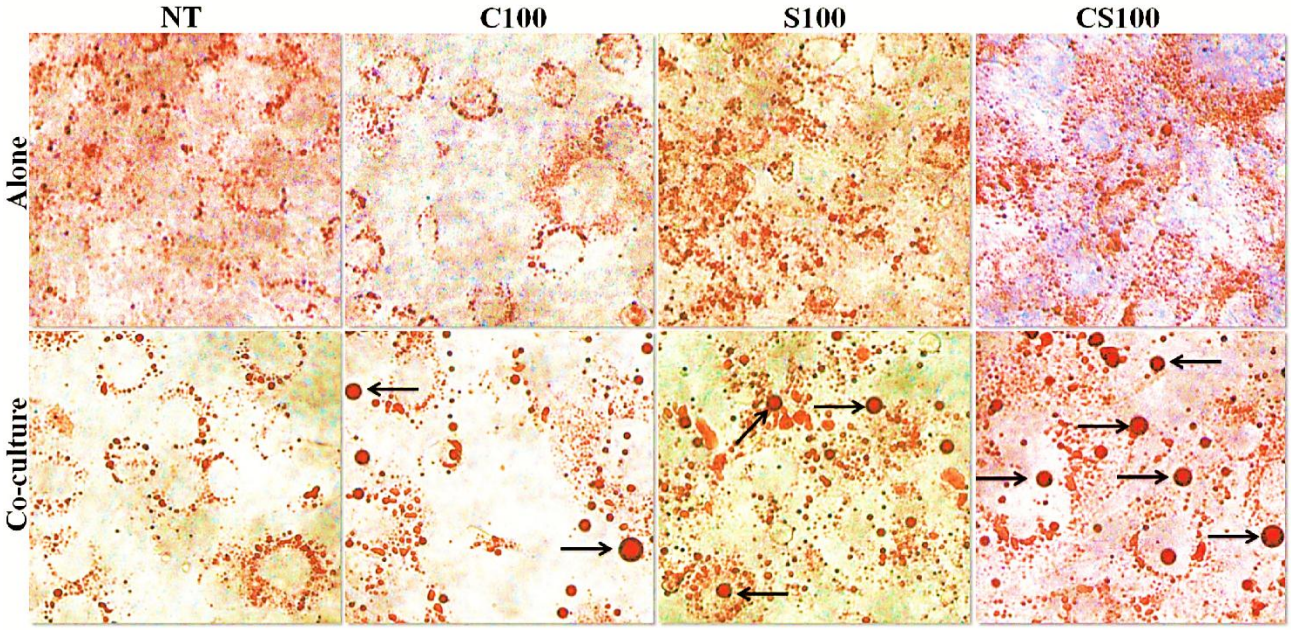


Figure 3.6: Light Microscopyof ORO-stained adipocytes grown alone and adipocytes co-cultured with macrophages.Oil-Red-O assay of untreated adipocytes as well as treated with C100, S100 and CS100 assayed qualitatively by light microscopy (Round shaped solid red colored objects in the images are fat droplets as observed in adipocytes co-cultured with macrophages).

Defined lipid droplets are indicated by arrows (figure 3.6). An Oil-Red-O assay for each of the treatments confirmed that co-cultured adipocytes accumulated more lipids than adipocytes grown alone, while CS treatment resulted in higher lipid accumulation than any other treatment conditions (figure 3.7).

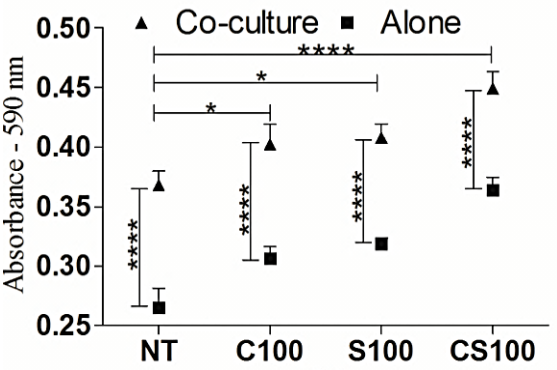


Figure 3.7: Lipid content in Oil Red O stained adipocytes grown alone and adipocytes co-cultured with macrophages untreated as well as treated with C100, S100 and CS100 assayed quantitatively by measuring absorbance at 590 nm.

It was also observed that the genes shown to enhance adipogenesis due to CS100 treatment in adipocytes grown alone were further up-regulated due to co-culturing with macrophages under same treatment conditions. In co-cultured adipocytes, significant up-regulation of HTR5a (8.6 folds), HTR2c (6.9 folds), PPARγ (7.8 folds), CD36 (6.8 folds) and OLR1 (4.8 folds) (figure 3.8a) was observed compared to lower expression levels in adipocytes grown alone. This corroborates well with higher lipid contents of co-cultured adipocytes treated with CS100 (figure 3.6 and 3.7).To check, whether this effect was indeed due to the treatment with cortisol and serotonin or due to co-culturing, the transcriptional profile of untreated adipocytes grown alone was also compared with untreated co-cultured adipocytes. It was seen that co-culturing contributed to increased transcription of OLR1, while PPARγ, AP2 and CD36 were suppressed in co-cultured adipocytes (figure 3.8b).The results clearly revealed that cortisol and serotonin acted together in inducing signaling through HTR5a, HTR2c and the accompanied increase in

transcription of adipogenic genes resulted in increased lipid content in co-cultured adipocytes treated with CS100.

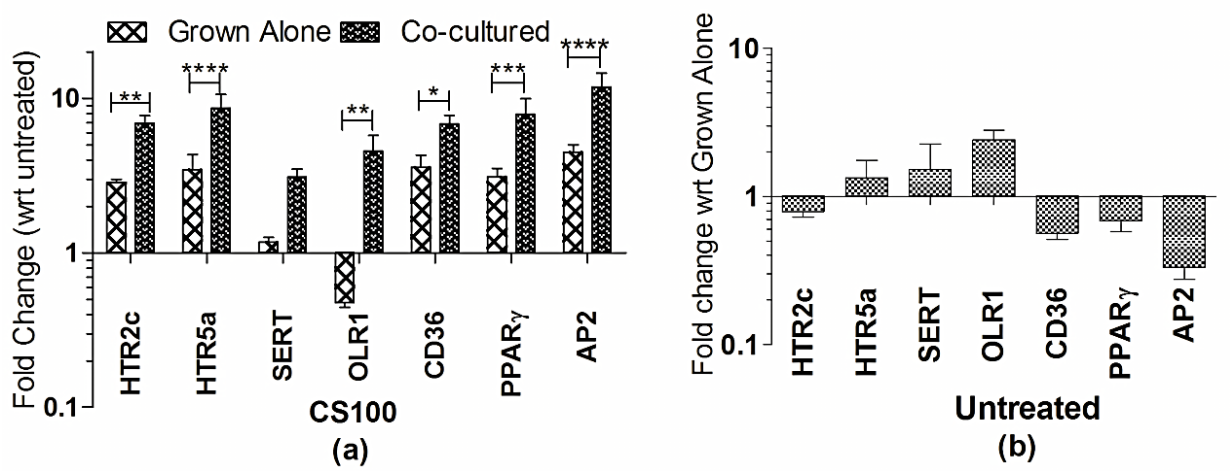


Figure 3.8: Effect of co-culturing on expression of HTR2c, HTR5a, SERT, PPARγ, AP2, OLR1, CD36 in co-cultured adipocytes. Relative transcriptional fold-changes of HTR2c, HTR5a, SERT, PPARγ, AP2, OLR1, CD36 in **(a)** CS100 treated adipocytes co-cultured as well as grown alone against time-matched untreated controls, and **(b)**untreated co-cultured adipocytes expressed as fold-changes against time-matched controls (untreated adipocytes grown alone).

To determine whether the adipogenicity observed in CS100 treatment condition is result of additive or synergistic effect of cortisol and serotonin, transcriptional profile of these serotonin receptor, transporter, adipogenic transcription factor, adipocyte differentiation marker and scavenger receptor genes in co-cultured adipocytes treated individually with C100 and S100 was evaluated. It was observed that treatment with CS100 up-regulated HTR5a, HTR2c, PPARγ, AP2, OLR1 and CD36 more than the additive effect of the individual C100 and S100 treatments (figure 3.9a, 3.9b), although AP2 transcription appeared to be more influenced by cortisol.

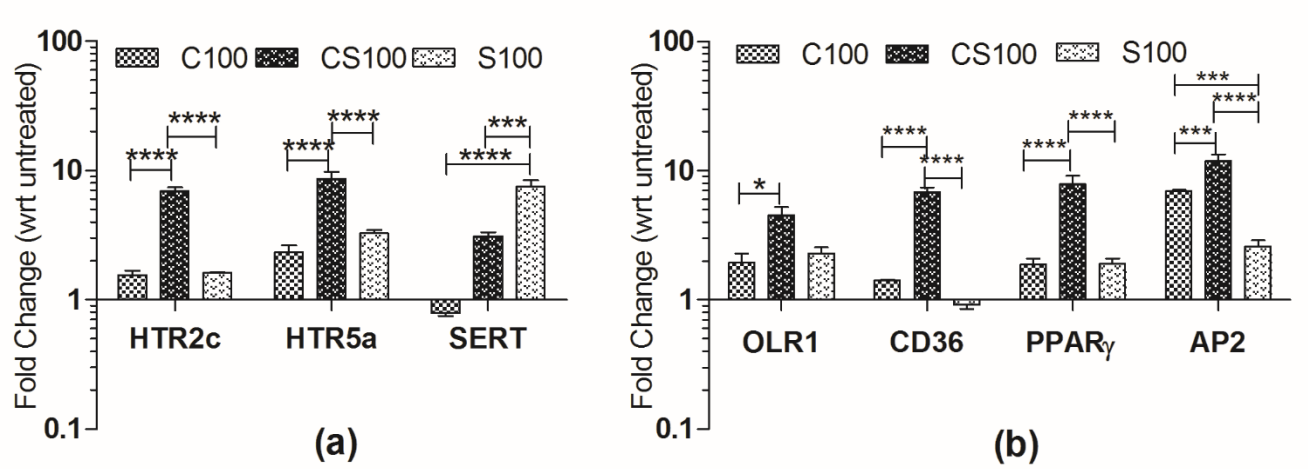


Figure 3.9: Synergistic action of cortisol and serotonin in mediating adipogenicity. Relative transcriptional fold-changes of (a) HTR2c, HTR5a and SERT and (b) OLR1, CD36, PPARγ, AP2 in co-cultured adipocytes treated with C100, S100 and CS100 expressed as fold-changes against time-matched untreated controls.

Comparison of the transcription profile of all these genes in adipocytes grown alone with those in co-cultured adipocytes treated individually with C100 and S100 can be seen in figure 3.10a and 3.10b.

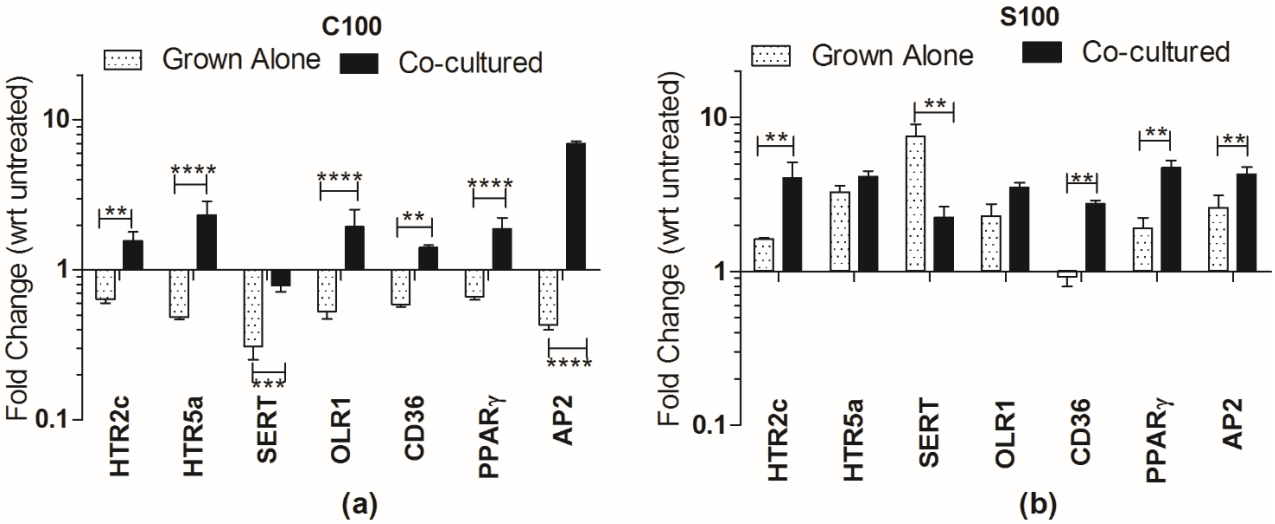


Figure 3.10: Comparison of effect of cortisol and serotonin in adipocytes grown alone and cocultured on the transcription ofHTR2c, HTR5a, SERT, OLR1, CD36, PPARγ and AP2. Relative transcriptional fold-changes of HTR2c, HTR5a, SERT, OLR1, CD36, PPARγ and AP2 in co-cultured adipocytes treated with **(a)** C100 and **(b)** S100 expressed as fold-changes against time-matched untreated controls.

The results thus indicated that serotonin and cortisol act synergistically in co-cultured adipocytes to increase adipogenicity through HTR5a and HTR2c signaling.

3.1.2.4. Enhanced lipid accumulation in co-cultured macrophages treated with cortisol and serotonin is independent of HTR5a and HTR2c signaling

Increased obesity in stress is often accompanied by adipose tissue inflammation, which attracts macrophages into inflamed adipose tissue. We, therefore, studied the effects of cortisol and serotonin on murine macrophage cell line RAW 264.7 grown in absence and presence of 3T3L1 adipocytes. It was observed that macrophages grown with adipocytes in indirect co-culture lost their spindle shape and showed dendrite-like projections (figure 3.11).

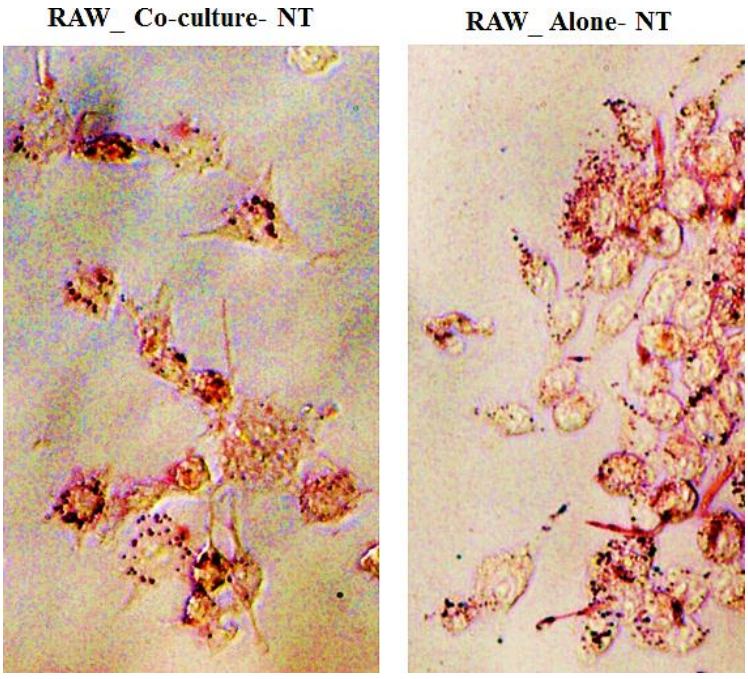


Figure 3.11: Light microscopy with Oil-Red-O staining for macrophages grown alone and macrophages co-cultured with adipocytes.

Since macrophages and adipocytes were co-cultured, macrophages were treated with the same concentration of cortisol and serotonin as adipocytes. Confocal fluorescence imaging was done to estimate the lipid content in them. It was found that like adipocytes, treatment of co-cultured macrophages with CS100 enhanced the lipid storing capacity of macrophages and lipid accumulation per cell in macrophages revealed that though C100 and S100 individually too contributed to enhanced lipid accumulation, treatment with CS100 resulted in maximum lipid accumulation (figure 3.12 and 3.13).

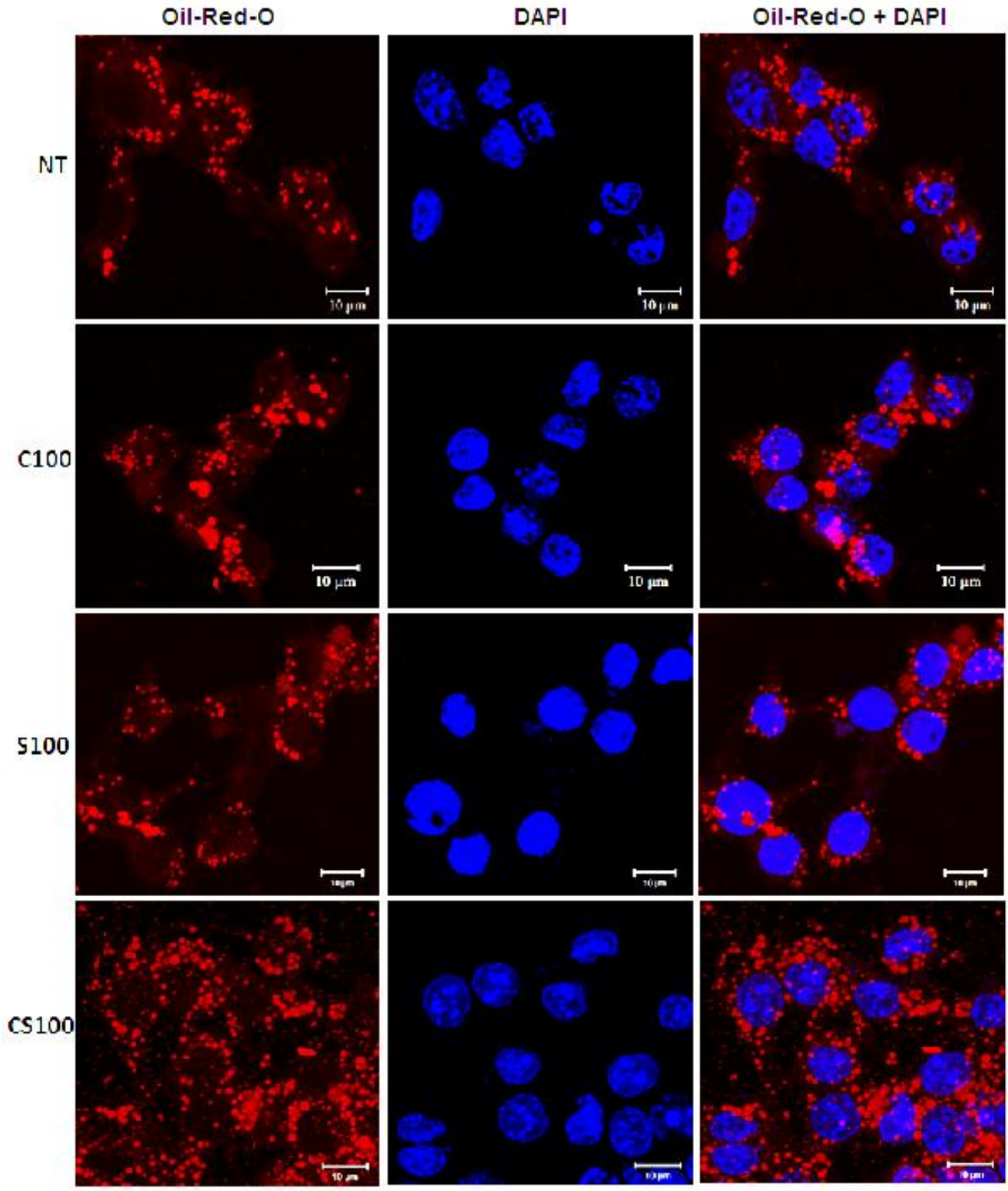


Figure 3.12: Confocal image of co-cultured untreated macrophages and treated with C100, S100, and CS100. First column indicates data for staining with Oil-Red-O, second column staining of nucleus with DAPI and third column as the merged data of first two columns.

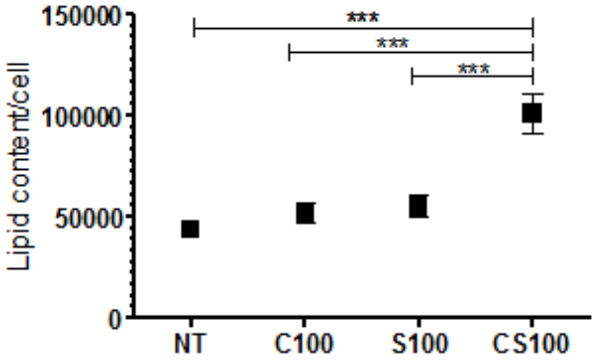


Figure 3.13: Estimation of differential effects of cortisol and/or serotonin treatment in co-cultured macrophages through imaging. Lipid content of co-cultured untreated macrophages as well as treated with C100, S100, CS100 determined from microscopy images as described in methods section.

Transcription profile of the serotonin receptors in macrophages showed that unlike adipocytes, increase in transcription of HTR2c and HTR5a genes was only 2.2 fold and 1.5 fold respectively (figure 3.14a); however, in co-cultured macrophages, HTR5a and HTR2c were highly down-regulated due to CS100 treatment. All other HTRs and SERT remained unaffected in macrophages unlike adipocytes. Interestingly, it was observed that PPARγ expression was slightly higher in macrophages in co-culture than when grown alone (figure 3.14a).

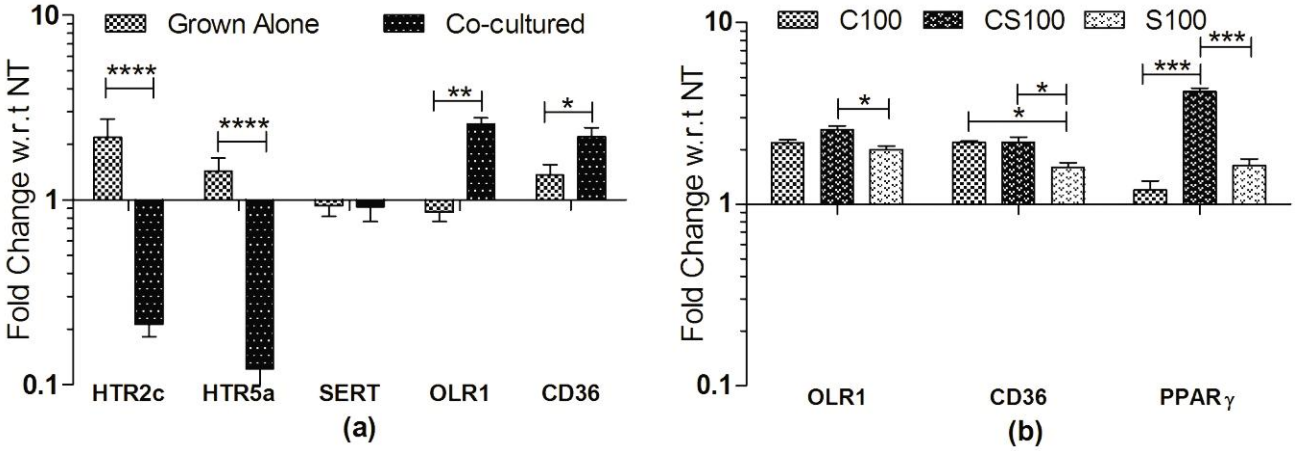


Figure 3.14: Effect of C100, S100 and CS100 on expression of HTR5a, HTR2c, SERT, OLR1, CD36 and PPARγin macrophages. **(a)**Relative transcriptional fold-changes of HTR5a, HTR2c, SERT, OLR1 and CD36 in CS100 treated macrophages grown alone and co-cultured expressed as fold changes w.r.t time matched untreated controls; **(b)** Relative transcriptional fold-changes of OLR1, CD36 and PPARγ in CS100 treated co-cultured macrophages expressed as fold changes w.r.t time matched untreated controls.

Transcriptional profile of co-cultured macrophages showed that adipogenic transcription factor, PPARγ2 was up-regulated 4.2 fold, when treated with CS100, compared to S100 or C100

treatment alone (less than 2 fold) (figure 3.14b). Co-cultured macrophages also showed up-regulation of CD36 (2.2 fold) and OLR1 (2.5 fold) scavenger receptors when treated with both cortisol and serotonin, but these changes did not differ significantly from treatments with either serotonin or with cortisol alone (figure 3.14b). Higher expression of PPARγ gene at 48h, therefore, could not be accounted for, unless up-regulation is caused by uptake of free fatty acids by scavenger receptors which are endogenous ligands for orphan receptor PPARγ.

3.2. Modulation of the metabolic and immune balance in adipose tissue by stress hormones: An *in vitro* simulation.

Adipose tissue, as a potent endocrine organ as well as the body's primary energy storage reserve, plays key roles in systemic metabolism by modulating metabolic and immune homeostasis.. Although regulation of adipocyte homeostasis by endocrine factors has been extensively studied, the involved signaling events are not completely understood. Glucocorticoids are well-established hormones, known to be involved in mediating psychological stress responses. Serotonin, produced by enterochromaffin cells in the gut-lining, is carried by platelets and mast-cells to site of inflammation. In case of chronic psychological stress, stress hormones like cortisol can induce adipocyte hypertrophy and adipose tissue inflammation, making adipose tissue an ideal site for serotonin dumpage by mast cells and platelets. Thus, in a chronic stress condition, inflamed adipose tissue would be subject to very high levels of cortisol and serotonin. These two hormones might thus also modulate immunological and metabolic functions of adipose tissue during chronic stress.

3.2.1. Materials and Methods

3.2.1.1. Cell culture

Same as described in section 3.1.1.1

3.2.1.2. Quantitative PCR

Same as described in section 3.1.1.4

3.2.1.3. Microarray

Total RNA was extracted using RNAeasy Kit (Qiagen). The RNA samples for gene expression were labeled using Agilent Quick-Amp labeling Kit (p/n5190-0444). 1000ng each of the time matched nontreated and C, S and CS treated RNA samples were incubated with reverse transcription mix at 40 °C and converted to double stranded cDNA primed by oligodT with a T7 polymerase promoter. Synthesized double stranded cDNA were used as template for cRNA generation. cRNA was generated by in vitro transcription. In the kinetic study and the dyes Cy3 CTP (time matched adipocytes grown alone) and Cy5 CTP (adipocytes co-cultured with macrophages) was incorporated, while in the comparison of adipocytes and macrophages the dyes Cy3 CTP (pooled cDNA from 48h untreated adipocyte and macrophages grown alone) and Cy5 CTP (co-cultured adipocytes and co-cultured macrophages treated with C, S and CS) was incorporated, during this step. The cDNA synthesis and in vitro transcription steps were carried out at 40 °C. Labeled cRNA was cleaned up and quality assessed for yields and specific activity. The labeled cRNA samples were hybridized to 4x44k microarray slides. 825 ng each of Cy3 and Cy5 labeled samples were fragmented and hybridized. Fragmentation of labeled cRNA and hybridization were done using the Gene Expression Hybridization kit of Agilent (Part Number 5188–5242). Hybridization was carried out in Agilent's Surehyb Chambers at 65 °C for 17 h. The hybridized slides were washed using Agilent Gene Expression wash buffers (Part No: 5188–

5327). Data extraction from Images was done using Feature Extraction software Version 10.7 of Agilent. Feature extracted data were analyzed in online webserver Arraypipe v 2.7. Significantly up- and down-regulated genes showing fold change of 1.5 and above were identified and parsed with InnateDb (http://www.innatedb.com/) and KEGG pathways to find out the pathways populated with the differentially regulated genes. Gene Ontology enrichment was done using Panther (http://pantherdb.org/). Kinetics of few selected genes also was also confirmed through qRT-PCR.The details of the experimental procedure can be found in the Annexure 2.

3.2.1.4. Cytokine Detection

TNF-α, IL-1β, IL-6, IL10, IL12 and TGFb in cell culture supernatants were also measured by ELISA. Briefly, the ELISA plates were coated with supernatant overnight at room temperature. After 1h of blocking, antibodies were added to each well and were incubated at room temperature for 6h. Wells were washed three times with PBS supplemented with 0.5% Tween 20 (PBST). Biotin-conjugated detection antibodies were added and incubated at room temperature for 2h. Alkaline phosphatase-conjugated streptavidin was then added and incubated at room temperature for 1h. After three washes, the substrate was added to the wells. Within 45 min, the reaction was stopped by the addition of 50µl of 1 N H_2SO_4, and absorbance was assessed using a Bio-Rad micro-plate reader, model 680 (Bio-Rad Laboratories) at 450 nm.

3.2.2. Results

The transcription kinetics of adipocytes co-cultured with macrophages was compared with adipocytes grown alone at 6h, 24h and 48h for each treatment to identify the genes responsible for interactive modulation of inflammatory and metabolic adipocyte function and phenotype in co-culture. Figure 3.15revealskinetic profile of significantly enriched pathways using differentially transcribed genes under different treatment conditions at 6h, 24h and48h.

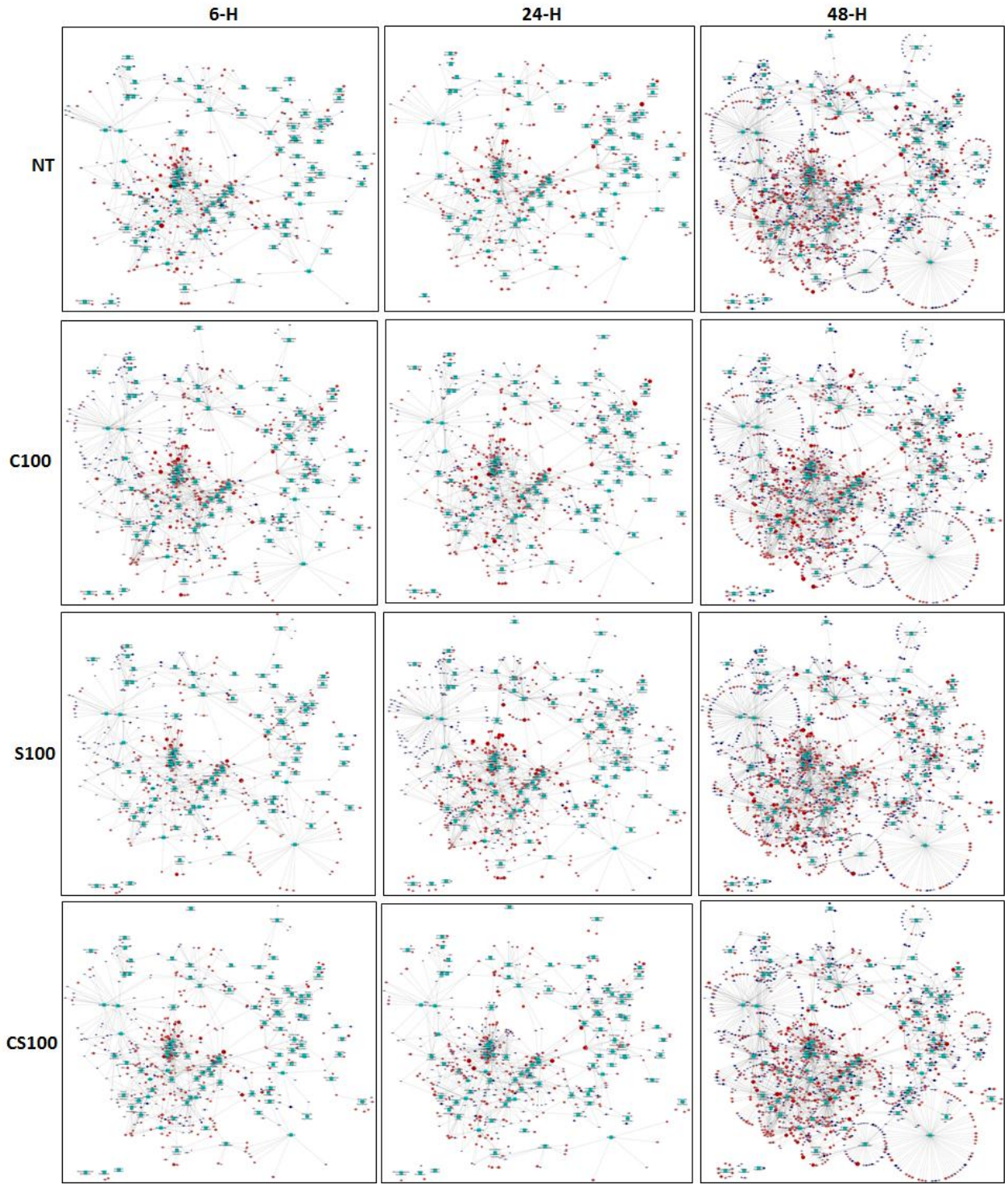


Figure 3.15: Gene network significantly enriched pathways and differentially regulated genes in co-cultured adipocytes when compared against adipocytes grown alone at 6h, 24h, and 48h under the following treatment conditions – NT, C, S and CS.

It was observed that the total number of differentially regulated genes at 48h time point was approximately double that of earlier time-points. Hence, 48h time point was chosen for further genome wide transcriptional profiling analysis to understand the effects of C, S and CS on adipocytes and macrophages when cultured together. Among various genes and pathways that were differentially expressed, following genes were worth mentioning in connection to the current objectives of the study.

3.2.2.1. Inflammatory regulation

It was observed that several inflammatory cytokines and chemokines such as IL1A, CSF3, CXCL5, CCL3, CCL4, CCL8, IL19, IL13RA, cAMP showed differential gene expression over time. Under no treatment condition IL19, cAMP and CCL4, showed increased transcription with time, however transcription of IL1A, CSF3, CXCL5, CCL3, CCL4, CCL8 and IL13RA either remained constant or dropped at 48h (figure 3.16a). Transcription of CSF3 increased with increasing time due to treatment with C, S or CS, although its transcription had appeared to decrease with time under NT condition. Cortisol however, appeared to suppress pro-inflammatory CCL4, and augment higher expression of anti-inflammatory IL13Ra and cAMP, which increased with time, though the effects become prominent only at 48h (figure 3.16b).

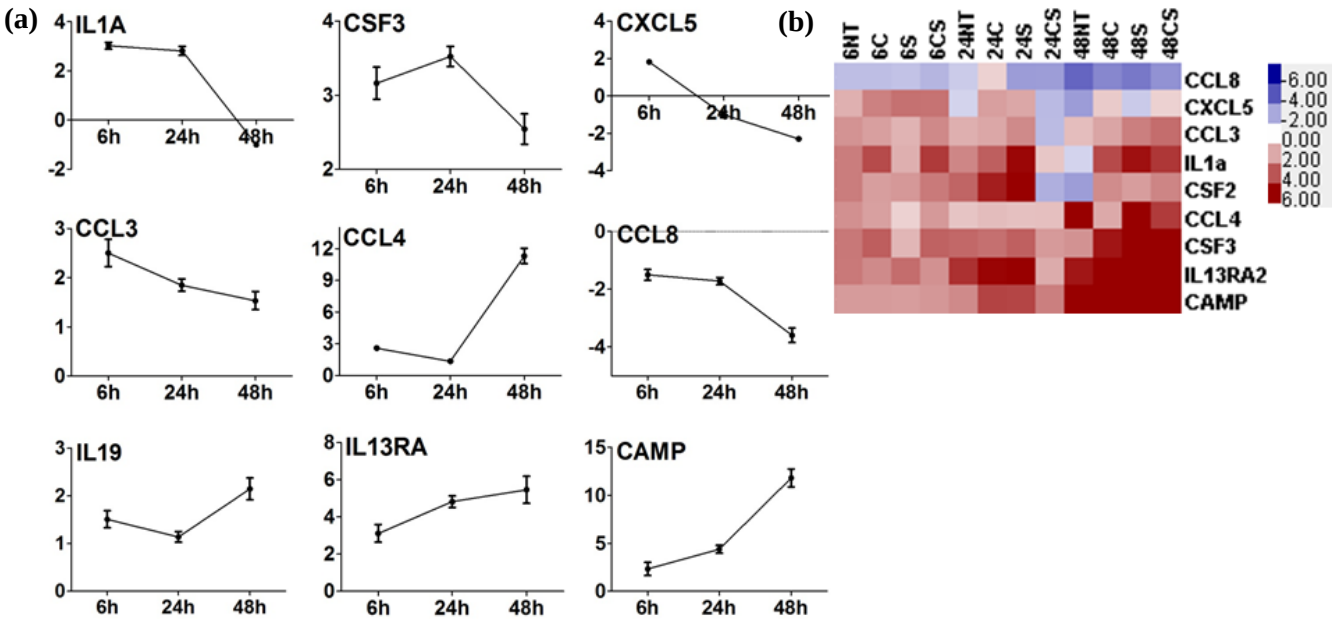


Figure 3.16: Kinetics of inflammatory changes due to co-culturing in adipocytes. Transcription kinetics of IL1a, CSF3, CXCL5, CCL3, CCL4, CCL8, IL13ra, IL19, and CAMP in adipocytes co-cultured with macrophages compared against adipocytes grown alone a) expressed as fold-changes under NT b) expressed as heatmap for NT- non treated, C- cortisol treated, S-serotonin treated, CS- treated with both cortisol and serotonin.

Among the inflammatory pathways that were significantly affected are NF-κB and TLR signaling pathway. It was observed that transcription of MIP1α and MIP1β, two known chemotactic agents for macrophages, also increased with time in co-cultured adipocytes with maximum transcriptional expression at 48h. This increase in transcription of MIPs was almost always accompanied by an increase in the transcriptional expression of TLR2/6 complex (figure 3.17).NF-κB signaling pathway showed gradual increase in activity with increasing time, with maximum activity at 48h. Although TLR2/6 mediated NF-κB pathway activation is known to induce apoptosis, our data showed that viability of the cells (as determined through MTT assay) was uncompromised even after 48h treatment. In fact, the apoptosis pathway was suppressed at 48h.

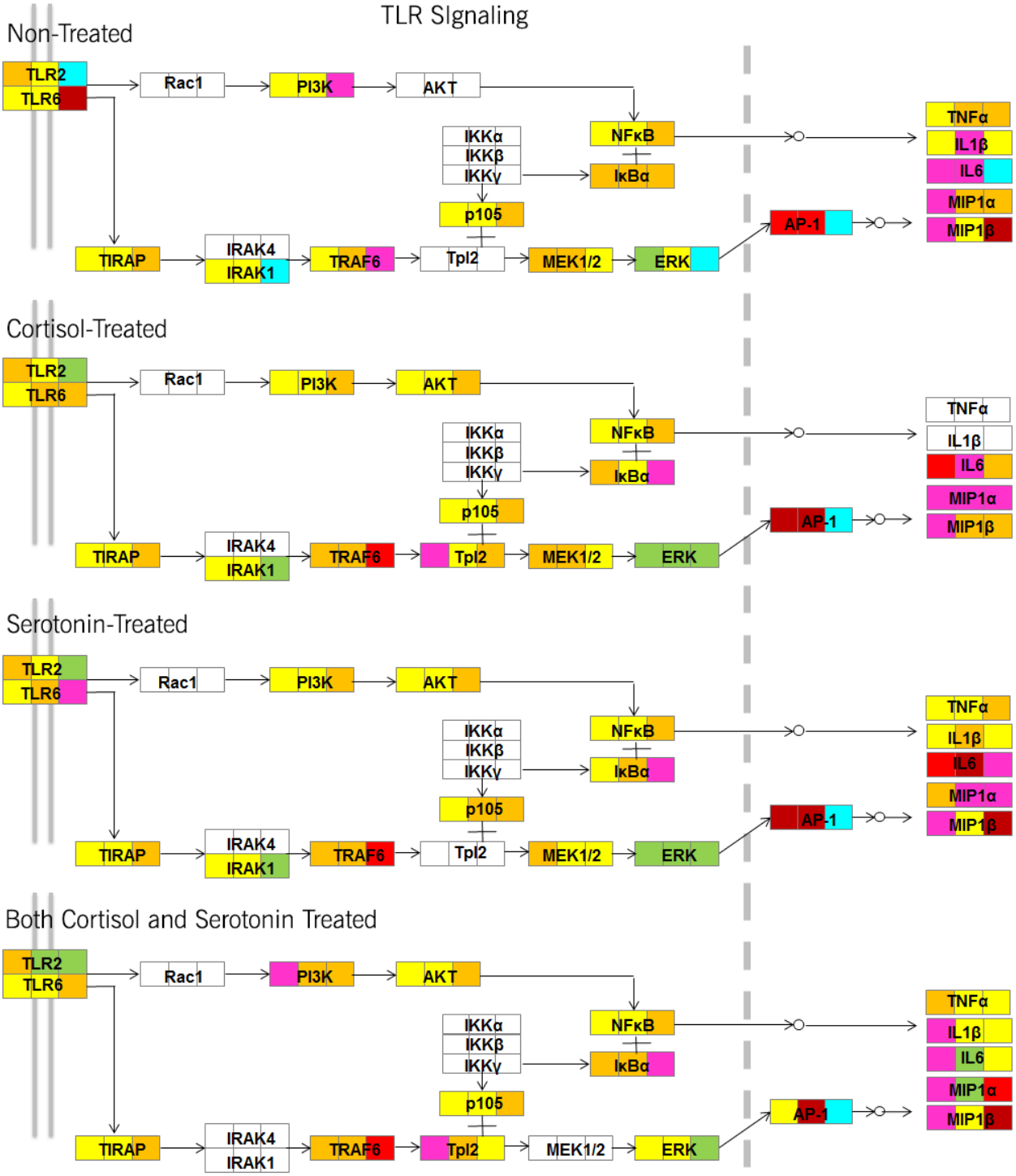


Figure 3.17: KEGG pathway representation of TLR signaling kinetics due to co-culturing in adipocytes. Pathway map of TLR signaling pathway using differential expression values from microarray of co-cultured adipocytes treated with NT, C, S and CS at 6h, 24h and 48h compared against time-matched adipocytes grown alone. Each gene is represented by a rectangle split into 3 compartments showing the fold-changes for that gene in the order 6h, 24h and 48h with warmer colours representing up-regulation and cooler colours representing down-regulation.

Many of these inflammatory proteins are known to be responsive to activation of Activator protein 1 (AP-1), which is a transcriptional regulator composed of members of the Fos and Jun families of DNA binding proteins[236]. Fos, an early response transcription factor, showed maximum transcription at 6h, which deceased gradually with time for all conditions. Both Cortisol and Serotonin enhanced its transcription from approximately 4 folds (NT) to 11 folds and 6 folds respectively at 6h (table 3.2). Similarly, Jun also showed increased transcription, due to treatment with C and S [237], although, the changes were not as pronounced as FOS.

Condition	Fos	Jun
6h-NT	3.8	1.6
24h-NT	3.8	1.7
48h-NT	-2.7	1.7
6h-C100	11.3	2.4
24h-C100	4.1	2.1
48h-C100	-2.8	2.5
6h-S100	5.8	2.4
24h-S100	5.9	3.3
48h-S100	-2.7	1.5
6h-CS100	1	1
24h-CS100	4.5	1
48h-CS100	-2.8	3.3

Table 3.2: Transcriptional fold changes of Fos and Jun at 6h, 24h, and48h for different treatment conditions.

It was also observed that most of the downstream TNF-signaling pathway genes, such as IL6, CXCL1, PTGS2, MMP3, CSF2, FOS, NOD2, TRAF1, TNFAIP3, showed differential expression pattern with time. Under NT condition, transcription of majority of these genes decreased at 48h, except TNFαip3 (which showed increased expression at 48h) (figure 3.18a). The temporal transcription pattern of these genes remained similar to NT following treatment with C, S and CS; although each of these treatments increased the transcription of these genes compared to non-treatment groups (figure 3.18b).

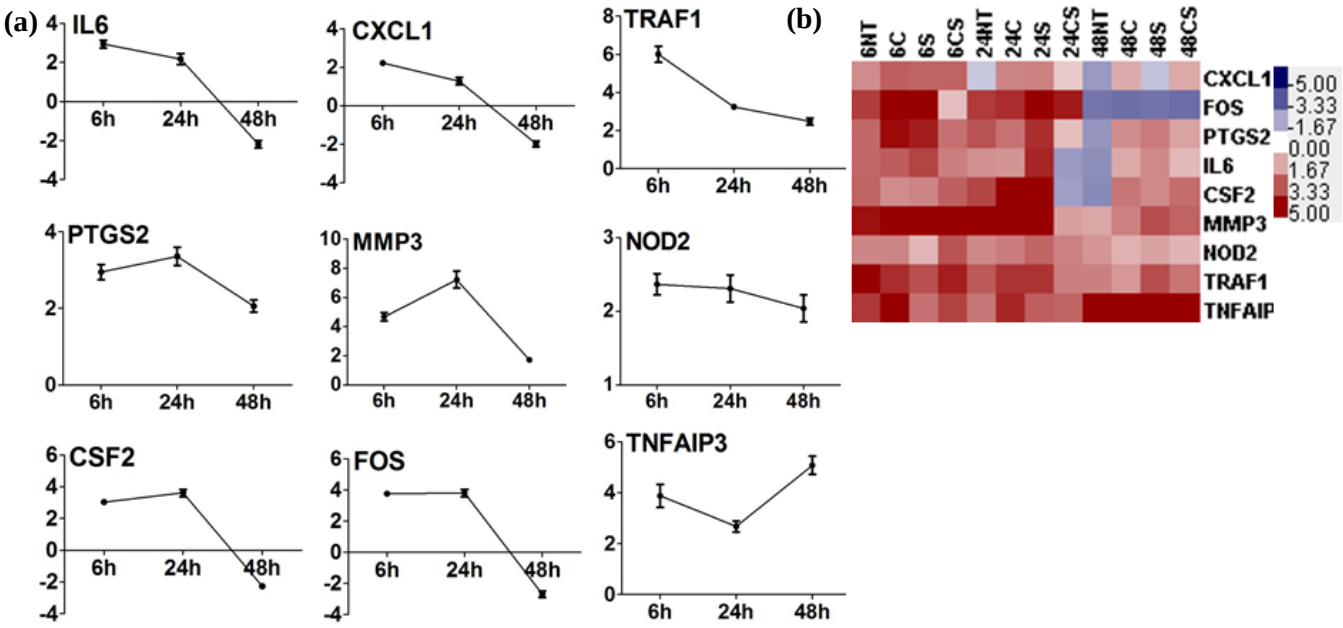


Figure 3.18: Transcription kinetics at 6, 24 and 48h of TNF-pathway down-stream genes. Transcription of IL6, CXCL1, TRAF1, PTGS2, MMP3, NOD2, TNFαip3, CSF2, FOS in adipocytes co-cultured with macrophages compared against adipocytes grown alone a) expressed as fold-changes under NT b) expressed as heatmap for NT-non treated, C- cortisol treated, S-serotonin treated, CS- treated with both cortisol and serotonin.

3.2.2.2. Metabolic regulation

Increase in lipid content of co-cultured adipocytes led us to believe that co-culturing either favoured import of fatty-acids into adipocytes or induced de novo lipid synthesis in them. Expression of TLR6, TLR2 appeared to increase at 48h for all the treatment conditions due to co-culturing and serotonin appeared to cause higher transcription of TLR6 compared to cortisol at 48h. At 48h, TLR13 (a newer endosomal receptor that recognizes bacterial 23S rRNA) too showed more than 4 fold increase in transcription in NT condition; treatment with C, S and CS however reduced the transcription of this gene to less than 2 folds(figure 3.19a). Analysis of the transcription profile of lipid transporters revealed that transcription of both OLR1 and FATP3 increased steadily with time. While cortisol and serotonin treatment up-regulated OLR1 transcription, FATP3 transcription appeared to be suppressed by treatment with C and S together

(figure 3.19b). We noticed that Slc5a transcription increased abruptly after 24h for all the treatment conditions (figure 3.19c).

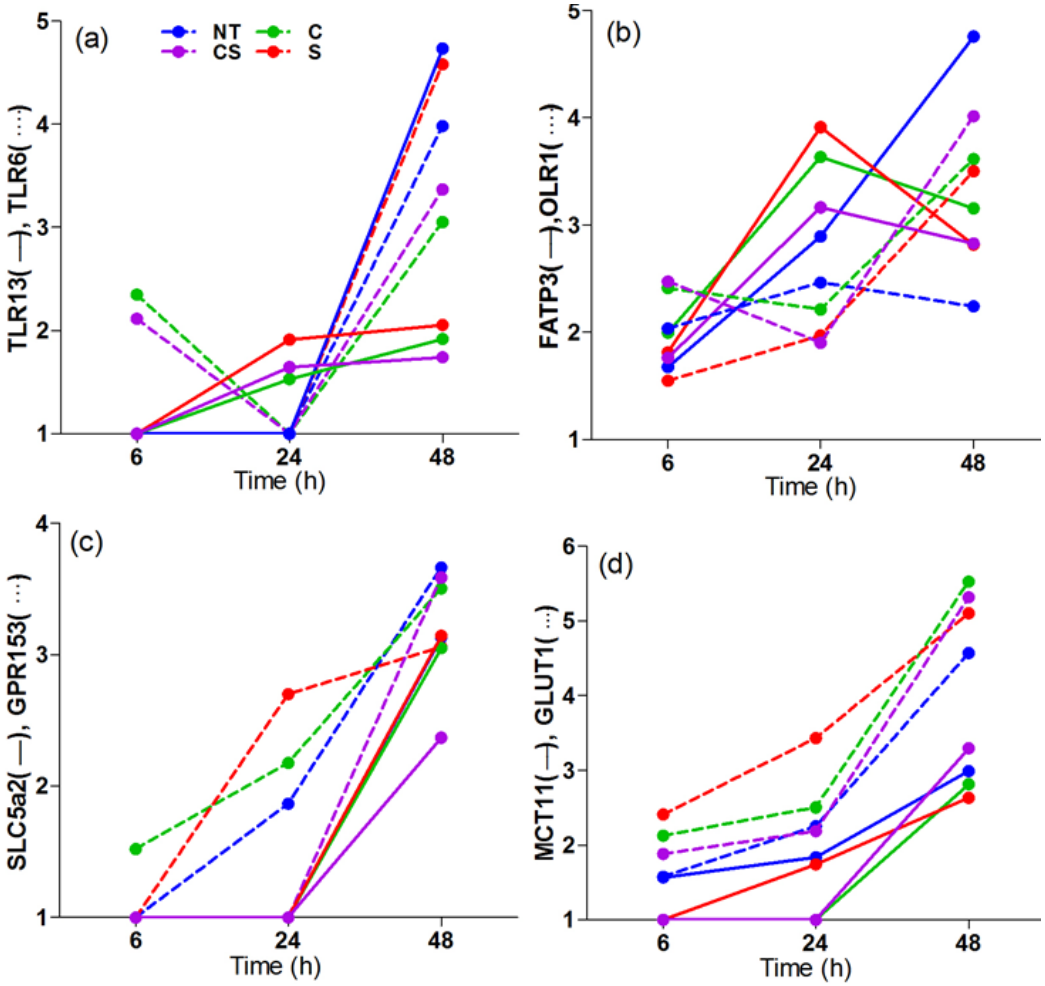


Figure 3.19: Differential transcription kinetics of metabolic regulator genes in adipocytes due to co-culturing.Transcription kinetics of at 6, 24 and 48h of a) TLR13 and TLR6 b) OLR1 and FATP3 c) SLC5a2 and GPR153 and d) GLUT1 and MCT11 in co-cultured adipocytes compared against adipocytes grown alone and treated for NT-non treated, C- cortisol treated, S-serotonin treated, CS- treated with both cortisol and serotonin.

Certain groups of G-protein receptors are known to bind to short chain fatty acids. GPR153, one of the genes of the same GPCR family too showed constant increase in transcription with time due to co-culturing (figure 3.2.2.2.1c). Like Slc5a, transcription of this gene too did not vary with different treatments. Cytoplasmic Acetyl-coenzyme A Synthetase 2 (ACSS2) was also up-regulated and its transcription increased with time. Besides, ACSL1 (Long-chain-fatty-acid-CoA

ligase 1), responsible for conversion of free long-chain fatty acids into fatty acyl-CoA esters was suppressed at 48h. However, ACSL4 which preferentially converts arachidonic acid to arachidonate was up-regulated at 48h. Thus, responsible for lipid import into the cells as well as those required for intra-cellular compartmentalization were affected. Apart from fatty acid transport, genes that could possibly contribute to de novo fat synthesis also increased due to co-culturing. GLUT1 transcription increased with more than 4-fold increase at 48h, and although treating with Cortisol and Serotonin increased transcription of this gene, yet there was no significant difference between C, S, and CS treatments (figure 3.19d). Increased transcription of GLUT1 was accompanied by a less pronounced, yet significant increase in the transcription of MCT11, a mono-carboxylate transporter (figure 3.19d). This suggested that co-culturing favored higher adipogenicity in these cells through lipid and glucose import, although this does not solely account for the entire metabolic regulation in these cells.

3.2.2.3. TNF Signaling in Metabolic regulation

Studies have associated inflamed obese tissue with higher secretion of TNFα, and its role in regulating adipocyte metabolism is already reported [238]. Our analysis revealed that TNFα did not show significant variation with time, all the while having comparatively low transcription; however TNF soluble receptors, known to initiate PI3K-AKT signaling pathway showed significant increase at 48h (figure 3.20a) in co-cultured adipocytes. TNFα signaling is already known to decrease transcription of LPL [5, 6] which is the rate-limiting enzyme for hydrolysis of triglycerides in Triglyceride Rich Proteins. Further analysis revealed that in co-cultured adipocytes LPL transcription decreased with time for all the treatments (figure 3.20b). It was also noted that CEBP transcription was less than 2 fold till 24h, however, the transcription increased to 4 fold at 48h for all treatment condition (figure 3.2c). The decrease in LPL transcription was

accompanied by increasing accumulation of lipid droplets in co-cultured adipocytes (figure 3.20d). Like CEBP, APOE transcription too increased to 4-5 folds at 48h (figure 3.20b), which correlated with the drop in TNF-signaling at 48h. APOE transcription is known to be affected by PPARγ.

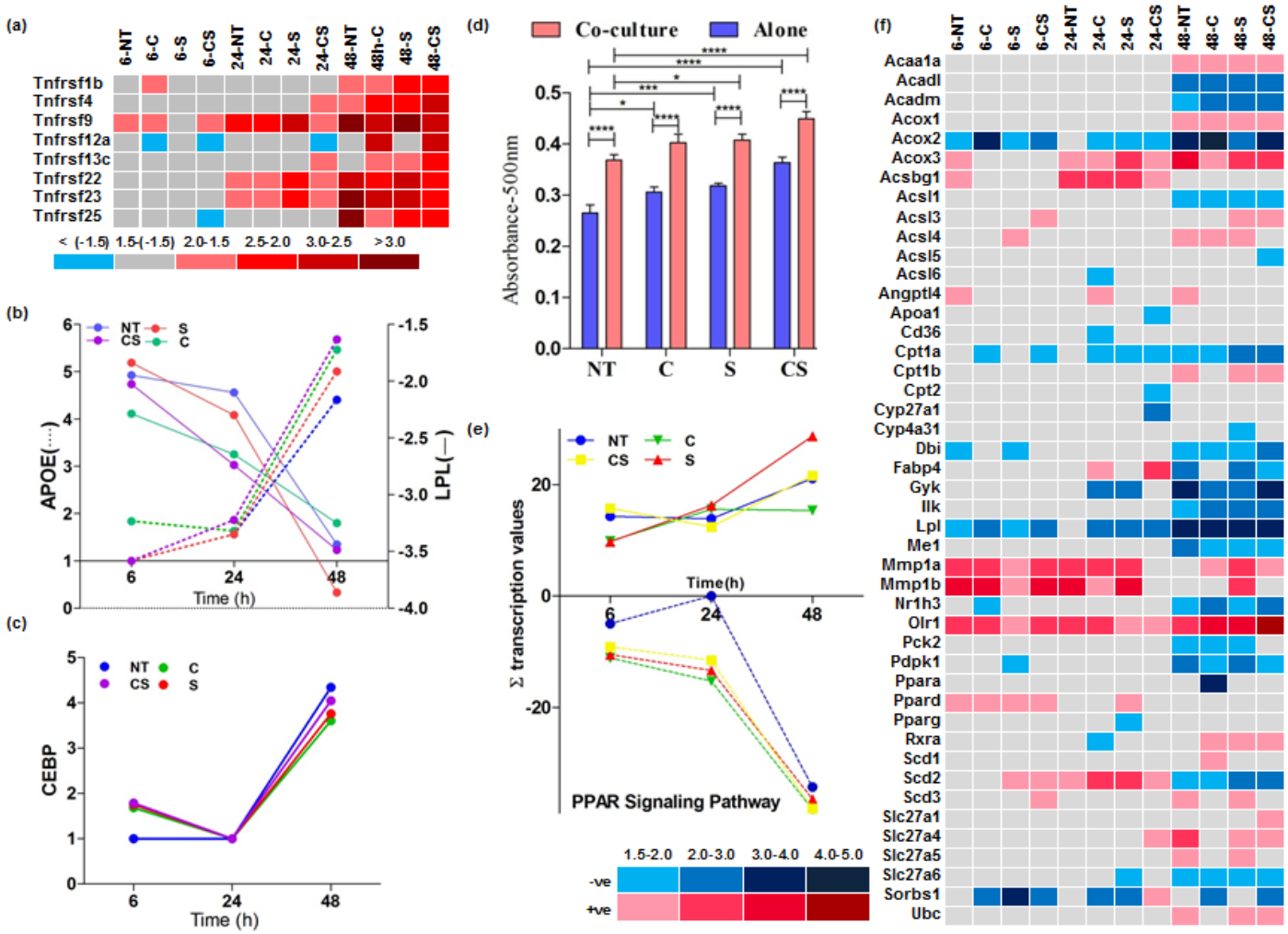


Figure 3.20: Relationship of TNF and PPAR pathway in metabolic regulation**.** Transcription kinetics at 6, 24 and 48h of a) TNF receptors expressed as heat-map b) APOE and LPL c) CEBP in adipocytes co-cultured with macrophages compared against adipocytes grown alone under NT-non treated, C- cortisol treated, S-serotonin treated, CS- treated with both cortisol and serotonin. d) Oil Red O absorbance of co-cultured adipocytes and adipocytes grown alone treated with NT, C, S and CS at 48h e) Summation of differential transcription values of all genes of PPAR signaling pathway in co-cultured adipocytes compared against adipocytes grown alone at 6h, 24h and 48h for each treatment condition d) Heatmap of differentially expressed genes in PPAR signaling pathway at 6h, 24h and 48h in co-cultured adipocytes compared against adipocytes grown alone

However, the analysis revealed that PPARγ transcription did not vary significantly due to co-culturing. In fact PDPK1, which activates PPARγ and promotes adipocyte differentiation [239] was down-regulated at 48h (figure 3.20d). PPAR signaling pathway however appeared to be affected after 24h concomitant with the decrease in TNF-signaling (figure 3.20e). It was observed that transcription of Stearoyl-CoA Desaturase 2 (SCD2), known to reduce adiposity, increased till 24h followed by a drop at 48h. Transcription of Acyl-CoA dehydrogenase genes (both long chain-ACADL and medium chain-ACADM) was suppressed at 48h, while CPT1a decreased with increasing time, indicating that mitochondrial beta-oxidation of fatty acids did not occur at 48h. Meanwhile, enzymes for peroxisomal oxidation of fatty acids like ACAA1a, ACOX1 and ACOX3 (help in desaturation of long chain fatty acids) were up regulated. Transcription of Acsbg1 (Acyl-CoA synthetase), responsible for activation and beta-oxidation of very long chain fatty acids [240][241] increased till 24h, followed by decrease at 48h (figure 3.20f). Glycerogenesis causes re-esterification of fatty acids, thus restraining the release of free fatty-acids. PPARγ/RXRα complex is required for activation of PCK2 (PEPCK2) [242, 243] which in turn is required for glycerogenesis in adipocytes for re-esterification of fatty acids to triglycerides was also down-regulated at 48h.

3.2.2.4. Effect of Cortisol and Serotonin in co-cultured adipocytes at 48h

We identified 167 genes that did not show significant differential expression till 24h, however their transcription increased from 2 to 18 folds only at 48h due to co-culturing (table 3.3 contains list of these genes, minimum cut-off of 2 fold-change was used to identify these genes).

Table 3.3: Differential transcription values of genes expressed as fold-changes obtained from microarray of co-cultured adipocytes treated with NT, C, S and CS at 6h, 24h and 48h compared against time-matched adipocytes grown alone; fold-change cut off used is +/- 2.

	6NT	6C	6S	6CS	24NT	24C	24S	24CS	48NT	48C	48S	48CS
LOC101055971	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	11.3	15.9	14.7	18.1
Gm4470	1.0	1.0	1.0	1.0	1.0	1.0	2.0	1.0	10.0	5.8	7.0	4.9
A830049F12Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	8.1	6.6	6.1	8.2
Glis1	1.0	1.0	1.0	1.0	1.0	1.0	2.2	1.0	7.1	7.2	5.7	6.8
Gm22	1.0	1.0	1.0	1.0	1.0	1.0	2.2	1.0	6.9	4.3	7.0	4.8
Pvrl2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	6.8	3.2	3.1	3.6
Lce3c	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	6.5	3.1	5.0	5.6
Galnt6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.7	2.7	3.8	1.0
Grb7	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.7	6.4	4.1	6.0
Hist1h2bc	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.7	5.6	6.9	4.8
Il4ra	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.6	3.5	3.8	3.7
1110002E22Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.4	2.6	3.5	4.8
Gm10605	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.4	6.2	8.2	7.4
Ost4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.4	2.1	4.0	2.2
Plch2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.3	3.0	3.3	3.2
Gzmm	1.0	1.0	1.0	1.0	1.0	1.0	2.1	2.5	5.2	5.0	5.4	5.2
Cdk5r1	1.0	2.2	1.0	1.0	1.0	1.0	1.0	1.0	5.2	7.9	6.6	9.0
Gm5088	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.2	5.7	5.4	5.5
BC100530	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.2	8.7	10.3	8.2
4930421J07Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.2	3.0	3.3	3.6
Gm2921	1.0	2.7	1.0	2.3	1.0	1.0	1.0	1.0	5.1	6.7	6.4	6.4
Nfyb	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.1	2.5	3.8	3.9
Atp9a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.0	5.4	7.3	7.1
Rab8b	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.9	1.0	3.6	2.4
Sik1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.9	5.3	7.1	5.3
Slc23a3	1.0	1.0	1.0	1.0	2.2	1.0	2.2	1.0	4.8	4.1	6.3	5.3
Gjb3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.8	4.6	5.6	5.5
AI606181	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.7	3.1	3.9	5.5
Atp8a1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.7	3.8	3.4	3.7
Dmkn	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.6	3.8	4.9	6.4
Dok7	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.6	3.8	3.3	3.7
Tnpo1	1.0	1.0	1.0	1.0	1.0	1.0	2.7	1.0	4.6	5.1	4.2	3.9
Lat2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.6	1.0	3.6	2.0
4631405J19Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.5	3.5	4.0	3.4
Sccpdh	1.0	1.0	1.0	1.0	1.0	1.0	2.0	1.0	4.5	3.0	3.9	3.0
Tbx3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.5	4.0	5.4	4.4
Tmem191c	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.4	3.5	5.8	3.8
Mgarp	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.4	2.2	3.9	2.3
Slc17a9	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.4	5.5	5.0	5.7
Nrn1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.4	3.9	4.9	3.9
Sec24a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.3	3.6	3.8	4.0
Cspg5	1.0	1.0	1.0	1.0	1.0	1.0	2.3	1.0	4.3	5.1	5.8	6.5
Lrrc8d	1.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0	4.3	4.5	4.9	5.2
Tram2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.2	3.4	4.2	3.6
Rpgrip1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.2	3.1	3.2	2.8

Gm8457	1.0	1.0	1.0	2.2	1.0	1.0	1.0	1.0	4.1	3.5	5.1	4.3
Sox9	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	4.0	3.5	3.8	4.0
Rps6ka1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.9	4.1	4.0	4.4
Dnahc6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.8	3.0	3.8	3.4
Id2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.4	3.8	4.4	4.4	5.0
2900097C17Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.8	3.9	4.4	3.7
A830073O21Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.8	2.2	3.2	3.0
Pdk4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.8	2.8	3.1	2.3
Emilin1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.8	4.2	4.4	4.4
Uap1	1.0	2.1	1.0	1.0	1.0	1.0	1.0	1.0	3.7	4.8	3.8	4.1
Rabgef1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.7	3.8	3.0	2.9
Arhgap22	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.7	1.0	3.6	3.0
Nmb	1.0	1.0	1.0	1.0	1.0	1.0	2.2	1.0	3.7	4.0	4.2	3.9
Coq10b	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.7	2.6	2.9	2.5
Gm14168	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.7	2.8	3.1	2.8
Myo15	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.6	3.8	4.5	3.3
Maff	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.3	3.6	4.0	4.4	5.0
Cfp	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.6	3.1	3.1	2.9
Mical3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.6	2.9	3.7	2.9
Erp27	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.5	2.6	3.2	3.0
6330416G13Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.5	3.0	3.5	3.0
Shd	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.5	3.5	3.8	3.2
Hist2h3c2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.5	3.6	5.4	4.2
Traf6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.5	3.2	3.3	3.8
Ppapdc1b	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.4	1.0	3.0	2.3
Crabp2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.4	3.5	3.5	3.2
Wdr26	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.4	3.7	3.4	3.7
BC062258	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.4	3.3	3.3	3.1
Kif5a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.4	3.5	3.8	3.4
Napsa	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.4	2.9	3.4	3.3
5430431A17Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.4	3.4	3.8	3.5
Zbtb1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.3	4.5	3.8	3.8
Soga1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.3	4.6	5.0	3.7
Mreg	1.0	1.0	1.0	1.0	1.0	1.0	2.1	1.0	3.3	2.7	4.4	2.7
Arrb1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.3	6.0	7.6	7.7
1700019N12Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.3	4.7	4.6	4.1
Gpd1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.3	2.5	3.3	2.6
Gdf9	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.2	2.6	2.9	3.4
Gal3st2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.2	4.2	4.6	4.2
Lrrc31	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.2	3.1	3.0	3.3
Fbxw10	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.2	3.1	3.0	3.3
Matk	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.2	3.0	4.7	3.4
Bin3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.2	2.5	3.6	2.2
Arid3a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.2	4.1	4.4	5.7
Gdap10	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.2	3.0	3.7	3.2
Id4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.2	3.7	4.6	4.0
Fosl2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.1	3.9	4.1	3.9
Lce1c	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.1	3.1	3.1	2.4
Jhdm1d	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.1	3.1	3.0	3.2
Xpo7	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.1	4.7	4.3	5.6

Stfa1	1.0	1.0	1.0	1.0	1.0	2.1	1.0	1.0	3.1	3.1	2.9	2.7
Gpi1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.1	3.0	3.3	2.9
Gm10631	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.0	3.5	3.2	3.1
Scml4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.0	3.0	3.1	2.6
Tmem184a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.0	3.9	3.7	3.7
Lce1g	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.0	4.5	4.4	4.2
5330406M23Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	3.0	3.6	4.0	4.6
Car15	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.9	1.0	3.5	2.2
5330426P16Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.9	2.0	3.0	2.1
Cebpb	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.9	4.4	4.6	4.8
Gm11412	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.9	2.1	3.2	2.5
Procr	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.9	3.5	3.7	3.5
2310058N22Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.9	2.7	3.0	2.4
Acrbp	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.9	2.5	2.9	2.6
LOC100861916	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.9	2.5	2.8	3.0
Best2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.9	2.8	3.4	3.1
Cnih2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.9	3.9	3.2	3.1
1110017D15Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.9	3.0	3.0	2.9
Cdh2	1.0	2.1	1.0	2.0	1.0	1.0	1.0	1.0	2.9	4.5	6.5	5.6
Slc22a13b-ps	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.9	2.4	3.3	2.8
Apol8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.8	5.3	5.9	5.4
Plcd4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.8	1.0	3.3	2.6
Dip2b	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.8	3.0	2.9	2.9
Vasn	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.8	3.5	3.5	3.4
5830469G19Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.8	3.3	3.3	2.6
Dnahc10	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.8	3.4	4.9	4.0
Evpl	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.7	3.5	3.6	3.7
Adssl1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.7	4.2	4.5	4.1
Pde4c	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.7	2.8	3.3	3.6
Tspan33	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.7	4.1	4.0	5.1
Tubb2a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.1	2.7	4.0	3.7	4.3
Mettl6	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.7	3.4	3.1	2.5
Tpsb2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.7	4.1	5.5	4.7
Mansc1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.7	6.2	6.2	5.7
1700109H08Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.7	1.0	2.8	2.5
D030002E05Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.7	3.1	2.9	3.3
Lmbr1l	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.6	2.6	3.1	2.9
Csrnp1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.6	2.8	3.2	3.5
Flcn	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.6	3.0	3.0	3.3
Rusc2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.6	3.0	3.1	3.2
Smg7	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.6	2.6	3.3	3.6
Khdrbs1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.6	2.5	3.4	2.9
Tigd3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.6	2.5	3.1	2.4
Gramd1c	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.6	3.1	2.9	3.6
Tbkbp1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.6	3.3	3.3	3.2
Taf8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.6	4.4	3.1	4.0
Cryab	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.5	3.1	3.5	3.4
Gm7092	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.5	2.8	2.8	3.0
Inhba	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.5	3.4	4.0	3.8
Fubp1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.5	2.5	3.3	3.0

1600020E01Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.5	4.4	3.9	4.5
B230303O12Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.5	2.4	2.8	3.5
Efnb2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.4	4.8	6.7	4.1
Ifnar1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.4	3.1	3.3	3.4
Chd7	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.4	2.6	3.3	3.2
Elavl1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.4	2.5	3.0	3.3
Acp5	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.4	3.3	3.5	3.6
1700001J03Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.3	2.1	3.3	3.7
Ids	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.3	3.0	3.2	2.9
Mslnl	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.3	1.0	3.5	1.0
Zbtb32	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.3	3.6	3.7	4.0
Fbxo28	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.3	2.5	2.9	1.0
Ppfia4	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.3	4.5	5.5	5.0
Ovgp1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.3	2.4	2.8	3.0
Cyp4f16	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.3	2.3	2.4	2.3
Hbp1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.3	3.6	3.0	2.8
BB166591	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.3	3.0	2.9	3.6
Pvrl1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.2	4.3	4.0	3.1
Safb	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.2	2.8	3.5	3.6
Elavl3	1.0	1.0	1.0	2.3	1.0	1.0	2.1	1.0	2.2	2.8	3.1	2.5
Gm10094	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.2	3.1	4.2	3.4
1700101I11Rik	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.2	2.6	3.0	3.6
Vwce	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.2	2.8	2.9	2.7
Serpinf2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.2	2.6	3.3	2.1
Fbxo30	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.2	2.6	3.0	2.9
Aqp11	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.2	3.5	3.1	3.2
Chpf2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.1	2.6	3.7	2.9
Sgms2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.1	2.5	4.1	3.9
Mafk	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.1	2.8	2.9	2.6
Stac3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.1	2.6	2.9	3.2
Mef2a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	2.0	2.9	3.7	2.5

Thus at 48h major shift in transcriptional activation occurred. Hence, to further explore the metabolic and immune regulatory changes in adipocytes co-cultured with macrophages at 48h, a whole genome transcriptional study of adipocytes after 48h of co-culturing with macrophages was carried out to see the effect of stress hormones on co-cultured adipocytes, in which co-cultured adipocytes treated with C, S and CS each, were compared with untreated co-cultured adipocytes. Analysis revealed that certain group of genes were significantly ($p \leq 0.05$) and differentially expressed ≥ 2 fold-changes w.r.t untreated [NT] adipocytes) i.e. either up-regulated or down-regulated only due to treatment with both Cortisol and Serotonin i.e. CS (these genes

remained suppressed when treated individually with C or S, figure 3.21a) shows the differential expression of these genes as a heat-map (the details are provided in table 3.4a and 4b).

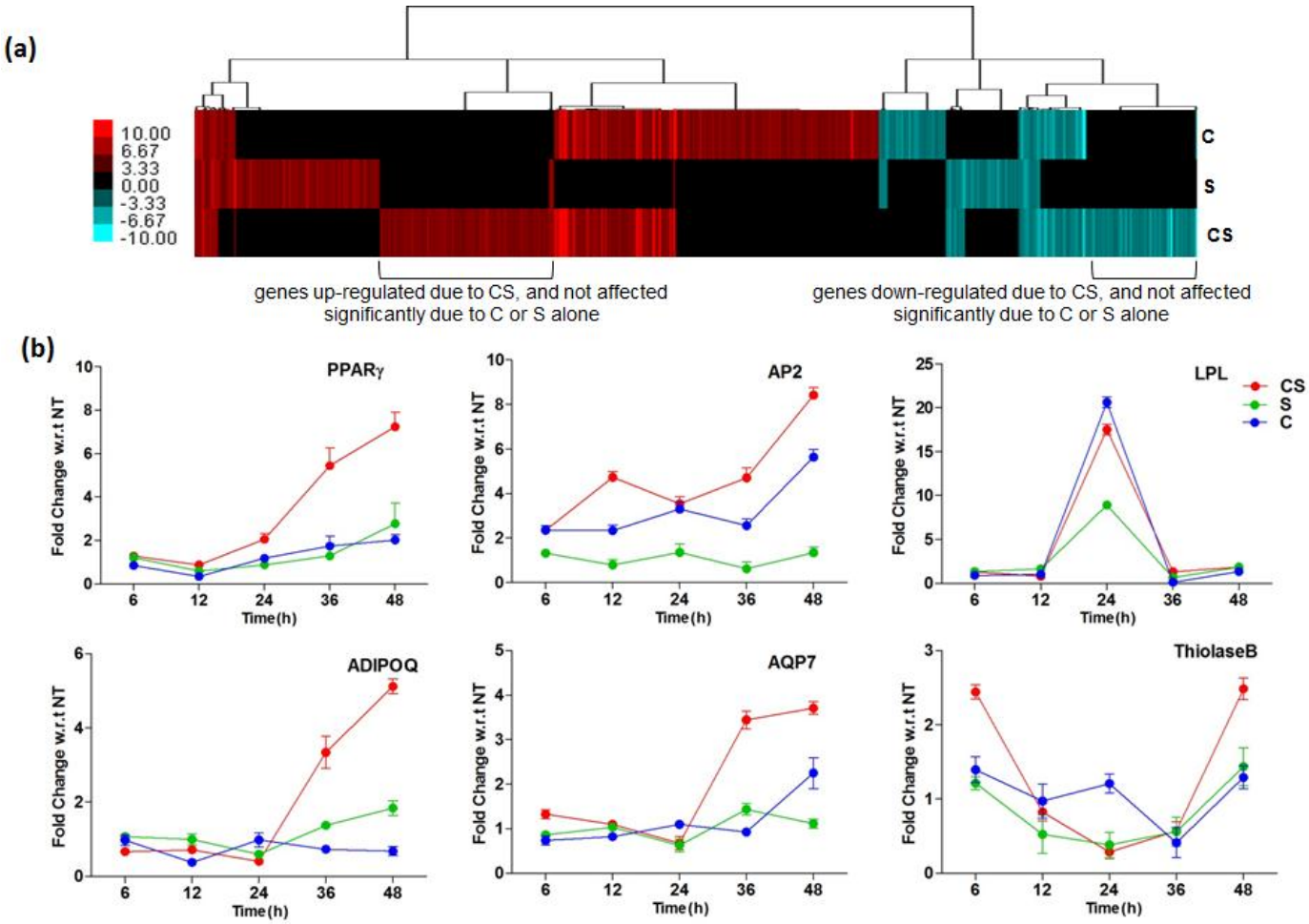


Figure 3.21: Differential effect of Cortisol and Serotonin on the transcriptional profile co-cultured adipocytes at 48h.
a) Heat-map of all differentially expressed genes in co-cultured adipocytes treated with C, S and CS compared against NT with fold change >2.0 b) RT-PCR determined relative transcription of PPARγ, AP2, LPL, ADIPOQ, AQP7 and ThiolaseB in co-cultured adipocytes treated with cortisol (C), serotonin (S), and both cortisol and serotonin (CS) expressed as fold-changes against time-matched untreated controls at 6, 12, 24, 36, and 48h. Relative fold changes are shown as mean ± SD. Significant differences are indicated by asterisks [* ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$) and **** ($p<0.0001$)].

Table 3.4: Genes affected only by cortisol or serotonin at 48h.(a) Genes up-regulated/down-regulated by S, but suppressed due to either C or CS, and (b) genes up-regulated/down-regulated by C and suppressed by both S and CS.

Table 3.2.2.4.2a

	CS	C	S	type
Tmem192	13.6	9.3	1.2	S-down
Nfatc3	10.1	8.1	1.6	S-down
Olfr791	7.2	6.3	1.4	S-down
Sfn	6.9	4.9	-1.1	S-down
Uqcrc2	6.5	4.4	-1.2	S-down
Fam134c	5.7	5.3	1.2	S-down

Table 3.2.2.4.2b

	CS	C	S	type
Mmp28	1.1	5.5	-1.0	C-up
AY761185	-1.0	4.8	1.0	C-up
Tbx20	2.6	3.9	3.0	C-up
Il20rb	3.0	3.9	3.0	C-up
Rheb	2.8	3.8	2.3	C-up
Krt13	2.7	3.5	1.1	C-up

Il31ra	5.4	3.7	1.1	S-down
Olfr150	5.4	4.5	-1.0	S-down
Arhgef15	5.2	4.1	-1.0	S-down
Chst13	4.9	4.7	1.0	S-down
Pkd2	4.9	6.1	1.3	S-down
Tcf4	4.9	2.6	1.1	S-down
Irgm2	4.8	4.2	1.1	S-down
Gm10091	4.5	4.6	1.0	S-down
Sdr16c6	4.3	2.5	-1.0	S-down
Mtor	4.1	3.8	1.1	S-down
Gm3669	3.8	3.1	1.1	S-down
Hps4	3.7	4.2	1.3	S-down
Sipa1l1	3.4	2.9	1.6	S-down
Kif2c	3.2	3.7	1.0	S-down
Fbxo34	3.2	2.5	1.5	S-down
Fut7	3.2	2.4	-1.0	S-down
Chst10	3.1	2.4	1.1	S-down
Kera	3.1	2.9	-1.1	S-down
Krt73	3.1	2.0	-1.6	S-down
Mocs3	3.0	2.7	1.0	S-down
1700128E19Rik	2.8	3.0	-1.2	S-down
Plekhh2	2.8	2.7	-1.0	S-down
Mrpl51	2.7	2.8	1.3	S-down
Trcg1	2.7	2.7	-1.5	S-down
Daam2	2.6	2.1	1.2	S-down
Aldob	2.6	2.5	-1.3	S-down
Nid1	2.6	2.5	1.1	S-down
Olfr917	2.5	3.0	1.3	S-down
Setbp1	2.5	2.1	1.2	S-down
5430439M09Rik	2.5	3.0	-1.4	S-down
Yipf1	2.4	2.2	1.2	S-down
Slc7a15	2.4	2.5	-1.0	S-down
Oprd1	2.4	2.6	-1.1	S-down
1110017D15Rik	2.4	2.4	1.1	S-down
Hras1	2.3	2.2	1.0	S-down
Gm10561	2.3	2.5	-1.0	S-down
Zdhhc2	2.3	2.4	1.4	S-down
Efcab9	2.3	2.5	-1.2	S-down
Gm5927	2.3	2.7	2.0	S-down
4932442L08Rik	2.3	2.1	1.0	S-down
Tmem61	2.3	2.7	1.1	S-down
2700005E23Rik	2.3	2.2	-1.1	S-down
Susd4	2.2	2.2	-1.0	S-down
Litaf	2.2	2.3	-1.0	S-down
Med28	2.1	2.0	1.2	S-down
Six3os1	2.1	3.0	1.9	S-down
41338	2.1	2.3	1.2	S-down
Tnfsf18	2.1	2.3	1.3	S-down
Gpr12	2.1	2.1	-1.0	S-down
H2-M10.2	2.1	2.5	1.3	S-down
Mthfd1l	2.0	2.3	1.3	S-down
Dcaf12l2	2.0	2.1	1.4	S-down
Lhfpl2	2.0	2.4	1.1	S-down
1200007C13Rik	2.0	2.2	1.1	S-down
Scfd2	2.2	2.3	8.4	S-up
Olfr582	2.2	2.0	4.0	S-up
Klhl23	1.1	-1.1	3.8	S-up
Wfdc6b	-1.3	-1.2	3.4	S-up
Psmc5	-1.1	1.2	3.3	S-up
Cylc1	-1.4	-2.3	3.3	S-up
ATP8	-1.3	-1.4	3.2	S-up
Nlk	-1.3	-1.4	3.0	S-up

Rab2b	1.2	3.4	1.2	C-up
Mapk4	1.4	3.4	1.6	C-up
Dhrsx	1.2	3.0	1.1	C-up
Rbm3	1.5	2.9	1.5	C-up
Prr14l	-1.0	2.9	-1.0	C-up
Mrpl52	1.0	2.8	1.2	C-up
Oraov1	-1.1	2.7	1.0	C-up
Aqp9	-1.0	2.7	1.0	C-up
Olfr539	-1.1	2.7	-1.0	C-up
Ak8	1.2	2.6	1.2	C-up
Decr1	1.8	2.6	1.4	C-up
Ankrd34b	-1.1	2.6	-1.1	C-up
Gbf1	-2.1	2.6	1.3	C-up
Hfe2	-1.1	2.6	1.1	C-up
Armcx3	1.4	2.6	1.2	C-up
Raver2	1.1	2.6	-1.2	C-up
Olfr458	1.0	2.6	1.1	C-up
Ptprk	-1.1	2.6	-1.0	C-up
Sftpd	-1.0	2.5	-1.0	C-up
Mis18a	-1.1	2.5	1.0	C-up
Nudt13	-1.1	2.5	1.2	C-up
Ccdc149	-1.0	2.5	1.1	C-up
Pmm1	1.1	2.5	-1.1	C-up
1700047E10Rik	1.1	2.5	1.2	C-up
Ace2	-1.0	2.5	1.0	C-up
Uxs1	1.1	2.4	1.1	C-up
Dpyd	1.1	2.4	-1.1	C-up
Smpd5	-1.1	2.4	-1.1	C-up
5730471H19Rik	1.4	2.4	1.3	C-up
Zfp442	-1.0	2.4	1.1	C-up
Epm2aip1	-1.0	2.4	-1.2	C-up
Txndc2	-1.0	2.4	1.0	C-up
Rmi1	1.2	2.4	1.5	C-up
Ppfia4	-1.2	2.4	1.0	C-up
Gm534	-1.1	2.3	-1.3	C-up
Onecut1	1.2	2.3	-1.2	C-up
4930435E12Rik	-1.0	2.3	1.0	C-up
Fam228b	-1.0	2.3	-1.1	C-up
Rnf220	1.2	2.3	1.2	C-up
Osbpl3	-1.2	2.3	1.0	C-up
BC048671	1.2	2.3	1.1	C-up
Daglb	-1.0	2.3	1.4	C-up
Alg14	-1.0	2.3	-1.1	C-up
Rpf1	1.1	2.2	1.1	C-up
Fam173b	1.0	2.2	1.0	C-up
Ebpl	-1.0	2.2	1.0	C-up
Gm5475	-1.2	2.2	-1.0	C-up
Stx6	1.4	2.2	1.1	C-up
Ppm1b	-1.1	2.2	1.0	C-up
Zfp92	-1.1	2.2	1.2	C-up
Ahcy	1.1	2.2	1.2	C-up
4930413E15Rik	1.0	2.1	1.0	C-up
A030012G06Rik	-1.0	2.1	1.0	C-up
Tmem144	1.1	2.1	1.5	C-up
Clca4	-1.0	2.1	1.0	C-up
Catsperd	-1.4	2.1	1.1	C-up
Carhsp1	1.1	2.1	1.1	C-up
Rhoa	1.2	2.1	-1.0	C-up
Zzz3	-1.0	2.1	-1.1	C-up
Adad1	-1.2	2.1	-1.1	C-up
Inmt	-1.1	2.1	-1.1	C-up
Txndc11	-1.3	2.1	-1.1	C-up

Racgap1	1.2	-1.1	3.0	S-up
Sbds	1.1	-1.1	3.0	S-up
Mettl7a3	-1.5	-3.2	2.8	S-up
Zfp653	-1.7	-2.0	2.8	S-up
Fam69c	-1.0	1.1	2.7	S-up
Slc25a26	-1.2	1.0	2.7	S-up
Pnlip	1.7	1.4	2.7	S-up
Slc16a8	1.2	-1.0	2.6	S-up
Reep4	1.1	1.0	2.6	S-up
Gm1082	-1.0	1.0	2.6	S-up
Slc37a4	1.0	-1.2	2.6	S-up
Odf2	-1.0	1.0	2.6	S-up
Nrp	1.4	1.1	2.5	S-up
Yipf3	1.2	-1.1	2.5	S-up
Nalp1b	-3.2	-2.6	2.5	S-up
Sema3c	-1.0	1.1	2.5	S-up
4933415E08Rik	1.1	-1.1	2.5	S-up
Lsm6	1.9	1.1	2.4	S-up
Olfr498	1.4	1.5	2.4	S-up
Sirt2	1.2	1.2	2.4	S-up
Pitx3	-1.1	1.1	2.3	S-up
Lepr	-1.0	1.1	2.3	S-up
Dctn1	-1.1	-1.3	2.3	S-up
Gm13125	1.1	1.2	2.2	S-up
Kdm4b	1.3	1.2	2.2	S-up
Mboat1	1.2	-1.1	2.2	S-up
Zfp189	1.1	-1.0	2.2	S-up
Pdcd7	-1.1	1.2	2.2	S-up
Cblb	1.1	1.2	2.2	S-up
Alpi	1.0	1.1	2.2	S-up
Pdcd1	-1.1	-1.2	2.2	S-up
Fdx1l	-1.0	-1.1	2.1	S-up
Krtap16-1	-1.2	-1.0	2.1	S-up
Gm10037	-1.2	-1.1	2.1	S-up
Cd1d1	1.0	-1.1	2.1	S-up
Dpm1	1.1	1.1	2.0	S-up
Qdpr	-1.2	1.3	2.0	S-up
Fabp12	1.1	1.1	2.0	S-up
Fbxl17	-1.1	-1.1	2.0	S-up
AI132709	1.2	1.0	2.0	S-up
Hist1h1c	1.2	2.1	1.3	C-up
Mpo	1.1	2.1	-1.0	C-up
Olfr691	-1.1	2.1	-1.1	C-up
Pax3	-1.0	2.0	-1.0	C-up

It was also observed that Arachidonic acid metabolism was affected in CS as well as S treated co-cultured adipocytes, however, it was not affected due to C treatment. Fatty acid elongation pathway was also affected due to serotonin. However, no pathways relevant to the context were sufficiently populated with high transcription values due to these treatments. The details of the genes up/down regulated due to CS treatment only can be found in table 3.5.

Table 3.5: Differential transcription values of genes that were uniquely up-regulated or uniquely down-regulated due to treatment with **(CS).F**old-changes obtained from microarray of co-cultured adipocytes treated C, S or CS at 48h compared against time-matched untreated co-cultured adipocytes as compared against untreated adipocytes after 48h of co-culturing with macrophages. Fold-change cut off used is +/- 2.

Genes	Up	Genes	down
Olfr381	3.2	Pbrm1	-8.3

1785C21Rik	3	Htr3a	-3.6
Lypd6b	3	Col19a1	-3.2
Vpreb3	3	Slc16a5	-3.1
Phactr3	3	4930517G19Rik	-3.1
D1379A8Rik	3	Pex7	-3.0
Gm5148	2.9	Slc22a13b-ps	-3.0
Supt6	2.9	Mrpl36	-2.9
Pemt	2.9	C80998	-2.8
Hcn2	2.9	Gm15753	-2.7
Loxl3	2.8	2600006L11Rik	-2.7
Olfr566	2.8	Fgb	-2.6
Gm1576	2.8	Olfr324	-2.6
Tgfb3	2.8	Arhgef26	-2.5
Mettl3	2.8	Dsn1	-2.5
Dcdc2c	2.8	Abca3	-2.5
S1a4	2.7	Mphosph8	-2.4
Zfp352	2.7	Gltscr1l	-2.4
Slc36a2	2.7	B3galt2	-2.4
Man1a	2.7	Fam71a	-2.4
Klk6	2.7	BC039771	-2.4
Mfsd1	2.6	Serpinb3b	-2.4
Zfp111	2.6	Creb3l3	-2.3
Ccdc59	2.6	9530077C14Rik	-2.3
Tmem74b	2.6	A930007D18Rik	-2.3
AU1612	2.6	Cilp2	-2.3
Rbm7	2.5	Ech1	-2.3
AI31464	2.5	Myt1l	-2.3
Cdnf	2.5	Socs1	-2.3
Des	2.5	Prlr	-2.3
Zfp182	2.5	Arid2	-2.3
Prss1	2.4	9230116L04Rik	-2.2
Cldn2	2.4	A430028G04Rik	-2.2
94378K24Rik	2.4	Dpp10	-2.2
493458A3Rik	2.4	Nr1i2	-2.2
Sstr3	2.4	Ubac2	-2.2
22146H18Rik	2.4	Map3k2	-2.2
Dlx1	2.4	Cd300lb	-2.2
Gna15	2.4	Snupn	-2.2
Grm8	2.4	LOC171588	-2.2
493347I5Rik	2.4	Ankzf1	-2.2
Nos3	2.4	Rrp9	-2.2
172I14Rik	2.4	Huwe1	-2.1
Mrps35	2.3	D930032P07Rik	-2.1
Gm13298	2.3	Gdf3	-2.1
Npm1	2.3	Slc10a3	-2.1
Mgat3	2.3	Znrd1	-2.1
Enc1	2.3	Pla2g4c	-2.1
Bicc1	2.3	LOC668306	-2.1
Map4k5	2.3	Gbf1	-2.1
Il1f6	2.3	Fam163a	-2.1
Gm5458	2.3	Icam4	-2.1
LOC1156153	2.3	Gm7903	-2.1
P2rx3	2.3	Serpina7	-2.1
Tdrd12	2.3	Defb7	-2.1
Synm	2.3	Gm15441	-2.0
Vstm5	2.3	Adamts20	-2.0
Olfr1436	2.3	Sipa1l2	-2.0
Tspo	2.3	Ccdc19	-2.0
Schip1	2.3	4930593A02Rik	-2.0
Arl1	2.3	Olfr355	-2.0
Kdsr	2.2	Hsd17b6	-2.0
Eif3c	2.2	Akirin1	-2.0
Micall1	2.2	LOC100504500	-2.0
Mrps17	2.2	Olfr775	-2.0
Gpsm1	2.2	Canx	-2.0

933184L24Rik	2.2
Gm242	2.2
Fam222a	2.2
Adamts9	2.2
Cox5b	2.2
493412O13Rik	2.2
Olfr713	2.2
Bad	2.2
Kcna5	2.2
Scarf1	2.2
Nfyc	2.2
Zfp354a	2.2
1729M2Rik	2.2
Fam63b	2.2
Psmb1	2.2
Chadl	2.2
Rybp	2.2
Galntl6	2.2
Chrnd	2.2
D7Ertd143e	2.2
Mrpl34	2.2
Ccdc92	2.1
4933411G6Rik	2.1
Zfp74	2.1
Ublcp1	2.1
Tmem39a	2.1
Nlrp9c	2.1
Smad2	2.1
Gm111	2.1
Gm5465	2.1
Mlh1	2.1
Nfkbil1	2.1
Ceacam-ps1	2.1
Ccdc24	2.1
Preb	2.1
Cela2a	2.1
Slc6a9	2.1

Although treatment with a mixture of cortisol and serotonin increased the lipid content of co-cultured adipocytes compared to non-treated ones, microarray analysis did not show significant change in PPARγ transcription in C, S, and CS treated co-cultured adipocytes. qRT-PCR based transcriptional profiling of PPARγ and AP2 across multiple time-points however revealed that transcription of both the genes increased with increasing time in co-cultured adipocytes, and treatment with CS caused higher transcription than C or S treatment. It was also observed that transcription of AQP7 (Aquaporin) and ADIPOQ (Adiponectin) increased after 24h and treatment with CS caused highest transcription of these 4 genes. CS treatment caused maximum transcription of Thiolase B at 6h, which gradually decreased with time till 36h, and again increased at 48h. C or S treatment however did not significantly affect the transcription of any of

these genes (except AP2, which was upregulated due to cortisol treatment at 48h) beyond 2 folds compared to non-treated adipocytes. LPL showed no increase in transcription due to treatment with C, S or CS at any time point, except at 24h, where its transcription was very high (figure 3.21b).

3.2.2.5. Cytokine profiling

The percentage increase of anti-inflammatory (IL10 and TGFβ) and pro-inflammatory genes (IL1b, IL6, IL12 and TNF) in macrophages grown alone as well as co-cultured with adipocytes was accessed through ELISA. It was seen that both the pro and anti-inflammatory cytokines were expressed in C, S and CS treated macrophages grown alone as well as co-cultured (figure 3.22).It was also observed that the secretion of these inflammatory cytokine decreased with time.

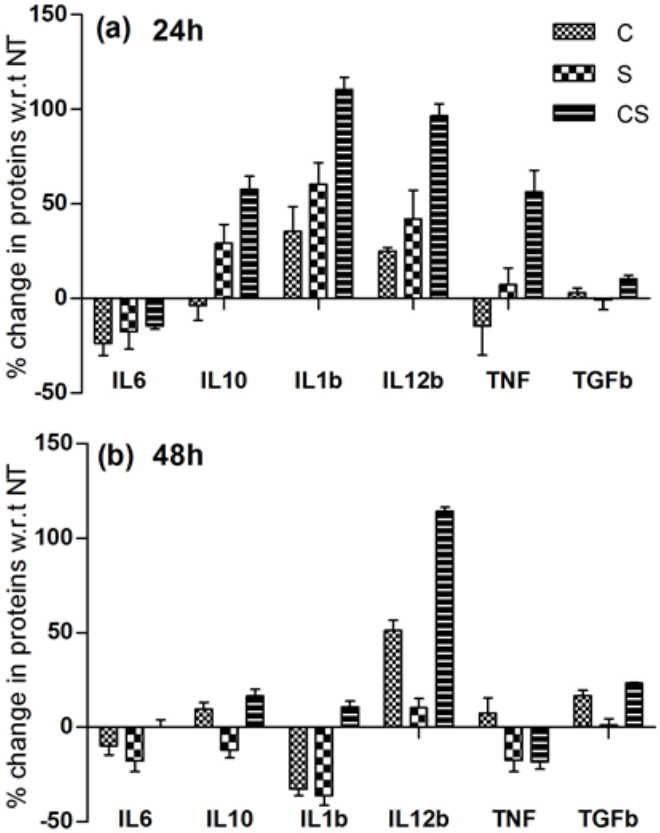


Figure 3.22: Relative fold changes of IL6, IL1b, IL10, IL12b, TNF, TGFb at 24h and 48h in co-cultured adipocytes treated with C, S and CS compared against untreated time-matched controls, determined through ELISA.

3.3. Discussion

Chronic psychological stress is known to regulate visceral obesity [244] and adipose tissue inflammation [245], which is marked by infiltration of ATMs (Adipose Tissue Macrophages) [226, 245-248]. Although adipose tissue comprises of components other than adipocytes and macrophages like pre-adipocytes, monocytes, granulocytes, mast cells, however the major players in regulating adipogenesis and immunity are adipocytes and macrophages. Glucocorticoids are already known to have adipogenic effect on adipocytes. Although one of the mechanisms by which glucocorticoids enhance adipogenesis in adipocytes is by promoting differentiation [230, 249], yet the molecular basis of adipogenic action of is not fully understood; hence, other mechanisms of glucocorticoid induced adipogenicity cannot be altogether be ruled out.To elucidate the underlying signaling events of chronic stress situation, where localized serotonin content in adipose tissue is high due to glucocorticoid induced adipose tissue inflammation in an in vitro simulation, adipocyte-macrophage co-culture system was treated with both serotonin and cortisol.

3.3.1. Adipogenic signaling through HTR2c and HTR5a in co-cultured adipocytes due to action of stress hormones cortisol and serotonin

Results from current study revealed that adipocyte differentiation (marked by adipocyte differentiation marker, AP2 gene) was contributed largely by cortisol alone and this differentiation was enhanced by co-culturing with macrophages. Increase in gene expression of PPARγ and the scavenger receptors (CD36 and OLR1), however, were synergistically induced by cortisol and serotonin. This result can be attributed to the fact that while serotonin or cortisol individually up-regulate HTR2c and HTR5a by less than 3 folds, their synergistic action up-regulates these receptors by more than 8 folds. Exact mechanism of this apparent synergistic effects of cortisol and serotonin on upregulation of two specific serotonin receptors (HTR2C and

HTR5A) is not known; however, studies have identified Glucocorticoid Responsive Elements (GREs) in the promoters of a number of serotonin receptors [250]. Bioinformatic analysis revealed that the consensus GRE sequence (ANAACANNNTGTTNT) occurs 12 times in the intron regions of HTR2c gene and only once in the exon of HTR5a gene as shown in figure 3.23a and b.

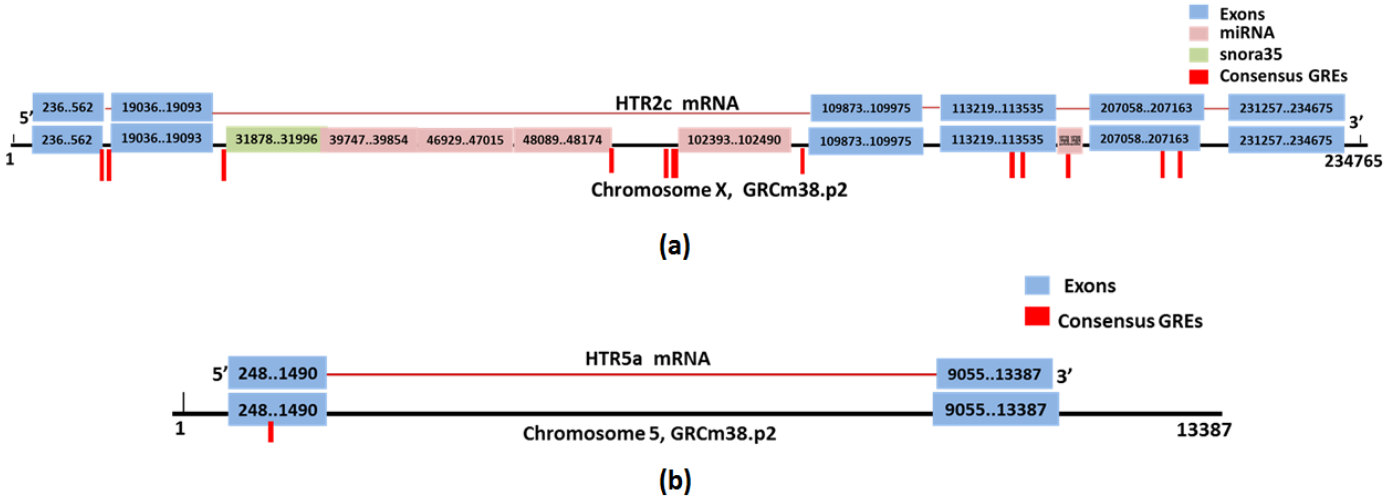


Figure 3.23 : Graphical mapping of genes showing the exons, introns, miRNAs and predicted GRE consensus sequences (shown as red vertical bars) in (a) HTR2c gene (b) HTR5a gene

It is possible that cortisol-binding could lead to binding of activated Glucocorticoid Receptors (GRs) to these GREs and enhance their transcription. Thus presence of GREs in HTR5a and HTR2c genes could be a possible explanation of cortisol induced up-regulation of these two genes (HTR2C and HTR5A). In addition, when treated with their natural ligand (serotonin), the transcription of these serotonin receptors might be naturally enhanced. This could account for the synergistic up-regulatory action of cortisol and serotonin on these two receptors.Synergistic action of cortisol and serotonin was not limited to HTR5a and HTR2c genes. Similar pattern of their action was also seen on PPARγ and the scavenger receptors. This observation led to the assumption that serotonergic signaling through these two receptors might cause their up-regulation. It was observed that by blocking HTR5a and HTR2c receptors signaling by their

respective antagonists transcription of PPARγ, OLR1 and CD36 genes were indeed significantly attenuated, and simultaneously resulted in significant reduction in adipogenesis. As expected, it was also observed that antagonist of HTR2C or HTR5A receptor did not affect the serotonin transporter, SERT, unlike HTR5a and HTR2c. This observation clearly reveals that glucocorticoids are capable of stimulating serotonergic signaling in adipocytes through HTR5a and HTR2c receptors, which in turn increases their lipid content by activating adipogenic transcriptional activity. Apart from activation of adipogenic transcription factors, there could also be other possible mechanisms that might contribute to enhanced adipogenesis in co-cultured adipocytes. Scavenger receptors are known for internalization of oxLDL, which are then acted upon by LPL (Lipoprotein lipase) in adipocytes to generate free fatty acids (FFAs). FFAs are long known ligands of PPARγ (adipogenic transcription factor) that increase lipid accumulation by increasing adipogenesis. The triglycerides generated by LPL might also be stored as lipid droplets in adipocytes. Cortisol has already been reported to cause up-regulation of OLR1 and CD36 scavenger receptor in earlier studies [251, 252]. Serotonin is associated with oxidation of fatty acids which may explain increase in expression of scavenger receptors OLR1 and CD36 in adipocytes treated with cortisol and serotonin. Current reports show that expression of these scavenger receptors, is also dependent on signaling from HTR5a and HTR2c, since blocking signaling from HTR2C and HTR5A decreases the transcription of OLR1 and CD36. It has been observed that apart from the established overlap of function between adipocytes and macrophages, in chronic obesity, metabolic dysfunction and inflammation [227-229], adipocytes and macrophages communicate with each other, and their interaction affects both adipocyte differentiation and macrophage function [253]. Similarly, adipocytes too exert modulatory effects on macrophages when co-cultured under similar conditions. Our study reveals that though

co-cultured macrophages have tendency to accumulate more lipids in presence of cortisol and serotonin through up-regulation of adipogenic transcription factor PPARγ. The synergistic effect of treatment, with cortisol and serotonin together, is however, independent of HTR5a, HTR2c and SERT signaling.

Microarray analysis shows that even when adipocytes and macrophages are not in direct contact with each other, co-culturing causes transcription activation of large number of genes in adipocytes, and the numbers increase sharply with time. Recent studies have indicated that during energy surplus adipocyte hypertrophy is associated with reduced adipocyte turnover and may promote inflammation induction of hypoxia and aberrant extracellular matrix remodeling. Here, it was found that metalloproteinases like MMP10 and MMP3 that have already been implicated in extra cellular tissue remodeling in adipocytes[254] were transcribed higher in adipocytes due to co-culturing. This suggests that crosstalk between adipocytes and macrophages in adipose tissue contributes to adipose tissue expansion and remodeling [255] and that there might be large number of paracrine factors that exert inflammatory and metabolic regulation in obese adipose tissue.

3.3.2. Both de-novo lipid synthesis and triglyceride acquisition contribute to increased lipid content in co-cultured adipocytes

The high lipid content of co-cultured adipocytes could be directly attributed in part to increased activity of genes like CEBP, APOE and OLR1 that have established roles in adipogenesis and partly to decrease in LPL expression. During lipid excess triglycerides become associated with apolipoproteins. Triglyceride rich lipoproteins become enriched in APOE and are lipolyzed on the surface of endothelial cells by Lipoprotein Lipase (LPL) that mediates release of fatty acids from circulating lipoproteins It is established that differentiated adipocytes are also capable of

producing APOE [256] which is capable of modulating adipocyte lipid and lipoprotein metabolism. Earlier studies along with this report show correlation of increase in APOE mRNA with the cellular lipid content. It is also reported that LPL shows early expression which gradually decreases with time, while APOE is late expressing and increases with time, which is also supported by this report. Apart from actions of CEBP and APOE, it appears that adipocyte-macrophage co-cultures are rich in extra-cellular oxidized lipids, which are directly transported into as is evident by higher expression of OLR1 gene in co-cultured adipocytes. Higher expression of APOE and OLR1 in CS treated adipocytes also corroborates with higher lipid content of these cells. Apart from theses, there are also other mechanisms that contribute to adipogenesis e.g. de novo synthesis of fatty acids (FA) from cytoplasmic pool of Acetly-CoA. Transport of glucose into co-cultured adipocytes increases as evidenced by increase in GLUT1 expression with time. Glucose after being converted to Acetyl CoA, can either undergo oxidation through mitochondrial TCA cycle, or can be transported through ATP Citrate lyase to cytoplasm and get converted to Malonyl-CoA for subsequent Long-Chain Fatty Acid (LCFA) synthesis. ACCA1 (acetyl-CoA carboxylase 1), which catalyzes conversion of Acetly-CoA to Mal-CoA, is upregulated, while carnitinepalmitoyltransferase 1 (CPT-1) is down-regulated at 48h, implying that import and oxidation of long chain fatty-acids is mitochondria is blocked. This might contribute to increased triglyceride synthesis in co-cultured adipocytes at 48h. It is also established that expression of SLC16A11 (MCT11), is capable of altering lipid metabolism, most notably causing an increase in intracellular triacylglycerol levels [257]. MCT11 expression too peaked at 48h in co-cultured adipocytes, implying that monocarboxylate transporters mediated pyruvate import too contributes to the increased lipid content of co-cultured adipocytes.

3.3.3. Role of adipogenic mediators in inflammation

Once inside the cytoplasm, the selective FAs are carried to different subcellular locations by Fatty Acid Binding Proteins (FATPs) for fatty acid synthesis or oxidation. Although FATP3 was up-regulated due to co-culturing, Acyl-CoA dehydrogenases and CPT1 (responsible for transport and oxidation of LCFAs in mitochondria) were suppressed suggesting that LCFAs are transported into co-cultured adipocytes and are targeted to peroxisome (has preference for very long chain fatty acids) rather than mitochondria for oxidation. Differential regulation of ACSL1, ACSL3 and ACSL4 in co-cultured adipocytes at 48h further indicated that differ types of fatty acids are present in the adipocytes. Suppression of ACSL1 indicated that although there is fatty-acid intake, long chain fatty acids are not activated to esters thereby blocking their metabolism. Up-regulation of ACSL4, known to have a preference for transport of Arachidonic acid Acid (AA) and its conversion to arachidonate indicates active arachidonic acid metabolism in co-cultured adipocytes. This is further supported by up-regulation of arachidonate metabolizing enzyme PTGES. Possible ω-hydroxylation of arachidonic acid and LTB_4 by CYP4f groupof enzymes further support the fact that AA metabolism is enhanced at 48h in co-cultured adipocytes. Stress hormones appear to down-regulate FATP3 at 48h, implying that cortisol and serotonin probably contribute to higher lipid accumulation either by dampening the oxidative degradation of LCFA or by reducing the transport of LCFAs into sub-cellular compartments like mitochondria or peroxisomes. Prostaglandins, by-products of arachidonate metabolism, bind to prostanoid receptor G-protein coupled receptors, leading to increase cyclic AMP concentrations and, and activation of a number of signaling transduction cascades. Decrease in PTGS2 (COX2) expression with time hints that prolonged co-culturing might induce AA-metabolism mediated anti-inflammatory state in adipocytes. However, stress hormones like cortisol and serotonin

cause higher expression of COX2 and therefore reinforce a pro-inflammatory state in co-cultured adipocytes. Recently it has been suggested that short chain fatty acids can bind to certain group of G-protein coupled receptors and trigger inflammation in adipocytes. In this report, up-regulation of GPR153 (a member of the same family) at 48h parallel with increasing adipogenicity and decreasing pro-inflammation pins down the potential role of short-chain fatty acid as well as this receptor in mediating inflammation. Apart from lipid oxidation by-products, increase in GLUT1 transcription also lays down possibility of high glucose oxidation. This can result in production of ROS (Reactive Oxygen Species), which is a potent inflammation causing agent and might also contribute to the inflammatory status of co-cultured adipocytes.

3.3.4. Crosstalk between adipocytes and macrophages causes a shift from pro-inflammatory to anti-inflammatory status with increasing time

Our results indicate that although co-cultured adipocytes secrete more pro-inflammatory proteins (IL1a, IL6, CXCL1, PTGS2, CXCL5, CSF2, CSF3, CCL3, CCL4, MMP3), their secretion decreases with more time spent in co-culture. The decrease in pro-inflammatory transcription profile was accompanied by increasing secretion of anti-inflammatory proteins like IL19 (a member of anti-inflammatory IL10 family), IL13Ra and cAMP with co-culture time (figure 3.16). It appears that co-culturing adipocytes with macrophages induces a shift from pro-inflammation to anti-inflammation secretory profile. This was also supported by decreasing transcription of FOS, NOD2 and TRAF1 with time due to co-culturing. Fos and Jun dimerise to form AP1 transcription factor, which is essential for activation of large number of pro-inflammatory cytokines. Similarly NOD2 is also among the DNA-binding proteins that are required for transcription activation of inflammatory proteins. However, in the presence of stress hormones Cortisol (C), it appeared that there is decrease in pro-inflammation, but higher

chemotraction for immune cells as supported by higher expression of CSF2, CSF2, CSF3 and CXCL5 or RANTES. Serotonin appeared to contribute more to adipose tissue remodeling though MMP3, but also contributed to pro-inflammation, which decreased with time (figure 3.16 and 3.18). This suggested that most probably in hypertrophied adipocytes, cortisol and serotonin act to aggravate the local inflammation by attracting macrophages and extra-cellular matrix remodeling.

Previous studies have reported the association of increased levels of TNFα with macrophage infiltration into adipose tissue. Supporting this observation, our results also show significant activation of TNF signaling pathway in adipocytes due to co-culturing. Although TNF-signaling pathway appeared to be activated due to co-culturing, the signaling however decreases with time as supported by an increase in the production of TNFAip3 parallel to the drop in the transcription of pro-inflammatory proteins. TNFAip3 (A20) is a negative feed-back regulator of TNF-signaling pathway and NF-κB, and plays essential roles in the homeostasis by preventing inflammation and apoptosis. Both cortisol and serotonin appear to boost secretion of TNFαip3 in adipocytes and thus prevent adipocyte death due to heightened inflammation. This also corroborates well with the anti-inflammatory boosting of cAMP and IL13Ra by stress hormone cortisol. TNFα and IL6 respond to immune and metabolic changes, and can be produced by activation of TLR-signaling through AP1 transcription factor, or through NF-κB. Though TLR signaling appears to contribute to the inflammatory profile of co-cultured adipocytes through TRAF6-mediated TLR2/TLR6 complex, yet the comparative transcriptional changes in TLR2 was not significant. Role of TLR13 has not been well explored yet, it however appears to be involved in the cross-talk between adipocytes and macrophages and interestingly is suppressed by stress hormones. This suggests a potential role for TLR13 in immune-metabolic regulation.

CHAPTER 4

DEVELOPMENT OF INTEGRATED PLATFORM FOR MINING AND ANALYSIS OF PSYCHOLOGICAL STRESS DATA

4.1. Current Structure of Data

There are numerous isolated investigations reporting complex data regarding genes, pathways affecting isolated biological signaling [258-261] with respect to psychological stress [262, 263]. As a result, current assembly of biological information largely comprises of low-throughput investigations of interactions among a list of very molecules. Over the last few years, information concerning the human interactome has grown rapidly (e.g. KEGG, HPRD, BIND, MINT, BIOMART, miRBase databases to name but a few). Although these efforts are enormously valuable, they are not without limitations. The identification and functional annotation of molecular/genetic network behind cross-regulation and interdependence between physiological functions still remains largely incomplete owing to lack of systematic information about connectivity at molecular level [264-266]. The arena of psychological stress and its effects on interaction between various physiological functions has not been studied extensively. Several large-scale efforts to identify all possible molecular interactions that make up the interactome are under way in several species[267-270], including human [271]. However, most of the investigations do not have a specific focus on stress, and in particular, not on psychological stress. Therefore, majority of interactions of relevance to psychological stress are not annotated by these efforts. Studies that do focus on investigations relevant to psychological stress, measure singular aspects of one or more physiological function affected/associated with it in an isolated manner, making annotation of their molecular inter-relationship difficult[264].

4.2. Role of Literature-mining

Psychological stress has been drawing attention lately owing to life-style or stress associated increase in mortality and diseases [50, 272-275]. Hence, in recent years, investigation of pathways and molecular interactions involved in stress has gained momentum, with an explosion

of publications reporting a large number of interactions in the biomedical literature. Although there may be only a few interactions reported in each publication, there are thousands of such publications, which are otherwise valuable sources of data for defining the molecular basis of stress. Manual curation of data from these sources require contextual considerations which includes cell and/or tissue type, environmental or experimental conditions including the presence of specific stimuli, the species, the time-point, etc. In addition, level of confidence, on actual occurrence of interactions, is also affected by whether the interaction has been independently reported by other research groups. The sheer scale of effort involved in curating such interactions from the literature manually, is huge and requires effort from massive consortiums. In fact, several databases barely manage to concentrate their curation efforts on papers published in fewer than ten journals. Literature mining approaches potentially provide a high-throughput, low-cost alternative to extracting context-dependent information and annotation from the literature [259].Rich contextual information on the interaction between molecules/pathways can be mined from these publications, which can prompt critical leads to exploring these molecular networks through several different experimental validations (as has been shown in this study).

4.3. Necessity of building associations among the mined data

Although mining annotation on such interactions from literature can be extremely valuable, it is important to first have a catalogue of the components of the system and how they interact with each other. Next it is important to identify candidate genes associated with physiological stress in each component, and understand the interaction between them by analyzing the molecular causative functional relationships among apparently unrelated candidate genes. Generating such a catalogue is complicated by the fact that the interactome for each species is a dynamic owing to new additions with research advances, and interactions that are reported too are dependent on

their individual contexts. Many resources can identify candidate genes associated with a given biological context of interest (let's say a physiological function), by mining the available interactome data (e.g. SUSPECTS, PROSPECTR, GeneSeeker, Endeavour) [262, 276] but they do not provide the required gene networking which is essential in understanding the relationships involved in a complex regulation of various cross-talking physiological functions. Although there are a few databases like STRING [277] that do provide gene networks based on select candidate genes, they lack robust user-controlled query systems which are required for association of genes to the typical biological context under study. Few network-based methodologies have been developed for the prediction of genes associated with diseases [278, 279], however, these are limited by lack of exhaustive disease-gene annotation for many diseases in OMIM [280-282]. A few recently developed methods [283] exist that identify gene-phenotype associations using BIOMART instead of gene-disease association for pulling out the causative genes and associating them with the phenotypes. Despite these improvements in the available tools, developing a systematic understanding of data connectivity is still only partial. This is due to the fact that all disease phenotypes are not completely annotated and many are yet to be listed in OMIM, on which the learning framework of these tools largely depends. To address the above challenges, literature based data-sets have also been used [284-286] as an aid since they are much richer in interaction data compared to the annotated databases.

4.4. Methods

4.4.1. Data Synthesis

The huge repertoire of already existing data can address the need to develop a global understanding of psychological stress and its association with varied many physiological functions in order to better understand stress-induced disease susceptibility. We have, therefore,

used a strategy that establishes connectivity in the information scattered across the pertinent literature through an unique systematic-analysis approach through the following steps: 1) Identifying candidate genes for a given biological context based on manually curated training genes which have already been established to be associated with those processes from the human interactome by data-mining, 2) Screening for associations of candidate genes, within the biological contexts of interest, using user controlled context-based information in the published literature, 3) Building of an association among the elements of the context-dependent screened data. Thus, molecular interaction data have been generated for psychological stress, associated physiological functions and diseases for many species including human.

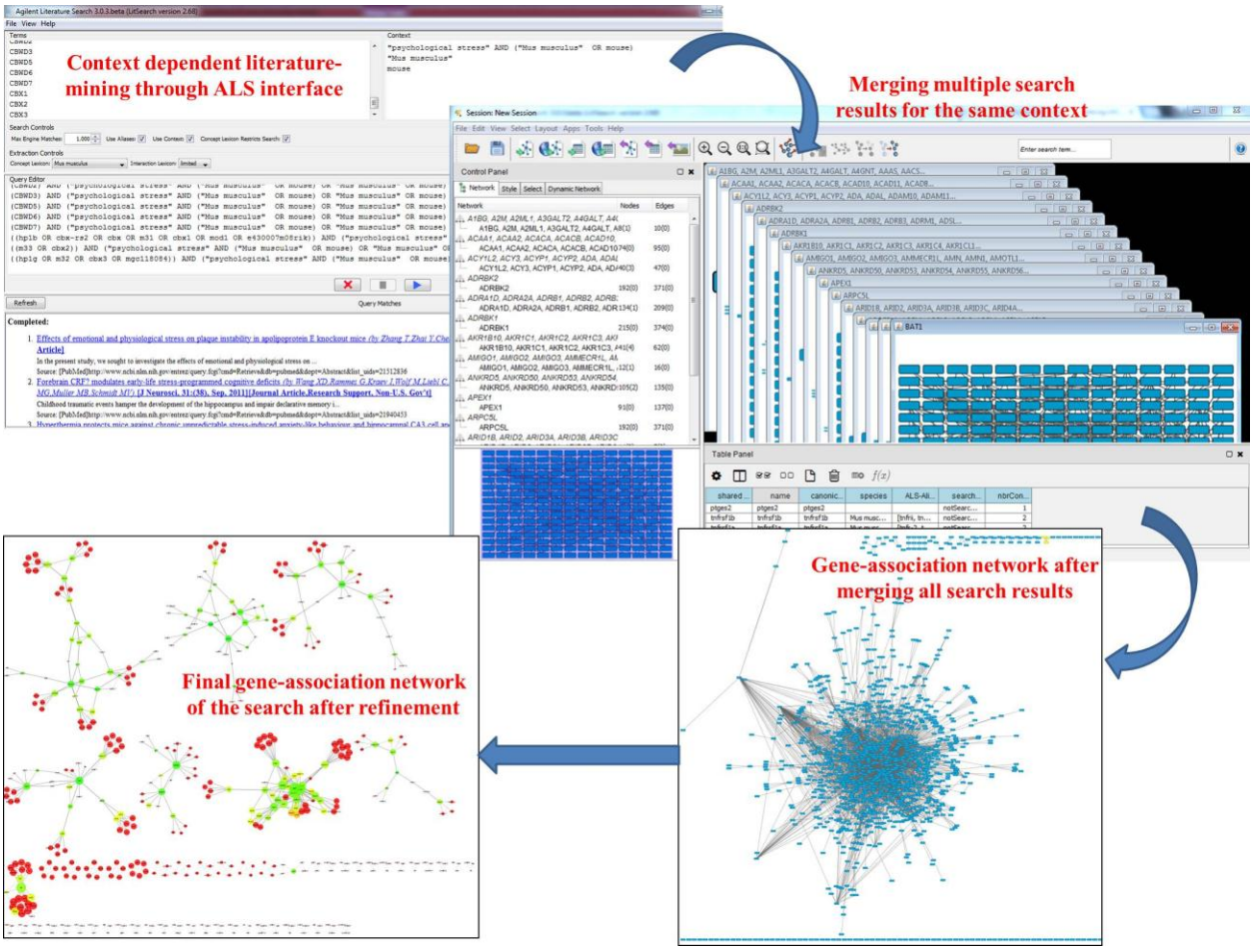


Figure 4.1: Schema of data synthesis for each literature-mining based gene-association network in the database

4.4.2. Technology

Wamp Server 2.4 [Apache Server version: 2.2.4, PHP version: 5.4.12, MySQL version: 5.6.12] was used to create the database and data each of the species and physiological process in the following table was used to populate the database.

4.5. Results

As described in previous section, gene-association networks based on literature mining were generated for ageing, digestion, metabolism, immunity, respiration and reproduction in human, mouse, rat, cattle and pig. To check whether stress could be associated with the genes in these association networks, gene-association network for psychological stress was developed for each of these species and provided in the database (table 4.1), where user can find whether each gene associated with a physiological process is a stress gene or not (figure 4.2 shows a snapshot of the interface containing gene-data of a physiological process and its stress status, in terms of literature-based association).

Table 4.1: The number of genes in each of the gene-association networks for each species.

	Human	**Mice**	**Rat**	**Cattle**	**Pig**
Immunity	1721	1604	485	83	161
Metabolism	2108	2806	1538	299	294
Ageing	2496	1483	444	34	161
Reproduction	499	1761	460	110	228
Digestion	1175	329	329	44	65
Development	385	289	286	42	31
Respiration	1361	453	281	75	86

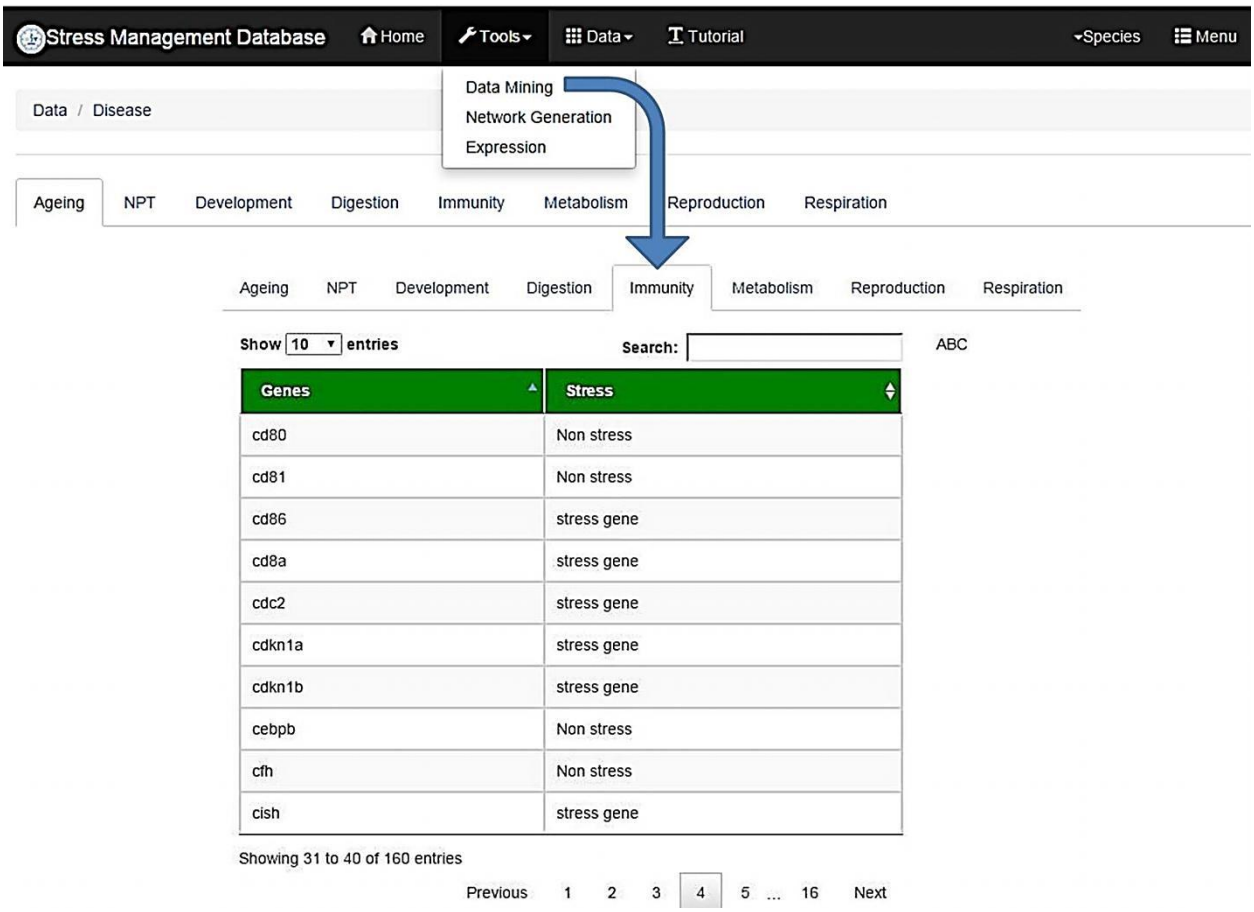
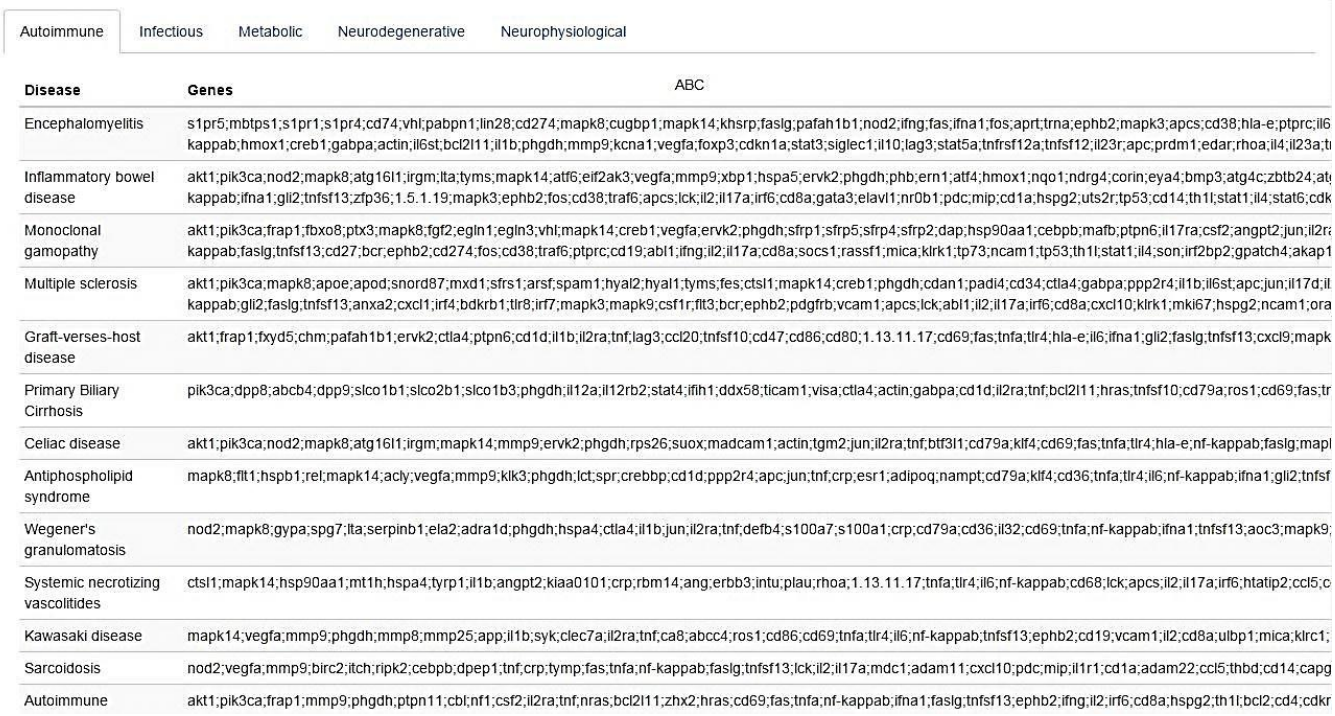


Figure 4.2: Snapshot of database interface for mining genes for each physiological process and its stress status based on literature mining.

To develop a preliminary understanding of stress and associated disease susceptibility and identify important molecular interactions/associations hitherto hidden, the genes-association networks thus established have also been made available in the database along with gene-association networks for 152 diseases (figure 4.3).



Autoimmune | Infectious | Metabolic | Neurodegenerative | Neurophysiological

ABC

Disease	Genes
Encephalomyelitis	s1pr5;mbtps1;s1pr1;s1pr4;cd74;vhl;pabpn1;lin28;cd274;mapk8;cugbp1;mapk14;khsrp;faslg;pafah1b1;nod2;ifng;fas;ifna1;fos;aprt;trna;ephb2;mapk3;apcs;cd38;hla-e;ptprc;il6 kappab;hmox1;creb1;gabpa;actin;il6st;bcl2l11;il1b;phgdh;mmp9;kcna1;vegfa;foxp3;cdkn1a;stat3;siglec1;il10;lag3;stat5a;tnfrsf12a;tnfsf12;il23r;apc;prdm1;edar;rhoa;il4;il23a;t
Inflammatory bowel disease	akt1;pik3ca;nod2;mapk8;atg16l1;irgm;lta;tyms;mapk14;atf6;eif2ak3;vegfa;mmp9;xbp1;hspa5;ervk2;phgdh;phb;ern1;atf4;hmox1;nqo1;ndrg4;corin;eya4;bmp3;atg4c;zbtb24;at kappab;ifna1;gli2;tnfsf13;zfp36;1.5.1.19;mapk3;ephb2;fos;cd38;traf6;apcs;lck;il2;il17a;irf6;cd8a;gata3;elavl1;nr0b1;pdc;mip;cd1a;hspg2;uts2r;tp53;cd14;th1l;stat1;il4;stat6;cdk
Monoclonal gamopathy	akt1;pik3ca;frap1;fbxo8;ptx3;mapk8;fgf2;egln1;egln3;vhl;mapk14;creb1;vegfa;ervk2;phgdh;sfrp1;sfrp5;sfrp4;sfrp2;dap;hsp90aa1;cebpb;mafb;ptpn6;il17ra;csf2;angpt2;jun;il2r kappab;faslg;tnfsf13;cd27;bcr;ephb2;cd274;fos;cd38;traf6;ptprc;cd19;abl1;ifng;il2;il17a;cd8a;socs1;rassf1;mica;klrk1;tp73;ncam1;tp53;th1l;stat1;il4;son;irf2bp2;gpatch4;akap1
Multiple sclerosis	akt1;pik3ca;mapk8;apoe;apod;snord87;mxd1;sfrs1;arsf;spam1;hyal2;hyal1;tyms;fes;ctsl1;mapk14;creb1;phgdh;cdan1;padi4;cd34;ctla4;gabpa;ppp2r4;il1b;il6st;apc;jun;il17d;il kappab;gli2;faslg;tnfsf13;anxa2;cxcl1;irf4;bdkrb1;tlr8;irf7;mapk3;mapk9;csf1r;flt3;bcr;ephb2;pdgfrb;vcam1;apcs;lck;abl1;il2;il17a;irf6;cd8a;cxcl10;klrk1;mki67;hspg2;ncam1;ora
Graft-verses-host disease	akt1;frap1;fxyd5;chm;pafah1b1;ervk2;ctla4;ptpn6;cd1d;il1b;il2ra;tnf;lag3;ccl20;tnfsf10;cd47;cd86;cd80;1.13.11.17;cd69;fas;tnfa;tlr4;hla-e;il6;ifna1;gli2;faslg;tnfsf13;cxcl9;mapk
Primary Biliary Cirrhosis	pik3ca;dpp8;abcb4;dpp9;slco1b1;slco2b1;slco1b3;phgdh;il12a;il12rb2;stat4;ifih1;ddx58;ticam1;visa;ctla4;actin;gabpa;cd1d;il2ra;tnf;bcl2l11;hras;tnfsf10;cd79a;ros1;cd69;fas;tr
Celiac disease	akt1;pik3ca;nod2;mapk8;atg16l1;irgm;mapk14;mmp9;ervk2;phgdh;rps26;suox;madcam1;actin;tgm2;jun;il2ra;tnf;btf3l1;cd79a;klf4;cd69;fas;tnfa;tlr4;hla-e;nf-kappab;faslg;mapl
Antiphospholipid syndrome	mapk8;flt1;hspb1;rel;mapk14;acly;vegfa;mmp9;klk3;phgdh;lct;spr;crebbp;cd1d;ppp2r4;apc;jun;tnf;crp;esr1;adipoq;nampt;cd79a;klf4;cd36;tnfa;tlr4;il6;nf-kappab;ifna1;gli2;tnfsf
Wegener's granulomatosis	nod2;mapk8;gypa;spg7;lta;serpinb1;ela2;adra1d;phgdh;hspa4;ctla4;il1b;jun;il2ra;tnf;defb4;s100a7;s100a1;crp;cd79a;cd36;il32;cd69;tnfa;nf-kappab;ifna1;tnfsf13;aoc3;mapk9;
Systemic necrotizing vascolitides	ctsl1;mapk14;hsp90aa1;mt1h;hspa4;tyrp1;il1b;angpt2;kiaa0101;crp;rbm14;ang;erbb3;intu;plau;rhoa;1.13.11.17;tnfa;tlr4;il6;nf-kappab;cd68;lck;apcs;il2;il17a;irf6;htatip2;ccl5;c
Kawasaki disease	mapk14;vegfa;mmp9;phgdh;mmp8;mmp25;app;il1b;syk;clec7a;il2ra;tnf;ca8;abcc4;ros1;cd86;cd69;tnfa;tlr4;il6;nf-kappab;tnfsf13;ephb2;cd19;vcam1;il2;cd8a;ulbp1;mica;klrc1;
Sarcoidosis	nod2;vegfa;mmp9;birc2;itch;ripk2;cebpb;dpep1;tnf;crp;tymp;fas;tnfa;nf-kappab;faslg;tnfsf13;lck;il2;il17a;mdc1;adam11;cxcl10;pdc;mip;il1r1;cd1a;adam22;ccl5;thbd;cd14;capg
Autoimmune	akt1;pik3ca;frap1;mmp9;phgdh;ptpn11;cbl;nf1;csf2;il2ra;tnf;nras;bcl2l11;zhx2;hras;cd69;fas;tnfa;nf-kappab;ifna1;faslg;tnfsf13;ephb2;ifng;il2;irf6;cd8a;hspg2;th1l;bcl2;cd4;cdkr

Figure 4.3: Snapshot of the database interface for mining genes for their association with diseases.

Various tools have also been integrated with the database for data-analysis. The gene-association networks, described above, have been provided in sif format (simple information format), which can be easily downloaded from the database and viewed in Cytoscape for further analysis (refer chapter 2 for different methods used for analyzing gene-association networks). A major challenge that users face during this type of analysis is conversion of gene identifiers. Although many gene identifier conversion tools already exist, they usually accept the genes in list form as input for conversion; gene networks are not directly accepted by these tools for ID conversion. Hence, we have provided facility that allows conversion of gene-lists as well as gene-networks (in sif format) among the following identifiers: Official Gene Symbol, Entrez Id, Ensembl Id, Refseq Id and Agilent Ids (User has to specify Agilent Grid No. if using Agilent Ids since these vary from grid to grid). Apart from that we have also provided a basic text-mining facility that allows to form word-cloud along with frequency chart from multiple literature corpus provided by user through over-representation analysis of the words used (figure 4.4)

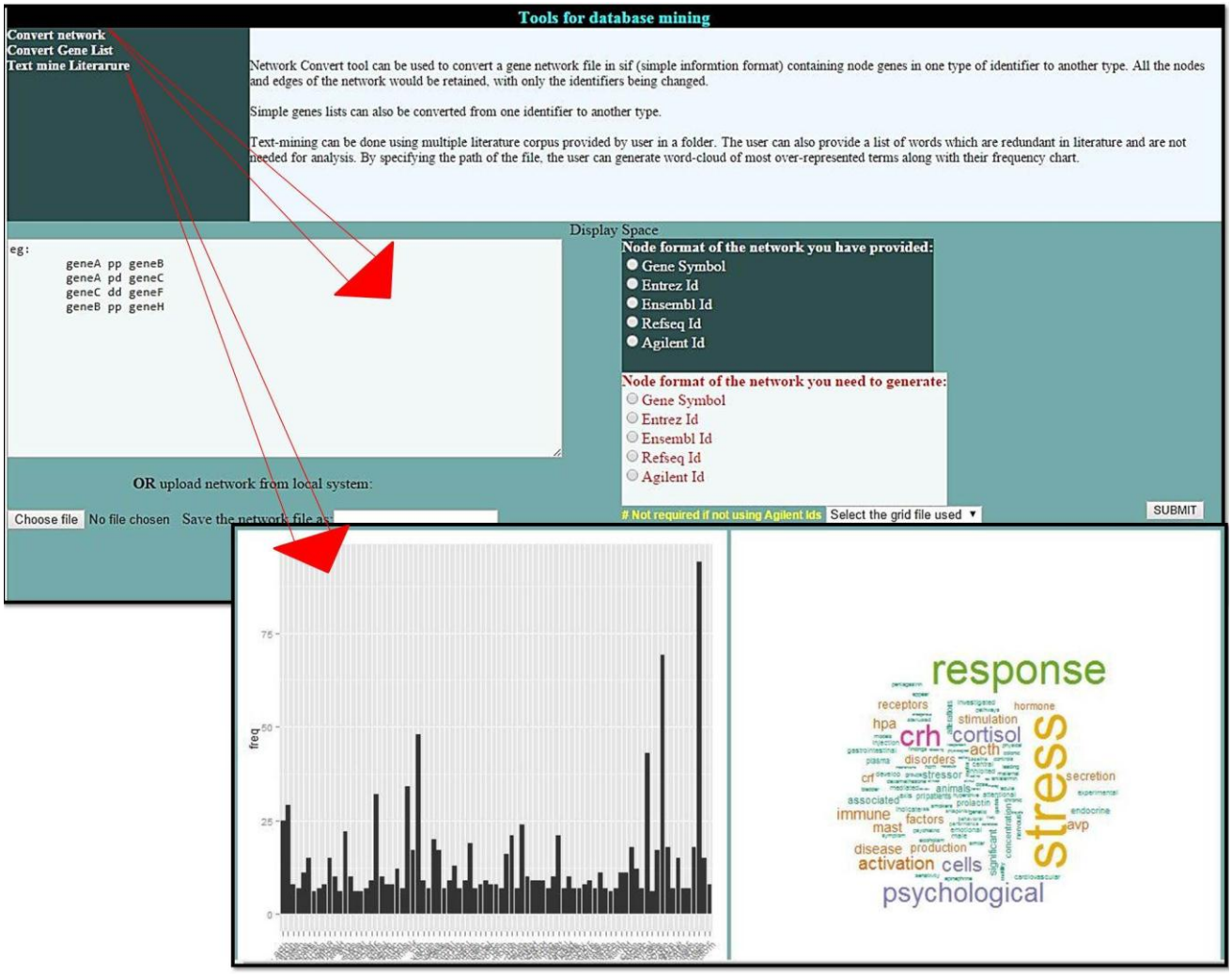


Figure 4.4: Snapshot of database interface for Gene-Id conversion and Text-mining tool.

We have also provided tools for heatmap-generation and hierarchical clustering on a user provided matrix of genes and values, which can be used for enrichment analysis based on association scores for each gene in the gene-association networks (genes with warmer color represent higher association values), as well as for usual expression analysis of expression data. Figure 4.5 shows a heat-map generated using a snippet of microarray expression values.

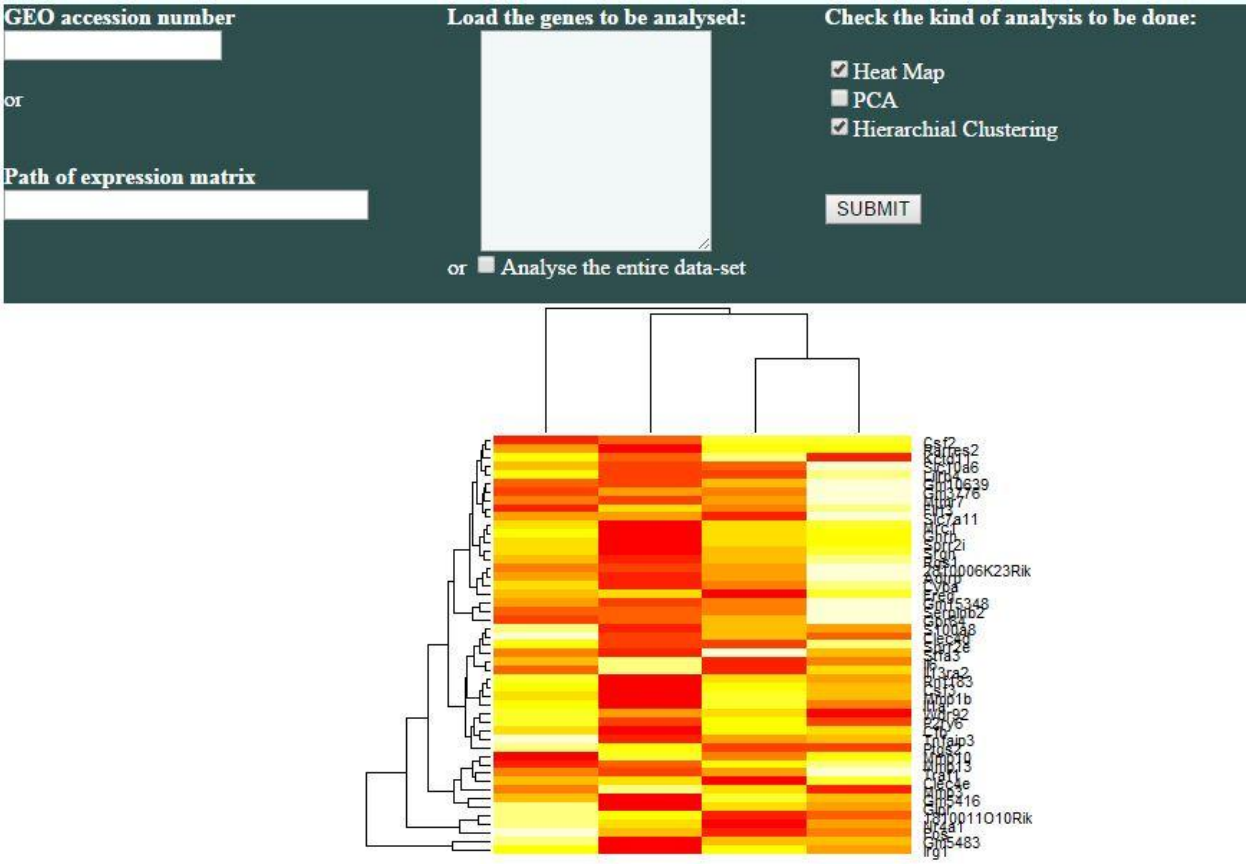


Figure 4.5: Snapshot of database interface for enrichment/expression analysis through heat-map and clustering.

4.6. Implications

Information from this database could be used for elucidating, in part or in whole, many more predictive mechanisms which might serve critical roles in answering key biological questions pertaining to stress biology. The database server ensures flexibility with respect to further development and increase in literature regarding psychological stress, including the potential of inclusion of more tools for analysis of gene-association networks.

CHAPTER 5

SUMMARY
AND
CONCLUSION

5.1. Summary and Conclusions

In this thesis we have attempted to develop a molecular basis of stress and its effects on various physiological processes. We have also established direct role of serotonin receptors (HTRs) in metabolic regulation in adipocytes under stress conditions. So far, HTR5a receptor had been studied in the purview of neuronal signaling, but its role has not been reported in adipocytes [287-290]. The systematic analysis of text-mining based gene-association network predicted HTR5a and HTR2c as strongly associated with metabolism and stress. *In-vitro* validation in the current study provides evidence for the first time that two serotonin receptors, HTR5a and HTR2c, could act as link between glucocorticoid and serotonergic signaling system in adipocytes, which is highly intensified when adipocytes are grown together with macrophages; thereby proving the robustness of such analysis. It was also seen that adipocytes treated with stress hormones, in presence of macrophages, express higher PPARγ and have higher lipid accumulation than non-treated ones hinting that paracrine signaling from macrophages definitely contributes to adipogenesis in adipocytes. It appears that co-culturing alone does not contribute to adipocyte differentiation or PPARγ-mediated adipogenesis, however synergism of cortisol and serotonin induce higher differentiation and adipogenesis in co-cultured adipocytes. Cortisol and serotonin act together to mobilize lipids and glycerol from macrophages through lipolysis, apart from initiating oxidative reactions. The FFAs thus liberated from macrophages, can be either scavenged by adipocyte scavenger receptors of adipocytes directly contributing to high lipid content, or glycerol released from the macrophages can be taken up by them for de-novo fat synthesis. The metabolic by-products of redox reactions can also act as potential PPARγ ligands, signaling through which can cause adipogenecity in co-cultured adipocytes in presence of stress hormones. The work presented in this thesis suggests that adipose tissue inflammation induced

serotonin localization (either gut derived or by mast cell homing and degranulation) and following serotonergic signaling could be one of the factors responsible for increased visceral obesity observed in chronic stress. Despite establishing molecular basis of stress and its effects on important physiological processes, it is also important to further establish these concepts *in vivo* and especially in humans for therapeutic interventions.

5.2 Future Prospects

In this thesis, associations between stress, physiology and disease have been comprehensively documented with a window for association based predictions. Data from these associations can be used for building prediction-models, followed by hypothesis testing to validate this candidate compensatory regulatory mechanism e.g. in this study, apart from role of serotonin in compensatory regulation during stress response, the ECS was also identified as potential player in stress response, which can be further used for generating testable hypothesis and can thus add to the repertoire of mechanistic insights to stress. Information in the current report could also be used for elucidating multitudes of possible predictive mechanisms which would serve a critical role, in part or whole, in answering key biological questions pertaining to stress physiology. This will add to the scientific findings being made in the areas of cognitive-emotional and clinical psychology for developing therapeutic interventions to cope with stress and improve health. Understanding stress psychology through subjective assessment as has been reported here can also lead to development of cognitive and behavioral interventions for coping with stress.

Chapter 6

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ANNEXURE 1

Clinical Trial Title	Conditions	Interventions
Copeptin During a Standardized Psychological Stress Test	Stress	-
Response of Urticaria to Stress Intervention	Chronic Urticaria	Behavioral: Psychological Stress Intervention|Behavioral: General Supportive Therapy
Effect of Mental Stress on Platelet Function	Mental Stress	Behavioral: 2-minute Bondet test
Developing Field Tools for Real-Time Assessment of Exposure to Psychosocial Stress and Drug Use in an Outpatient Treatment Population	Psychological Stress|Drug Abuse	-
Management and Treatment of Stress-related Disorders (INTERSTRESS)	Psychological Stress	Behavioral: Coping skills and relaxation training with new technologies|Behavioral: Traditional coping skills and relaxation training
Mechanisms of Mindfulness and Stress Resilience: A Mobile App Mindfulness Training Study	Psychological Stress|Mindfulness	Behavioral: Mindfulness Training|Behavioral: Mindful Attention Only Training|Behavioral: Analytic Thinking Training
Effect of Mental Stress on Glucose Control in Patients With Diabetes Mellitus	Diabetes Mellitus|Mental Stress	Behavioral: stress
Mental Stress Reduction in Defibrillator Patients	Cardiomyopathy, Dilated|Arrhythmia|Anger|Stress	Behavioral: Cognitive Behavioral Stress Management (CBSM)|Other: Patient Education
Effect of Mental Stress on Platelet Function in Healthy Subject	Healthy	-
Effect of Acute Psychological Stress on Glucose Concentrations in Patients With Type 2 Diabetes	Type 2 Diabetes	Behavioral: Trier Social Stress Test (TSST)
Life-Stress Interview for Women With Chronic Urogenital Pain Conditions	Chronic Urogenital Pain	Behavioral: Life-Stress Interview
Mindful Hearts Study	Cardiovascular Disease|Psychological Stress|Stroke|Coronary Artery Disease	Behavioral: Mindfulness Based Stress Reduction|Behavioral: Health Education Program
A Study On The Effect Of Vitano® On Physiological And Psychological Responses To Psychological Stress	Mild Stress/Anxiety	Drug: Vitano
Effects of an Immunosuppressant Mycophenolate Mofetil or MMF on the Urinary Sodium Excretion Response to Mental Stress	Psychological Stress|Hypertension, Renal	Drug: mycophenolate mofetil (MMF)|Drug: Placebo
Responses of Myocardial Ischemia to Escitalopram Treatment	Myocardial Ischemia	Drug: Escitalopram|Drug: Placebo
Effects of Cognitive Behavioural Therapy and Exercise on Stress and Cognitive Deficits in Multiple Sclerosis	Multiple Sclerosis|Cognitive Deficits|Stress, Psychological	Behavioral: Cognitive Behavioural Therapy|Behavioral: Aerobic Exercise
Mechanisms of Mindfulness Training and Stress Reduction	Psychological Stress|Mindfulness	Behavioral: Mindfulness
Waitlist-controlled Trial of a Psychological Education Program for Nurses	Psychological Stress	Behavioral: Psychological education program for nurses

Traits Associated With Early Life Stress Among Treatment-Seeking Alcoholics	Fear|Stress|Alcohol Dependence	-
Effects of an Intervention to Enhance Resilience in Physical Therapy Students	Psychological Stress	Other: Resilience Curriculum
Program of Exercise, Diet and Control of Psychological Stress in Cancer Survivors	Cancer Survivors	Behavioral: Exercise|Behavioral: Exercise + Diet counseling|Behavioral: Exercise + Diet counseling + psycho-emotional support
Effect of Counseling on Psychological Stress Amongst Women Felt to be at High Risk for Breast Cancer Development	Breast Cancer	Behavioral: Physician Counseling
Study on the Effects of a Probiotic on Autonomic and Psychological Stress	Stress, Psychological	Dietary Supplement: ProbioStick|Other: Placebo
The Effects of Physical Training on Physiological and Psychological Stress-reactions and Cognitive Function.	Healthy Sedentary Individuals	Other: Exercise
The Relationship Between the Response to Mental Stress and Vascular Endothelial Function	Coronary Endothelial Dysfunction|Apical Ballooning Syndrome|Myocardial Infarction	-
The Effect of Beta-Blockers and Aspirin on Hemostasis and Endothelial Function After Acute Mental Stress	Arteriosclerosis|Stress, Psychological	Drug: inderal (drug), acetylsalicylic acid (drug)
Effectiveness of Prazosin on the Urinary Sodium Excretion Response to Mental Stress	Hypertension	Drug: Prazosin|Drug: Placebo
Tea Components, Mental Stress and Blood Pressure	Healthy	Other: Combination of caffeine and theanine|Other: Caffeine
Psychological Group Intervention to Reduce Stress and Burnout Among Cardiac Intensive Care Nurses	Stress, Psychological	Behavioral: short-term group intervention program
With-Me - Technology-Aided Phone Coaching for Occupational Health Study	Psychological Stress	Behavioral: Technology-aided phone wellness counselling|Behavioral: Phone wellness counselling
The Efficacy of a Brief Resilience Training Program for Hurricane Sandy Disaster Responders	Psychological Stress	Behavioral: Disaster Worker Resiliency Training
Study of Effectiveness of Different Lifestyle Interventions for Health and Wellbeing	Metabolic Syndrome Risk Factors|Overweight|Psychological Stress	Behavioral: Wellbeing intervention (ACT)|Behavioral: Wellbeing intervention (Internet)|Behavioral: No intervention
Study on Therapeutic Effect of Treating Mental Stress Induced Myocardial Ischemia	Theraphy of Chinese Traditional Medicine	Drug: Xinkeshu tablets|Other: placebo
Effects of Antioxidants on Cardiovascular Risk Measures (Spice Study)	Cardiovascular|Psychological Stress	Dietary Supplement: High Antioxidant Spice Blend|Behavioral: Trier Psychological Stressor|Other: Placebo antioxidant concentrate
Programs to Support You During Chemotherapy (Pro-You)	Depressive Symptoms|Fatigue|Psychosocial Effects of Cancer and Its Treatment at various stages	Behavioral: Yoga Skills Training (YST)|Other: questionnaire administration|Other: fatigue and depressive symptom assessment and management|Other: psychological stress assessment|Other: laboratory biomarker analysis|Other: Attention Control
Strengths-Based Family Psychoeducation for Youth Psychosis	Psychological Stresses	Behavioral: Strengths-based family psychoeducation

Myocardial Infarction - Stress Prevention Intervention	Stress Disorders, Post-Traumatic|Myocardial Infarction	Behavioral: Minimal behavioral intervention|Behavioral: Control intervention
Does Fluoxetine Have an Effect on the CNS CRF Systems in Women Abused in Childhood?	Major Depressive Disorder	Drug: Fluoxetine
Cardiac Autonomic Function in Women With Microvascular Coronary Dysfunction	Microvascular Coronary Dysfunction	Behavioral: Mental Stress Testing|Other: Peripheral Arterial Tonometry (PAT) Testing|Other: Heart Rate Variability (HRV)|Other: RESPeRATE Breathing Trial|Procedure: SPECT cardiac scan with 123I-mIBG and Myoview
An Investigation of Early Life Stress and Depression	Major Depressive Disorder (MDD)|History of Childhood Sexual Abuse (CSA)	Drug: Amisulpride|Drug: Placebo
Girls In Recovery From Life Stress (GIRLS) Study	Posttraumatic Stress Disorder|PTSD	Behavioral: Trauma Adaptive Recovery Group Education and Therapy|Behavioral: Enhanced Treatment As Usual
Stress Intervention for Chronic Urticaria	Chronic Urticaria	Behavioral: Stress intervention
Physiological Assessment of the Endothelium - Circadian Rhythm and Role of the Sympathetic Nervous System	Endothelial Function	Procedure: Mental stress|Other: no intervention|Procedure: Myogenic activation|Procedure: Cold pressure test
The Effect of Exercise Training on Mental Stress-Induced Silent Ischemia	Myocardial Ischemia	Behavioral: exercise
Fitness, Aging, and Stress Study	Aging|Stress|Disease Risk	Behavioral: Aerobic Training Intervention
Programs To Support You During Chemotherapy	Depression|Fatigue|Various stages of Cancer	Other: Laboratory Biomarker Analysis|Other: Questionnaire Administration|Other: Daily Survey Administration|Behavioral: Yoga Skills Training|Behavioral: Attention Control|Other: Actigraphy Assessment
Neural Correlates of Stress Reduction	Stress|Psychological Stress|Life Stress|Emotional Stress|Social Stress	Behavioral: Stress Reduction
A Trial of Multi-convergent Therapy for Functional Symptoms and Stress in Patients With Inflammatory Bowel Disease	Inflammatory Bowel Disease	Behavioral: Multi-Convergent Therapy
Psychosocial Determinants of Medication Adherence in Hypertensive African Americans	Hypertension	Behavioral: Self affirmation and positive affect induction vs. control
Nitric Oxide Bioavailability and Early Life Stress (NO-Stress)	Cardiovascular Disease	Dietary Supplement: Antioxidant cocktail|Other: Biopsy|Other: Placebo|Drug: NMD; 0.4mg sub-lingual nitroglycerin spray
Atomoxetine Effects in Humans	Physiological Stress	Drug: Atomoxetine
Mindfulness-Based Stress Reduction and Myocardial Ischemia	Arteriosclerosis	Behavioral: Mindfulness Based Stress Reduction Class|Behavioral: Cardiac Education Class
Trial of Multidisciplinary Team Stress and Performance in Immersive Simulation for Management of Infant in Shock	Stress	Other: MDT simulation sessions of management of life-threatening events over 1 year
Biological Biomarkers in MOMS Partnership	Psychological Stress	Other: Biological Sample Collection

Improving Parental Psychosocial Functioning and Early Developmental Outcomes in Children With Sickle Cell Disease	Sickle Cell Disease|Cognitive Ability, General|Psychological Stress|Parenting	Behavioral: Play therapy|Other: Problem solving skills
Relationship Between the Gut Microbiota and Stress	Psychological Stress	Other: Observation
Resiliency Program for Medical Interpreters	Psychological Stress	Behavioral: Behavioral: Resiliency Intervention
Effects of Sitting Meditation and Hatha Yoga in Adolescents	Anxiety|Psychological Stress	Behavioral: Sitting Meditation|Behavioral: Hatha yoga
Acute Effects of Cortisol on Alcohol Craving in Alcohol Dependence	Alcohol Craving|Psychological Stress|Physiological Stress	Drug: Cortisol 20mg|Drug: Placebo Mannitol
Descriptive Study of Biological Stress and Perceived Stress at the Center 15	Physiological Stress|Psychological Stress	Other: salivary sampling by a biomnis swab
Optimizing Resilience and Coping in HIV Via Internet Delivery	HIV Disease|Depression|Affect|Psychological Stress	Behavioral: Intervention
Online Positive Emotion Skills Intervention for Symptoms of Depression	Depression|Psychological Stress|Affect	Behavioral: Positive Affect Skills Training
Stress Free Now, a Mind-body Reduction Program for Nurses	Psychological Stress|Anxiety|Depression	Behavioral: Stress Free Now online program|Behavioral: Group support session
Effectiveness of Telephone Intervention for Colorectal Cancer Caregivers	Psychological Stress	Behavioral: Telephone intervention
Does Emotional Support Decrease In Vitro Fertilization Stress?	In Vitro Fertilization|Psychological Stress	Behavioral: Phone calls
The Effect of the Duty Loading on the Stress Response of Physician	Physiological Stress|Psychological Stress	Procedure: 24hour EKG and blood pressure monitor|Other: Blood and urine sampling
A Nursing Intervention to Enhance Child Comfort and Psychological Well-Being During and Following PICU Hospitalization	Psychological Stress	Other: Comfort Care|Other: Usual care
Conventional vs Mindfulness Intervention in Parents of Children With Disabilities	Psychological Stress|Depression|Anxiety	Behavioral: mindfulness intervention|Behavioral: Conventional parent Support Group
The Pandemic Stress Vaccine: A Resource to Enhance the Resilience of Healthcare Workers Facing an Infectious Outbreak	Psychological Stress	Behavioral: Interactive computerized learning resource|Behavioral: Didactic computerized learning resource
A Clinical Trial of the Transcendental Meditation (TM) Program on Blood Pressure, Psychological Distress, and Coping	High-normal Blood Pressure|Psychological Stress	Behavioral: Transcendental Meditation program
Moderating Impact of Various Emotion Personality Factors on Salivary Cortisol Response to a TSST	Psychological Stress	-
Stress Management Therapy in Patients Receiving Chemotherapy for Cancer	Cancer|Psychological Stress|Unspecified Adult Solid Tumor, Protocol Specific	Other: Self Administered Stress Management
The TLC2 (Teaching Healthy Lifestyles to Caregivers 2)/CALM (Counseling Advice for Lifestyle Management) Study	Health Behavior|Psychological Stress|Healthy	Behavioral: The Stanford Active Choices program
Health SMART (Stress Management and Relaxation Training) to Improve Vaccine Immune Response	Psychological Stress	Behavioral: Cognitive Behavioral Stress Management (CBSM) group intervention

Biomarker Analysis of Stress	Sepsis	-
Depression and Interleukin-6 Production in Patients With Ovarian Epithelial Cancer	Depression|Fatigue|Ovarian Cancer|Psychosocial Effects of Cancer and Its Treatment|Sleep Disorders	Genetic: polymorphism analysis|Other: laboratory biomarker analysis|Other: physiologic testing|Other: questionnaire administration|Procedure: conventional surgery|Procedure: fatigue assessment and management|Procedure: psychosocial assessment and care
Characterization of Sympathetic Nerve Activity in Stress Cardiomyopathy	Stress-induced (Takotsubo) Cardiomyopathy	Device: Sympathetic Nerve Activity|Behavioral: Mental Stress Test (Color Word Test)|Drug: The Modified Oxford Technique for Baroreflex Sensitivity|Other: Cold Pressor Test|Device: Echocardiographic evaluation
Transcranial Electrical Stimulation in the Treatment of Acute Anxiety Induced by Stressful Life Events: A Pilot Study	Anxiety	Device: NeuroConn DC Stimulator Plus tDCS
Qigong Intervention Program for Abused Chinese Women	Abused Women	Behavioral: Qigong training|Other: Wait-list control- Health talks
MBCGT and Psycho-physiological Stress Regulation Group for Depression and Psycho-physiological Stress Patient	Psychophysiologic Disorders|Stress, Psychological|Unipolar Depression	Behavioral: Mindfulness-based cognitive Group therapy|Behavioral: psycho-physiological stress regulation group
Pilot Study - Impact of Traditional Acupuncture on Menopause	Menopause	Procedure: TA|Other: AA|Other: WC
Cortisol Levels on Menopausal Symptoms - Ancillary (Addendum) Study to Protocol 16997	Menopause	Procedure: ACTH
Short-term Existential Behavioural Therapy for Informal Caregivers of Palliative Patients: a Randomised Controlled Trial	Depression	Behavioral: EBT|Behavioral: treatment-as-usual
Role of Sympathetic Overactivity and Angiotensin II in PTSD and CV	Stress Disorders, Post-Traumatic	Procedure: Microneurography|Behavioral: Combat virtual reality video clip|Procedure: Handgrip Exercise|Procedure: Cold Pressor Test (CPT)|Drug: Sodium Nitroprusside (SNP)|Drug: Phenylephrine|Drug: Losartan|Drug: Atenolol
Late-Life Stress and Inflammation	Depression	Drug: Escitalopram|Drug: Placebo
Mental Stress, Autonomic Function, and Heart Disease	Cardiovascular Diseases|Heart Diseases|Coronary Disease	-
Chronic Life Stress and Incident Asthma in Adult Women	Asthma|Lung Diseases	-
Promoting Health During Pregnancy: A Multiple Behavior Computer Tailored Intervention	Life Stress|Smoking Cessation|Poor Nutrition	Behavioral: Healthy Pregnancy: Step by Step
Multicentre, Double-blind Study Versus Placebo on Impact and Safety of Extramel® 140 IU on Perceived Stress, Physical and Intellectual Fatigue, Pain Perception if Present, and the Impact on the Quality of Life	Life Stress|Fatigue|Pain|Quality of Life	Dietary Supplement: Extramel 10 mg - 140 UI SOD|Dietary Supplement: Placebo - Excipient only
Task Focusing Strategy During a Simulated Cardiopulmonary Resuscitation	Mental Stress	Behavioral: instruction
Analysis of the Stress Induced by in Situ Simulation	Psychologic Stress|Heart Arrest	Other: distress analysis

Adaptation and Validation of Recent Life Changes Questionnaire for Measuring Stress Among Adults in Karachi, Pakistan	Life Change Events	-
The Relaxation vs. Retreat Study	Stress|Ageing|Wellbeing	Behavioral: Meditation Retreat|Behavioral: Active Comparator Relaxation Group
Psychophysical Aspects of Maximal Anaerobic Performance	Anaerobic Performance	Other: Anaerobic wingate test
Web-based Psycho-Educational Program to Support Carers of Alzheimer's Patients	Caregivers|Alzheimer Disease,|Psycho-educational Web-based Program,|Stress,|Self-efficacy,|Burden.	Behavioral: psycho-social intervention based on a web-based psycho-educational program, called Diapason.|Behavioral: Consultation in memory clinic
The Relationship Between Psychological Factors and Bell's Palsy	Bell's Palsy	-
Stress Reduction Training to Improve Sleep Quality, Stress Physiology	-	-
Effect of Paroxetine on Smokers' Cardiovascular Response to Stress - 1	Tobacco Use Disorder	Drug: Paroxetine|Drug: Placebo
Resiliency Training for Patients With Neurofibromatosis Via Videoconferencing With Skype	Neurofibromatosis|Neurofibromatosis I|Neurofibromatosis 2|Schwannomatosis	Behavioral: The Relaxation Response Resiliency Program (3RP) via Skype
Variability in Adrenergic Response	Vascular Reaction to Medications	Drug: phenylephrine and nitroglycerin
The Mental Health and Dynamic Referral for Oncology Protocol (MHADRO)	Cancer|Distress	Behavioral: personalized, motivational messages
High Resolution Phenotyping in Healthy Humans	Pressor Response|Baroreflex Sensitivity|Blood Pressure Variability|Heart Rate Variability|Arterial Catecholamines	Other: Physiological maneuvers
Effects of Relaxing Hydrotherapy in Third Trimester of Pregnancy	Pregnancy|Breech Presentation|Low Back Pain|Stress, Psychological	Procedure: WATSU
Depression, Epinephrine, and Platelet Function	Major Depressive Disorder	Drug: Escitalopram|Drug: Desipramine
Classical Conditioning to Treat Allergic Airway Diseases	Allergic Airway Disease	Drug: Corticosteroids|Behavioral: Classical conditioning to corticosteroid
Evaluating a Peer-Facilitated Skills-Based Intervention for Caregivers of Concurrent Disordered Youth	Caregiver Burden	Other: DBT Based Skills Groups for Families
Healing Touch in Treating Patients Receiving Chemotherapy for Acute Myeloid Leukemia or Acute Lymphocytic Leukemia	Fatigue|Leukemia|Psychosocial Effects of Cancer and Its Treatment	Procedure: therapeutic touch
Craving and Lifestyle Management Through Mindfulness Study	Obesity	Behavioral: Craving and Lifestyle Management through Mindfulness
Epidemiology of Stress and the Metabolic Syndrome	Cardiovascular Diseases|Heart Diseases|Obesity|Hypertension|Hyperinsulinism|Insulin Resistance|Metabolic Syndrome X	-

Asthma Symptom Management Through Mindfulness Training	Asthma	Behavioral: Mindfulness Based Stress Reduction (MBSR)|Behavioral: Healthy Living Course (HLC)
Quality of Life in Patients Undergoing Total Pelvic Exenteration	Total Exenteration,|Anterior or Posterior Pelvic Exenteration|Gynecologic Malignancies|Colorectal Malignancies|Urologic Malignancies	Behavioral: questionnaires/interviews
Effect of Tai Chi Vs. Structured Exercise on Physical Fitness and Stress in Cancer Survivors	Cancer|Cancer Survivor	Procedure: Tai Chi Chuan
Social Support and Myocardial Ischemia	Cardiovascular Diseases|Heart Diseases|Myocardial Ischemia|Coronary Disease	-
Copeptin as a Stress Marker During a Written Examination	Healthy	-
A Randomized, Double-Blind Comparison of Placebo and Atomoxetine Hydrochloride Given Once a Day in Adults With Attention-Deficit/Hyperactivity Disorder (ADHD)	Attention Deficit Hyperactivity Disorder	Drug: Atomoxetine Hydrochloride|Drug: Placebo
Implementation and Evaluation of a Family-based Intervention Program for Children of Mentally Ill Parents	Schizophrenia and Disorders With Psychotic Features|Mood Disorders|Neurotic Disorders|Personality Disorders|Substance-Related Disorders	Behavioral: CHIMPS intervention
The Effects of Stress Reduction on Surgical Wound Healing	Wound Healing|Stress|Surgery	Behavioral: Stress reduction intervention
Influence of Stress on Non Surgical Periodontal Treatment	Chronic Periodontitis	Other: Non surgical periodontal treatment
Stress and Sugar Synergy	Metabolic Syndrome|Insulin Resistance|Dyslipidemia	Other: orange juice|Other: sucrose
Pharmacokinetics of Hydrocortisone After Subcutaneous Administration in Chronic Adrenal Insufficiency	Primary Adrenal Insufficiency	Drug: Hydrocortisone intramuscular first|Drug: Hydrocortisone subcutaneously first
Quantification of Outcome Measures for Mind-body Interventions	Stress, Psychological|Stress, Physiological	Behavioral: Stress Management Group 1|Behavioral: Stress Management Group 2|Behavioral: Stress Management Group 3
A Study on the Effects of VR-3 Herbal Blend Intake on Acute and Chronic Stress in Healthy Adults	Healthy Adults	Dietary Supplement: VR-3 Herbal Blend|Dietary Supplement: Placebo
A Study Survey of Stress in Anesthesia Personnel	Stress|Anxiety|Depression	Other: 2 courseware databases.
Urinary Cytokines in Patients With Overactive Bladder (OAB)	Overactive Bladder|Urinary Tract Infection	-
Treatment of Faecal Incontinence With Sacral Nerve Stimulation - Improved Function With Stimulation Bilaterally?	Fecal Incontinence	Device: Medtronic INTERSIM II - 3058

Study of Luteinizing Hormone-Releasing Hormone Analog (LHRHa) in Pubertal Patients With Extreme Short Stature	Dwarfism|Growth Disorder	Drug: Deslorelin
Doula Combined Latent Phrase Epidural Analgesia in Primiparous Women	Labor Pain	Procedure: Doula combined analgesia|Procedure: Analgesia without doula
Nutrition Interventions to Support the Immune System in Response to Stress	Mitigation of Immune Function Decrements in Response to Stress	Dietary Supplement: Immune-enhancing nutritional beverage|Dietary Supplement: Probiotics (BB-12)
Mindfulness-based Psychotherapy for Drug-resistant Epilepsy	Epilepsy|Stress	Behavioral: Mindfulness-based therapy|Behavioral: Social support group
Impact of Physical Exercise on Quality of Life in Patients With Inflammatory Bowel Disease - a Pilot Study.	Quality of Life in Patients With Inflammatory Bowel Disease	Behavioral: physical exercise
Transplant and Addiction Project (TAP) - 1	End Stage Liver Disease|End Stage Renal Disease|Substance-Related Disorders	Behavioral: TAP|Other: TAU
Determining Relationships Among Maternity Stress	-	-
Understanding Pediatric Chest Pain and Other Symptoms	Chest Pain	-
Prospective Randomised Trial of Exercise and / or Antioxidants in COlorectal Cancer Patients Undergoing Surgery.	Colorectal Cancer	Behavioral: Exercise
Translational Research Evaluating Neurocognitive Memory Processes	Neurocognition	-
Visual Stress of the Open Urban Environment	Visual Stress	-
Hypnotherapy for Prevention of Relapse in Ulcerative Colitis: a Randomised, Single-blind, Controlled Clinical Trial	Ulcerative Colitis	Behavioral: Gut focussed hypnotherapy|Behavioral: Controlled educational sessions
Effects of Atomoxetine Treatment in Humans	Stress	Drug: Placebo|Drug: Atomoxetine
Immune Responses to the Flu Shot During Pregnancy	Pregnancy	Biological: influenza virus vaccination
Effect of Psychological Interventions on Maternal Outcomes Undergoing Cesarean	Perioperative Psychology	Other: Language|Other: Language
CVD Risk Reduction Trial	Cardiovascular Disease	Behavioral: Group-based motivational interviewing|Behavioral: Individual CVD-risk factor feedback|Behavioral: Education for protective health behavior change
Stress Management Intervention for Mothers of Children With Cancer	Stress|Depression	Behavioral: Stress management
Neural Changes Associated With a Mindfulness-based Intervention for Young Adults With Childhood Maltreatment	Depression|Anxiety|PTSD	Behavioral: Mindfulness based stress reduction
Diabetes Distress, Psychological Well-being and Family Hardiness in Parents of Children and Adolescents With T1D	Type 1 Diabetes	-
Interstitial Cystitis: Elucidation of the Psychophysiologic and Autonomic Characteristics (ICEPAC) Study	Interstitial Cystitis/Painful Bladder Syndrome|Myofascial Pelvic Pain	Drug: Bupivacaine

Antinociceptive Modalities on Ischemia Reperfusion Injury	End Stage Liver Disease|Lung Cancer	Drug: Patient controlled analgesia
Biological CVD Risk Factors in Older Depressed Patients	Depression|Hypertension|Hypercholesterolemia	Behavioral: Cognitive behavioral therapy
Nurse Practitioner Hospice Program for Patients With Terminal Metastatic Cancer and Their Families or Caregivers	Hematopoietic/Lymphoid Cancer|Unspecified Adult Solid Tumor, Protocol Specific	Other: counseling intervention|Other: questionnaire administration|Other: survey administration|Procedure: end-of-life treatment/management|Procedure: psychosocial assessment and care
Pilot Trial of CenteringPregnancy With Mindfulness Skills	Premature Birth|Low Birth Weight|Postpartum Depression	Behavioral: CenteringPregnancy|Behavioral: CenteringPregnancy with Mindfulness Skills
Intern Health Study	Depression	
Fast-track Perioperative Program for Laparoscopic Colorectal Surgery	Colorectal Cancer	Other: Fast-track perioperative program|Other: Traditional perioperative program
Impact of Complementary Medicine Techniques (Therapeutic Touch and Hypno Analgesia) on the Term of Delivery of Patients Hospitalized for Preterm Labor (Hypnorelax)	Premature Birth	Other: Touching relaxant|Other: Hypnoses
Zurcher Adolescent Screening for Mental Disorder	Mental Disorders	Other: Screening
RELAX Surgical: Effects of Environmental Design on Pre-surgical Relaxation	Surgery	Other: Enhanced clinical environment|Other: Other distraction
Early Intervention for Preterm Infants	Premature Birth	Behavioral: clinic-based intervention program and home-based intervention program
Mindfulness-based Stress Reduction in Multiple Sclerosis	Multiple Sclerosis	Behavioral: Mindfulness-based stress reduction (MBSR)|Other: waiting list control
Relationship Between Personality and Coping Styles in Bone Marrow Transplant Candidates	Adaptation, Psychological|Bone Marrow Transplantation	-
The Effect of Medical Clown on the Pain and Anxiety Perception During LRH Analog Treatment or GH Provocation Test	Precocious Puberty|Growth Hormone Tests	Behavioral: Presence of medical clown during endocrine test
An Integrated-Delivery-of-Care Approach to Improve Patient Outcomes, Safety, Well-Being After Orthopaedic Trauma	Musculoskeletal Injury Trauma	Procedure: Integrated care (ICare)|Procedure: Usual Care (UsCare)|Other: Patient-Reported Outcomes Measurement Information System|Other: Lower Extremity Gain Scale (LEGS)|Other: Dynamometer|Other: Active Range of Motion (AROM)|Other: Posttraumatic Stress Disorder (PTSD)|Other: Beck Depression Inventory-II|Other: State-Trait Anxiety Inventory (STAI)|Other: Tampa Scale of Kinesiophobia-11 (TSK-11)
Personality of Diabetic Patients Will Influence Their Medical compliance-a 5 Years Cohort Follow up With Type D Personality	Type 2 Diabetes Mellitus	-
Managing Stress and Social Ties for Health Aging	Stress	Behavioral: Tai Chi Chih|Behavioral: Stress Education Control
Oxidative Stress in Motor Neuron Disease: COSMOS Add-On Study	Motor Neuron Disease|Primary Lateral Sclerosis	
Effect of Tyrosine Supplementation on Cognitive Performance and Mood During	Reaction to Severe Stress, Unspecified	Other: Tyrosine-Containing Food Bar|Other: Placebo Bar

Military Stress		
Therapy With an Oxytocin Adjunct for Major Depression	Depressive Disorder	Drug: Oxytocin
Effect of Tyrosine on Behavioral, Physiological and Nutritional Status During Survive, Evade, Resist, Escape (SERE) School	Response to Severe Stress	Other: Tyrosine-Containing Bar|Other: Placebo Bar
Effects of Qigong on Type 2 Diabetic Patients	Diabetes Mellitus, Type 2	Other: Qigong therapy|Other: Progressive resistance training
Childhood Obesity: Variations in Management	Obesity	Behavioral: Identification/management of childhood obesity (behavior)
Impact of JuicePlus+ on the Health Status of an Overweight Stressed Population	Inflammation	Dietary Supplement: Juice Plus+|Other: Placebo
A Study of Infliximab for Treatment Resistant Major Depression	Depression	Drug: infliximab (remicade)|Drug: Placebo
Zinc, Iron, Vitamin A and Psychosocial Care for Child Growth and Development	Development|Iron Deficiency|Zinc Deficiency	Dietary Supplement: Zinc Alone|Dietary Supplement: Iron and Zinc|Dietary Supplement: Iron, Zinc and Vitamin A|Other: Placebo
Essential Hypotension and Adaptability Registry	Blood Pressure| Depression|Panic Attack| Fibromyalgia|POTS|Inappropriate Sinus Tachycardia| Coronary Heart Disease| Acute Coronary Syndrome (ACS)|Acute Myocardial Infarction (AMI)| Cerebrovascular Disease (CVD)|Transient Ischemic Attack (TIA)|Atrial Fibrillation| Diabetes Mellitus|Cancer|Systolic Heart Failure|Diastolic Heart Failure|Chronic Fatigue Syndrome| Syncope|Vasovagal Syncope	-
Stress-induced Drinking in Emerging Adults: the Role of Trauma History	Trauma|Posttraumatic Stress Disorder	Behavioral: Trier Social Stress Test (TSST)|Other: No stress condition
Hypertension in Black Americans: A Life Course Approach	Cardiovascular Diseases|Heart Diseases|Hypertension	-
Noradrenergic Activity, Cognition and Major Depressive Disorder	Major Depressive Disorder|Early Life Trauma	Drug: yohimbine|Drug: placebo
TODAY2 Phase 2 Follow-up	Type 2 Diabetes	Other: TODAY cohort
Physical Health in Midlife: Influences of Adversity and Relationships Over Time	Cardiovascular Disease|Type 2 Diabetes	-
A Study of Meditation as a Treatment for Epilepsy	Epilepsy	Behavioral: Meditation
Effectiveness of Avapro in Obese Normotensive/Hypertensive African Americans	Hypertension|Obesity	Drug: Irbesartan|Drug: Placebo

Development of a Post-Traumatic Stress Disorder (PTSD) Population Registry for Veterans	Stress Disorders, Post-Traumatic	-
ARCH II Study (Alcohol Research Center on HIV Study II)	Alcohol Consumption|HIV|Motivation	Behavioral: Motivational Interviewing|Other: Questionnaire assessment|Other: Neurocognitive assessments|Other: Blood specimens
HBPL Study of the Impact of the NK1 Antagonist Aprepitant	Cocaine|Alcohol Dependence	Drug: Aprepitant|Drug: Placebo
Conditioning, the Placebo Effect, and Psoriasis	Psoriasis	Behavioral: Partial schedule of pharmacotherapeutic reinforcement|Drug: Dose control for Arm B|Other: Standard pharmacotherapeutic protocol
Feldenkrais vs Back School for Treating Chronic Low Back Pain: a Randomized Controlled Trial	Chronic Low Back Pain	Other: Feldenkrais Method|Other: Back School
Effects of Diacetylmorphine (DAM) on Brain Function and Stress Response	Opiate	Drug: diacetylmorphine (DAM)|Other: Placebo
Prediction of Cardiovascular Events in Vulnerable Patients Following Acute Coronary Syndrome	Acute Coronary Syndrome	Other: EndoPAT testing (non invasive device)|Other: WatchPAT testing (non-invasive device)
Neuroendocrine and Immune Response to Stress in Schizophrenia	Schizophrenia|Schizoaffective Disorder	Behavioral: Trier Social Stress Test
Herpetic Eye Disease Study (HEDS) II	Keratitis, Herpetic|Ocular Herpes Simplex|Herpes Simplex	Behavioral: Stress|Drug: Acyclovir
Evaluation of PTSD Family Coach, a Mobile Phone App for Family Members of Individuals With PTSD	Stress Disorders, Post-Traumatic	Behavioral: PTSD Family Coach Full|Behavioral: PTSD Family Coach Basic
Stereotactic Directional Vacuum-Assisted Breast Biopsy	Breast Cancer|Stereotactic Directional Vacuum-assisted Breast Biopsy|Female|Adult Patients	-
Behavioral Neurocardiac Training and Hypertension	Hypertension	Behavioral: Behavioral neurocardiac training|Behavioral: Autogenic relaxation training
Effectiveness and Safety of Early-Stage Amputation and External Herbs Chitosan for Diabetic Foot Ulcer	Diabetic Foot Ulcer	Procedure: Early-stage amputation|Procedure: Amputation|Other: External herbs chitosan|Other: Traditional gauze
High-dose Antioxidants for Central Serous Chorioretinopathy	Central Serous Chorioretinopathy	Drug: antioxidants tablets
Teen Online Problem Solving (TOPS) - An Online Intervention Following TBI	TBI (Traumatic Brain Injury)|Brain Edema|Craniocerebral Trauma|Hematoma|Brain Concussion	Behavioral: Teen Online Problem Solving
Effect of Real-fire Training on Vascular Function	Vascular Function|Atherothrombosis	Procedure: Forearm Vascular Study|Procedure: Badimon Chamber Study
Isolated and Associated Effects of Physical Exercise and Estrogen Therapy on Climactercs Women	Women's Health	Drug: Estradiol valerate|Other: Aerobic training|Drug: Placebo|Other: Control
Tako-Tsubo Cardiomyopathy and Cardiac Syndrome X: New Insights Into the Pathophysiology	Syndrome X|Takotsubo Cardiomyopathy	Other: BaroReflex Sensitivity, endothelial function measurement

Comparison of Vascular Function in Emergency Service Professionals	Vascular Function|Atherothrombosis	Procedure: Forearm Vascular Study|Procedure: Badimon Chamber
Methylation Status of BDNF Gene After Dialectical Behavior Therapy in BPD	Borderline Personality Disorder	Behavioral: Dialectical behavior therapy
Epi-Genetic Modulators of Fear Extinction in Alcohol Dependence	Fear|Stress|Alcohol Dependence	-
Neurofeedback as a Method to Strengthen Mental and Emotional Resilience	Feedback, Psychological|Stress Disorders, Post-Traumatic	Other: EFP-NF|Other: Sham
A Comparison of Robot-assisted Single Site and Laparoscopic Single-incision Cholecystectomy for Benign Gallbladder	Benign Gallbladder Disease	Device: Da Vinci Single Site Robot-Assisted Cholecystectomy|Device: Single Incision Laparoscopic Cholecystectomy
Late Effects of Treatment for Sarcomas in Children	Sarcoma	-
Working Memory and School Readiness in Preschool-Aged Children With Sickle Cell Disease	Sickle Cell Disease	Other: Evaluation of cognitive and pre-academic skills|Other: Parent questionnaires|Other: Home Observation Measure
Stepped Care for Treating Obsessive-Compulsive Disorder	Obsessive-compulsive Disorder	Behavioral: Cognitive-Behavioral Therapy with EX/RP|Behavioral: Stepped-Care CBT
Closed Loop Stimulation, Cognitive Performance, and Quality of Life in Pacemaker Patients	Cardiac Pacing, Artificial	Device: Rate-adaptive pacemaker: accelerometer|Device: Rate-adaptive pacemaker: Closed Loop Stimulation
Step Down Colon Cancer Risk	Adenomatous Polyps	Behavioral: Walking
The Influence of Psychobiological Adversity to Children and Adolescents With Type 1 Diabetes	Type 1 Diabetes|Stress	Behavioral: Psychological background determinations|Genetic: Genetic susceptibility determination|Procedure: Cortisol release test|Procedure: Saliva cortisol measurement

ANNEXURE 2

1. Protocol for Agilent 2-color microarray of adipocytes at 6h, 24h and 48h

Steps:

Sample Preparation

Step 1. Preparation of Spike A Mix and Spike B Mix

Step 2. Preparation labeling reaction

Step 3. Purification of labeled/amplified RNA

Step 4. Quantification of cRNA

Hybridization

Step 1. Preparation of 10X Blocking Agent

Step 2. Preparation of hybridization samples

Step 3. Preparation of hybridization assembly

Step 4. Microarray Slide wash

Scanning and Feature Extraction

Step 1. Slides scanning

Step 2. Data extraction using Agilent Feature Extraction Software

Slide Set-up:

6 Hr	24 Hr	48 Hr
NT (A) vs NT (ccA)	NT(A) vs NT(ccA)	NT(A) vs NT(ccA)
S100 (A) vs S100 (ccA)	S100 (A) vs S100 (ccA)	S100 (A) vs S100 (ccA)
C100 (A) vs C100 (ccA)	C100 (A) vs C100 (ccA)	C100 (A) vs C100 (ccA)
C100/S100 (A) vs C100/S100 (ccA)	C100/S100 (A) vs C100/S100 (ccA)	C100/S100 (A) vs C100/S100 (ccA)

Cultured alone Adipocyte Samples: NT(A), S100(A), C100(A), C100/S100(A)- **Cy3** label

Co-cultured Adipocyte Samples: NT(ccA), S100(ccA), C100(ccA), C100/S100(ccA)- **Cy5** label

1A. Sample Preparation

Step 1. Spike A Mix and Spike B Mix preparation:

(RNA Spike-In Kit, Two-Color – Agilent p/n 5188-5279)

1. The stock solutions were mixed, heated at 37°C for 5 minutes and mixed on a vortex mixer once more.
2. The stock solutions were then centrifuged to drive contents to the bottom of the tube prior to opening.
3. 40 µl of Spike A mix and 40 µl of Spike B mix were prepared as per the calculations of Table1 in 1.5 ml labeled microfuge tubes.
4. The spike mixtures were mixed well with a briefly spin after each dilution.

Table 1

S. No	Sample ID	Starting amount of Total RNA (ng)	Serial dilution		
			First Dilution(1:20) (Spike + Dilution buffer)	Second Dilution(1:40) (First dilution + Dilution buffer)	Third Dilution(1:4) (Second dilution + Dilution buffer)
1	NT (A) – 6hr	3000	2µl Spike A + 38 µl Dilution buffer	2µl First Dilution s-A + 78 µl Dilution buffer	10µl Second Dilution s-A + 30µl Dilution buffer (Volume required per labeling reaction per sample = 2 µl)
2	S100 (A) – 6hr	3000			
3	C100 (A) – 6hr	3000			
4	C100/S100(A) – 6hr	3000			
5	NT (A) – 24hr	3000			
6	S100 (A) – 24hr	3000			
7	C100 (A) – 24hr	3000			
8	C100/S100(A) – 24hr	3000			
9	NT (A) – 48hr	3000			
10	S100 (A) – 48hr	3000			
11	C100 (A) – 48hr	3000			
12	C100/S100(A) – 48hr	3000			
	Total samples = 12			Total Spike-A (3rd dilution) mix required (for labeling reaction) = 2µl X 12 = 24µl	
13	NT (ccA) – 6hr	3000	2µl Spike B + 38 µl Dilution buffer	2µl First Dilution s-B + 78 µl Dilution buffer	10µl Second Dilution s-B + 30µl Dilution buffer (Volume required per labeling reaction per sample = 2 µl)
14	S100 (ccA) – 6hr	3000			
15	C100 (ccA) – 6hr	3000			
16	C100/S100(ccA) – 6hr	3000			
17	NT (ccA) – 24hr	3000			
18	S100 (ccA) – 24hr	3000			
19	C100 (ccA) – 24hr	3000			
20	C100/S100(ccA) – 24hr	3000			
21	NT (ccA) – 48hr	3000			
22	S100 (ccA) – 48hr	3000			
23	C100 (ccA) – 48hr	3000			
24	C100/S100(ccA) – 48hr	3000			
	Total samples = 12			Total Spike-B (3rd dilution) mix required (for labeling reaction) = 2µl X 12 = 24µl	

Step 2. Labeling reaction preparation

1. 5X first strand buffer was prewarmed at 80°C for 3 to 4 minutes to ensure adequate resuspensions of the buffer components.
2. 1000ng of RNA sample, spike mix (2 µl), T7 Promoter primer(1.2 µl) and nuclease free water were added as per Table2 such that the final volume is 11.5 µl for each sample.

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3. The primer and the template were denatured by incubating the reaction at 65°C in a circulating water bath for 10 minutes.
4. The reactions were put on ice and incubated for 5 minutes.
5. Immediately prior to use, the components listed in Table 3 were mixed gently for the cDNA Master Mix by adding in the order indicated, and put on ice.

Table 2

S.No	Sample ID	Total RNA (ng)	RNA volume (µL)	3rd Dilution Spike Mix amounts (µL)	T7 Promoter primer (µL)	Nuclease free water (µl)	Total volume (µL)
1	NT (A) – 6h	3000	5.58	2 (Spike A)	1.2	2.7	11.5
2	S100 (A) – 6h	3000	5.50	2 (Spike A)	1.2	2.8	11.5
3	C100 (A) – 6h	3000	5.59	2 (Spike A)	1.2	2.7	11.5
4	C100/S100(A) – 6h	3000	4.731	2 (Spike A)	1.2	3.6	11.5
5	NT (A) – 24h	3000	3.83	2 (Spike A)	1.2	4.5	11.5
6	S100 (A) – 24h	3000	4.08	2 (Spike A)	1.2	4.2	11.5
7	C100 (A) – 24h	3000	3.56	2 (Spike A)	1.2	4.7	11.5
8	C100/S100(A) – 24h	3000	5.49	2 (Spike A)	1.2	2.8	11.5
9	NT (A) – 48h	3000	3.07	2 (Spike A)	1.2	5.2	11.5
10	S100 (A) – 48h	3000	4.048	2 (Spike A)	1.2	4.2	11.5
11	C100 (A) – 48h	3000	3.649	2 (Spike A)	1.2	4.7	11.5
12	C100/S100(A) – 48h	3000	3.355	2 (Spike A)	1.2	4.9	11.5
	Total samples = 12						
13	NT (ccA) – 6h	3000	4.45	2 (Spike B)	1.2	3.2	11.5
14	S100 (ccA) – 6h	3000	3.65	2 (Spike B)	1.2	2.7	11.5
15	C100 (ccA) – 6h	3000	4.76	2 (Spike B)	1.2	3.6	11.5
16	C100/S100(ccA) – 6h	3000	5.76	2 (Spike B)	1.2	3.4	11.5
17	NT (ccA) – 24h	3000	6.84	2 (Spike B)	1.2	3.3	11.5
18	S100 (ccA) – 24h	3000	3.47	2 (Spike B)	1.2	3.7	11.5
19	C100 (ccA) – 24h	3000	4.68	2 (Spike B)	1.2	4.9	11.5
20	C100/S100(ccA) – 24h	3000	3.32	2 (Spike B)	1.2	3.0	11.5
21	NT (ccA) – 48h	3000	5.76	2 (Spike B)	1.2	3.2	11.5
22	S100 (ccA) – 48h	3000	4.65	2 (Spike B)	1.2	2.9	11.5
23	C100 (ccA) – 48h	3000	3.56	2 (Spike B)	1.2	2.9	11.5
24	C100/S100(ccA) – 48h	3000	3.21	2 (Spike B)	1.2	1.7	11.5
	Total samples = 12				28.8		

Table 3 : cDNA Master Mix

Component	Volume per reaction (µL)	Volume per 24 reactions (µL)
5X First Strand Buffer	4	96
0.1 M DTT	2	48
10 mM dNTP mix	1	24
MMLV-RT	1	24
RnaseOut	0.5	12
Total Volume	8.5	204

6. During incubation, 50% PEG solution was pre-warmed at 40°C for 1 minute, briefly mixed on a vortex mixer, centrifuged to drive down the contents from the tube walls and kept at room temperature until needed.
7. The samples were then shifted to a 65°C circulating water bath and incubated for 15 minutes.
8. The samples were then qransferred to ice and incubated for 5 minutes.
9. Samples were briefly centrifuged to drive down tube contents from the tube walls.
10. Components listed in Table 4 were mixed in the order indicated for the Transcription Master Mix by pipetting at room temperature and use immediately.
11. 60 µL of Transcription Master Mix was added to each sample tube and gently mixed by pipetting.
12. Samples were then incubated in a circulating water bath at 40°C for 2 hours.

Table 4 : Transcription Master Mix

Component	Volume per reaction (µL)	Volume per 24 reactions (µL)
Nuclease-free water	15.3	367.2
4X Transcription Buffer	20	480
0.1 M DTT	6	144
NTP mix	8	192
50% PEG	6.4	153.6
RNaseOUT	0.5	12
Inorganic pyrophosphatase	0.6	14.4
T7 RNA Polymerase	0.8	19.2
Cyanine 3-CTP/5-CTP	2.4	57.6
Total Volume	60	1440

Step 3. Purification of the labeled/amplified RNA

(Rneasy Mini Kits – Qiagen p/n 74104 or 74106)

1. 20 µL of nuclease-free water was added to the cRNA sample, for a total volume of 100 µL.
2. 350 µL of Buffer RLT was added and mixed well by pipetting.
3. 250 µL of ethanol (96% to 100% purity) was added and mixed thoroughly by pipetting.
4. 700 µL of the cRNA sample was transferred to an Rneasy mini column in a 2 mL collection tube and centrifuged at 4°C for 30 seconds at 13,000 rpm. (flow-through and collection tube were discarded)
5. Rneasy column was transferred to a new collection tube and 500 µL of buffer RPE (containing ethanol) was added to the column. The sample was centrifuged at 4°C for 30 seconds at 13,000 rpm (the flow-through was discarded).
6. Another 500 µL of buffer RPE was added to the column. The sample was centrifuged at 4°C for 60 seconds at 13,000 rpm and flow-through discarded.

7. The cleaned cRNA sample was eluted by transferring the Rneasy column to a new 1.5 mL collection tube. 30 µL Rnase-free water was added directly onto the Rneasy filter membrane. After 60 seconds, the column was centrifuged at 4°C for 30 seconds at 13,000 rpm.
8. The cRNA sample-containing flow-through was maintained on ice.

Step 4. Quantification the cRNA

1. The Cy3 and Cy5 dye incorporation and cRNA concentration was measured through NanoDrop ND-1000 UV-VIS Spectrophotometer version 3.2.1.
2. The cRNA yield, and specific activity if Cy3 and Cy5 were calculated as shown in Table 5.

Table 5

S.No	Sample ID	Cy3 dye concentration ©(pmol/µl)	260 /280	cRNA concentration (y)(ng/µL)	Specific activity (pmol Cy3 per µg cRNA) $\frac{(c \div y) \times 1000}{}$
1	NT (A) – 6h	21.9	1.4	554.6	39.4
2	S100 (A) – 6h	26.4	1.2	529.5	49.8
3	C100 (A) – 6h	28.4	1.15	520.1	54.6
4	C100/S100(A) – 6h	23.6	1.36	550.4	42.8
5	NT (A) – 24h	19.8	1.60	570.5	34.7
6	S100 (A) – 24h	15.2	1.97	567.4	26.7
7	C100 (A) – 24h	21.5	1.40	555.5	38.7
8	C100/S100(A) – 24h	24.9	1.25	539.6	46.1
9	NT (A) – 48h	21.0	1.48	570.9	36.7
10	S100 (A) – 48h	18.0	1.64	576.3	31.2
11	C100 (A) – 48h	18.5	1.65	571.2	32.3
12	C100/S100(A) – 48h	18.1	1.64	575.2	31.4
		Cy5 dye concentration ©(pmol/µl)	**260 /280**	**cRNA concentration (y)(ng/µL)**	**Specific activity (pmol Cy5 per µg cRNA)** $\frac{(c \div y) \times 1000}{}$
13	NT (ccA) – 6h	23.2	1.30	545.8	42.5
14	S100 (ccA) – 6h	22.7	1.25	515.6	44.0
15	C100 (ccA) – 6h	23.8	1.23	514.6	46.2

16	C100/S100(ccA) – 6h	22.5	1.28	527.6	42.6
17	NT (ccA) – 24h	22.5	1.27	526.2	42.7
18	S100 (ccA) – 24h	23.9	1.20	519.7	45.9
19	C100 (ccA) – 24h	16.0	1.82	576.4	27.7
20	C100/S100(ccA) – 24h	23.6	1.24	533.3	44.2
21	NT (ccA) – 48h	6.2	2.51	367.2	16.8
22	S100 (ccA) – 48h	7.7	2.45	431.5	17.8
23	C100 (ccA) – 48h	5.4	2.55	312.0	17.3
24	C100/S100(ccA) – 48h	5.3	2.58	314.2	16.8

1B. Hybridization

Step 1. 10X Blocking Agent preparation

(Gene Expression Hybridization Kit - Agilent p/n 5188-5242)

1. 500 µL of nuclease-free water was added to the vial containing lyophilized 10X Blocking Agent supplied with the Agilent Gene Expression Hybridization Kit and gently vortexed.
2. The mix was heated for 4 to 5 minutes at 37°C and centrifuged for 5 to 10 seconds.

Step 2. Preparation of hybridization samples

1. For each microarray, each of the components was added as indicated in the fragmentation mix table below to a 1.5 mL nuclease-free microfuge tube, and mixed gently on a vortex mixer.
2. Each of the hybridization mixture was incubate at 60°C in water bath for exactly 30 minutes to fragment RNA.
3. Equal volume (55 µl) 2x GEx Hybridization Buffer HI-RPM was added to the fragmentation mix at the appropriate volume to stop the fragmentation reaction and centrifuged for 1 minute at room temperature at 13,000 rpm.
4. The mixtures were on ice and loaded quickly onto the array.

Table 6: Fragmentation mix table

Components				Components			
Property	**Sample**	**Conc (ng/µl)**	**Vol (µL)**	**Property**	**Sample**	**Conc (ng/µl)**	**Vol (µL)**
cy3-labeled, cRNA (825 ng)	**NT(A) -6h**	554.6	**1.48**	**cy3-labeled, cRNA (825 ng)**	**S100(A) -6h**	529.5	**1.58**
cy5-labeled, cRNA (825 ng)	**NT(ccA) –6h**	545.8	**1.51**	**cy5-labeled, cRNA (825 ng)**	**S100(ccA) –6h**	515.6	**1.60**
10X Blocking Agent			**11**	**10X Blocking Agent**			**11**
Nuclease-free water (vol. make up)-38.81			**to 52.8**	**Nuclease-free water (vol. make up)-38.62**			**to 52.8**
25X Fragmentation Buffer			**2.2**	**25X Fragmentation Buffer**			**2.2**
Total Volume			**55**	**Total Volume**			**55**
Components				**Components**			
Property	**Sample**	**Conc (ng/µl)**	**Vol (µL)**	**Property**	**Sample**	**Conc (ng/µl**	**Vol (µL)**
cy3-labeled, cRNA (825 ng)	**C100(A) -6h**	520.1	**1.55**	**cy3-labeled, cRNA (825 ng)**	**C100/S100(A) -6h**	550.4	**1.49**
cy5-labeled, cRNA (825 ng)	**C100(ccA) –6h**	514.6	**1.60**	**cy5-labeled, cRNA (825 ng)**	**C100/S100(ccA) –6h**	527.6	**1.56**
10X Blocking Agent			**11**	**10X Blocking Agent**			**11**
Nuclease-free water (vol. make up)-38.65			**to 52.8**	**Nuclease-free water (vol. make up)-38.75**			**to 52.8**
25X Fragmentation Buffer			**2.2**	**25X Fragmentation Buffer**			**2.2**
Total Volume			**55**	**Total Volume**			**55**
Components				**Components**			
Property	**Sample**	**Conc (ng/µl)**	**Vol (µL)**	**Property**	**Sample**	**Conc (ng/µl)**	**Vol (µL)**
cy3-labeled, cRNA (825 ng)	**NT(A) -24h**	570.5	**1.45**	**cy3-labeled, cRNA (825 ng)**	**S100(A) -24h**	567.4	**1.48**
cy5-labeled, cRNA (825 ng)	**NT(ccA) –24h**	526.2	**1.57**	**cy5-labeled, cRNA (825 ng)**	**S100(ccA) –24h**	519.7	**1.43**
10X Blocking Agent			**11**	**10X Blocking Agent**			**11**
Nuclease-free water (vol. make up)-38.78			**to 52.8**	**Nuclease-free water (vol. make up)-38.89**			**to 52.8**
25X Fragmentation Buffer			**2.2**	**25X Fragmentation Buffer**			**2.2**
Total Volume			**55**	**Total Volume**			**55**

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Components			
Property	**Sample**	**Conc (ng/µl)**	**Vol (µL)**
cy3-labeled, cRNA (825 ng)	**C100(A) -24h**	555.5	**1.45**
cy5-labeled, cRNA (825 ng)	**C100(ccA) – 24h**	576.4	**1.58**
10X Blocking Agent			**11**
Nuclease-free water (vol. make up)-38.77			**to 52.8**
25X Fragmentation Buffer			**2.2**
Total Volume			**55**

Components			
Property	**Sample**	**Conc (ng/µl)**	**Vol (µL)**
cy3-labeled, cRNA (825 ng)	**C100/S100(A) -24h**	539.6	**1.53**
cy5-labeled, cRNA (825 ng)	**C100/S100(ccA) – 24h**	533.3	**1.54**
10X Blocking Agent			**11**
Nuclease-free water (vol. make up)-38.73			**to 52.8**
25X Fragmentation Buffer			**2.2**
Total Volume			**55**

Components			
Property	**Sample**	**Conc (ng/µl)**	**Vol (µL)**
cy3-labeled, cRNA (825 ng)	**NT(A) -48h**	570.9	**1.44**
cy5-labeled, cRNA (825 ng)	**NT(ccA) –48h**	367.2	**2.24**
10X B ocking Agent			**11**
Nuclease-free water (vol. make up)-38.12			**to 52.8**
25X Fragmentation Buffer			**2.2**
Total Volume			**55**

Components			
Property	**Sample**	**Conc (ng/µl)**	**Vol (µL)**
cy3-labeled, cRNA (825 ng)	**S100(A) -48h**	576.3	**1.44**
cy5-labeled, cRNA (825 ng)	**S100(ccA) –48h**	431.5	**2.64**
10X Blocking Agent			**11**
Nuclease-free water (vol. make up)-37.72			**to 52.8**
25X Fragm ntation Buffer			**2.2**
Total Volume			**55**

Components			
Property	**Sample**	**Conc (ng/µl)**	**Vol (µL)**
cy3-labeled, cRNA (825 ng)	**C100(A) -48h**	571.2	**1.43**
cy5-labeled, cRNA (825 ng)	**C100(ccA)–48h**	312.0	**1.91**
10X Blocking Agent			**11**
Nuclease-free water (vol. make up)-38.46			**to 52.8**
25X Fragmentation Buffer			**2.2**
Total Volume			**55**

Components			
Property	**Sample**	**Conc (ng/µl)**	**Vol (µL)**
cy3-labeled, cRNA (825 ng)	**C100/S100(A) -48h**	575.2	**1.43**
cy5-labeled, cRNA (825 ng)	**C100/S100(ccA)– 48h**	314.2	**2.63**
10X Blocking Agent			**11**
Nuclease-free water (vol. make up)-37.74			**to 52.8**
25X Fragmentation Buffer			**2.2**
Total Volume			**55**

Step 3. Preparation of hybridization assembly

1. A clean gasket slide was loaded into Agilent SureHyb chamber base with label facing up and aligned with the rectangular section of the chamber base.
2. 100 µl of each hybridization sample was slowly dispensed onto a gasket well in a "drag and dispense" manner.
3. An array "active side" down was placed onto the SureHyb gasket slide, properly aligned, and placed in the SureHyb chamber and hand-tightened.
4. The assembled slide chamber was vertically rotated with slight tapping to wet the gasket and the mobility of the bubbles was accessed.
5. The slide chamber was placed in the rotisserie in a hybridization oven set to 65^{o}C, with rotation of 10 rpm and for 17 hrs.

Step 4. Microarray Slide Wash

1. 2 ml of 10% Triton X-102 was added to Gene Expression wash buffer 1 and 2.
2. Gene Expression wash buffer 2 was pre-warmed overnight at 37 ^{o}C.
3. The slide rack and stir bar, assembled on the staining dish and filled with 100% acetonitrile, was placed on a magnetic stir plate and washed for 5 mins.
4. The acetonitrile was discarded and steps 4 and 5 were repeated, followed by air drying and a Milli-Q wash.
5. Slide staining dish #1 was filled with Gene Expression wash buffer 1 at room temp., and a slide staining rack was placed in slide staining dish #2 filled with Gene Expression wash buffer 1 at room temp. Entire set-up was placed on magnetic stir plate.
6. The hybridization chamber assembly was placed on a flat surface and the thumb-screw was loosened to slide off the clamp assembly with the chamber cover removed.

7. The array–gasket sandwich was transferred into staining dish #1 with the numeric barcode facing up.
8. Remove the microarray slide was removed with forceps (plastic) and placed in the slide rack in slide staining dish #2 with stirring for 1 min.
9. Staining dish #3 was filled with Gene Expression wash buffer 2 from 37^{o}C water-bath and placed on a magnetic stir plate with a magnetic stirrer in it.
10. The slide rack was removed from staining dish #2 and transferred to staining dish#3 and stirred 37^{o}C for 1 min.
11. The slide rack was removed minimizing droplets on the slides and slides were removed within 5-10 secs for scanning to minimize the impact of environmental oxidants.

1C. Scanning and Feature Extraction

Step 1. Slide Scanning

1. The scanner was set to ready 20 minutes in advance.
2. The slides were assembled into slide holder such that the numeric barcode was visible and placed in the scanner carousel with the following scan settings:

Scan region	**:**	Scan area(61 X 21.6mm)
Scan resolution(µm)	**:**	5
5 µm scanning mode	**:**	Single pass
Extended dynamic range	**:**	selected
Dye channel	**:**	Red and Green
Green PMT	**:**	XDR Hi 80% ; XDR Lo 20%
Red PMT	**:**	XDR Hi 80% ; XDR Lo 20%

Step 2. Data extraction using Feature extraction software

1. Feature extraction was done using the Agilent Feature Extraction Software.

2. Protocol for Agilent 2-color microarray of co-cultured adipocytes and macrophages at 48h

Steps:

Sample Preparation

Step 1. Prepare Spike A Mix and Spike B Mix

Step 2. Prepare labeling reaction

Step 3. Purify the labeled/amplified RNA

Step 4. Quantify the cRNA

Hybridization

Step 1. Prepare the 10X Blocking Agent

Step 2. Prepare hybridization samples

Step 3. Prepare hybridization assembly

Step 4. Microarray Wash

Scanning and Feature Extraction

Step 1. Scan the slides

Step 2. Extract data using Agilent Feature Extraction Software

Slide Set-up:

Macrophage(CC) 48 Hr	Adipocyte(CC) 48 Hr
NT(Mcc) vs Ref	NT(Acc) vs Ref
C100(Mcc) vs Ref	C100(Acc) vs Ref
S100(Mcc) vs Ref	S100(Acc) vs Ref
CS100(Mcc) vs Ref	CS100(Acc) vs Ref

Coculture Macrophage Samples : NT(Mcc), S100(Mcc), C100(Mcc), CS100(Mcc)- **Cy5** label

Coculture Adipocyte Samples : NT(Acc), S100(Acc), C100(Acc), CS100(Acc)- **Cy5** label

Ref : NT(M only) + NT(A only) – **Cy3** label

Sample Preparation

Step 1. Spike A Mix and Spike B Mix preparation: (same as in 1A)

Table 1

S. No	Sample ID	Starting amount of Total RNA (ng)	Serial dilution		
			First Dilution(1:20) (Spike + Dilution buffer)	Second Dilution(1:40) (First dilution + Dilution buffer)	Third Dilution(1:4) (Second dilution + Dilution buffer)
1	Ref -48h	2000	2µl Spike A + 38 µl Dilution buffer	2µl First Dilution s-A + 78 µl Dilution buffer	10µl Second Dilution s-A + 30µl Dilution buffer (Volume required per labeling reaction per sample = 2 µl)
	Total samples = 1			Total Spike-A (3rd dilution) mix required (for labeling reaction) = 2µl X 12 = 24µl	
2	NT (Acc)-48h	1000			
3	S100 (Acc) -48h	1000			

4	C100 (Acc) -48h	1000	2µl Spike B + 38 µl Dilution buffer	2µl First Dilution s-B + 78 µl Dilution buffer	10µl Second Dilution s-B + 30µl Dilution buffer (Volume required per labeling reaction per sample = 2 µl)
5	CS100(Acc) -48h	1000			
6	NT (Mcc) -48h	1000			
7	S100 (Mcc) -48h	1000			
8	C100 (Mcc) -48h	1000			
9	CS100(Mcc)-48h	1000			
	Total samples = 8			Total Spike-B (3rd dilution) mix required (for labeling reaction) = 2µl X 12 = 24µl	

Step 2. Labeling reaction preparation: (same as in 1A)

Table 2

S.No	Sample ID	Total RNA (ng)	RNA volume (µL)	3rd Dilution Spike Mix amounts (µL)	T7 Promoter primer (µL)	Nuclease free water (µl)	Total volume (µL)
1	Ref -48h	2000	2.7	2 (Spike A)	1.2	5.6	11.5
	Total samples = 1						
17	NT (Acc)-48h	1000	1.34	2 (Spike B)	1.2	6.96	11.5
18	S100 (Acc) -48h	1000	2.43	2 (Spike B)	1.2	5.87	11.5
19	C100 (Acc) -48h	1000	2.55	2 (Spike B)	1.2	5.75	11.5
20	CS100(Acc) -48h	1000	2.53	2 (Spike B)	1.2	5.77	11.5
21	NT (Mcc) -48h	1000	1.59	2 (Spike B)	1.2	6.71	11.5
22	S100 (Mcc) -48h	1000	1.75	2 (Spike B)	1.2	6.55	11.5
23	C100 (Mcc) -48h	1000	1.50	2 (Spike B)	1.2	6.8	11.5
24	CS100(Mcc)-48h	1000	1.72	2 (Spike B)	1.2	6.58	11.5
	Total samples = 8				10.8		

Table 3 : cDNA Master Mix

Component	Volume per reaction (µL)	Volume per 9 reactions (µL)
5X First Strand Buffer	4	36
0.1 M DTT	2	18
10 mM dNTP mix	1	9
MMLV-RT	1	9
RNaseOut	0.5	4.5
Total Volume	8.5	76.5

Table 4 : Transcription Master Mix

Component	Volume per reaction (µL)	Volume per 9reactions (µL)
Nuclease-free water	15.3	137.7
4X Transcription Buffer	20	180.0
0.1 M DTT	6	54.0
NTP mix	8	72.0
50% PEG	6.4	57.6
RNaseOUT	0.5	4.5
Inorganic pyrophosphatase	0.6	5.4
T7 RNA Polymerase	0.8	7.2
Cyanine 3-CTP/5-CTP	2.4	19.2
Total Volume	60	540.0

Step 3. Purification of the labeled/amplified RNA: (same as in 1A)

Step 4. Quantification the cRNA: (same as in 1A)

Table 5

S. No	Sample ID	Cy3 dye concentration(c) (pmol/µl)	260 /280	cRNA concentration (y)(ng/µL)	Specific activity (pmol Cy3 per µg cRNA) $(c \div y) \times 1000$
1	Ref -48h	44.7	1.48	751.6	59
		Cy5 dye concentration(c) (pmol/µl)	**260 /280**	**cRNA concentration (y)(ng/µL)**	**Specific activity (pmol Cy3 per µg cRNA) $(c \div y) \times 1000$**
2	NT (Acc)-48h	12.6	2.17	655.12	19.2
3	S100 (Acc) -48h	20.6	1.7	743.3	20.6
4	C100 (Acc) -48h	9.8	2.27	557.7	9.8
5	CS100(Acc) -48h	15.9	2.0	725.4	15.9
6	NT (Mcc) -48h	15.1	2.0	716.8	15.1
7	S100 (Mcc) -48h	15.3	2.02	710.4	15.3
8	C100 (Mcc) -48h	14.0	2.09	697.0	14.0
9	CS100(Mcc)-48h	11.5	2.19	634.1	11.5

2B. Hybridization

Step 1. 10X Blocking Agent preparation: (same as in 1B)

Step 2. Preparation of hybridization samples: (same as in 1B)

Table 6: Fragmentation mix table

Components			Volume (µL)
Property	**Sample**	**Conc(ng/µl)**	
cy3-labeled, cRNA (825 ng)	Ref -48h	751.6	1.098
cy5-labeled, cRNA (825 ng)	NT(Acc)-48h	716.8	1.1509
10X Blocking Agent			11
Nuclease-free water (vol. make up)			to 52.8
25X Fragmentation Buffer			2.2
Total Volume			55

Components			Volume (µL)
Property	**Sample**	**Conc(ng/µl)**	
cy3-labeled, cRNA (825 ng)	Ref -48h	751.6	1.098
cy5-labeled, cRNA (825 ng)	NT(Mcc)-48h	716.8	1.1509
10X Blocking Agent			11
Nuclease-free water (vol. make up)			to 52.8
25X Fragmentation Buffer			2.2
Total Volume			55

Components			Volume (µL)
Property	**Sample**	**Conc(ng/µl)**	
cy3-labeled, cRNA (825 ng)	Ref -48h	751.6	1.098
cy5-labeled, cRNA (825 ng)	C100(Acc)-48h	710.4	1.161
10X Blocking Agent			11
Nuclease-free water (vol. make up)			to 52.8
25X Fragmentation B ffer			2.2
Total Volume			55

Components			Volume (µL)
Property	**Sample**	**Conc(ng/µl)**	
cy3-labeled, cRNA (825 ng)	Ref -48h	751.6	1.098
cy5-labeled, cRNA (825 ng)	C100(Mcc)-48h	557.7	1.479
10X Blocking Agent			11
Nuclease-free water (vol. make up)			to 52.8
25X Fragmentation Buffer			2.2
Total Volume			55

Components			Volume (µL)
Property	**Sample**	**Conc(ng/µl)**	
cy3-labeled, cRNA (825 ng)	Ref -48h	751.6	1.098
cy5-labeled, cRNA (825 ng)	S100(Acc)-48h	697.0	1.183
10X Blocking Agent			11
Nuclease-free water (vol. make up)			to 52.8
25X Fragmentation Buffer			2.2
Total Volume			55

Components			Volume (µL)
Property	**Sample**	**Conc(ng/µl)**	
cy3-labeled, cRNA (825 ng)	Ref -48h	751.6	1.098
cy5-labeled, cRNA (825 ng)	S100(Mcc)-48h	743.3	1.109
10X Blocking Agent			11
Nuclease-free water (vol. make up)			to 52.8
25X Fragmentation Buffer			2.2
Total Volume			55

Components			
Property	**Sample**	**Conc(ng/µl)**	**Volume (µL)**
cy3-labeled, cRNA (825 ng)	**Ref -48h**	**751.6**	**1.098**
cy5-labeled, cRNA (825 ng)	**CS100(Acc) -48h**	**634.1**	**1.301**
10X Blocking Agent			**11**
Nuclease-free water (vol. make up)			**to 52.8**
25X Fragmentation Buffer			**2.2**
Total Volume			**55**

Components			
Property	**Sample**	**Conc(ng/µl)**	**Volume (µL)**
cy3-labeled, cRNA (825 ng)	**Ref -48h**	**751.6**	**1.098**
cy5-labeled, cRNA (825 ng)	**CS100(Mcc)-48h**	**725.4**	**1.137**
10 Blocking Agent			**11**
Nuclease-free water (vol. make up)			**to 52.8**
25X Fragmentation Buffer			**2.2**
Total Volume			**55**

Step 3. Preparation of hybridization assembly: (same as in 1B)

Step 4. Microarray Slide Wash: (same as in 1B)

2C. Scanning and Feature Extraction

Step 1. Slide Scanning: (same as in 1C)

Step 2. Data extraction using Feature extraction software: (same as in 1C)

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Homi Bhabha National Institute

Ph.D. Thesis Evaluation Report

1. **Name of the Constituent Institution:**
2. **Name of the Student:** Ms. SUSHRI PRIYADARSHINI
3. **Enrolment No.:** LIFE07201004006
4. **Title of the Thesis:** "Effect of psychological stress on physiological homeostasis and disease susceptibility"
5. **Name of the Board of Studies:**

Recommendations

Tick one of the following:

1. The thesis in its present form is commended for the award of the Ph.D. Degree. ☐
2. The thesis is commended for the award of the Ph.D. degree. However, my suggestions for improving the thesis may be considered at the time of the viva voce examination and if the viva voce board deems it appropriate, the same may be incorporated in the thesis based on the discussions during the viva voce examination. The revised thesis need not be sent to me. ☑
3. The thesis should be revised as per the suggestions enclosed. I would like to see the revised thesis incorporating my suggestions before I give further recommendations. ☐
4. The thesis is not acceptable for the award of the Ph.D. degree. ☐

Date:

(Signature):

Name of Examiner:
And affiliation:

Prof. Tapas K. Kundu, Ph.D., FNASc., FASc., FNA.
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Please give your detailed report in the attached sheet. You may use additional sheets, if required.

Version approved during the meeting of Deans held during 29-30 Nov 2013

Name of the student: Ms. Sushri Priyadarshini

Title of the Thesis: "Effects of psychological stress on physiological homeostasis and disease susceptibility"

DETAILED REPORT

The thesis entitled, "Effects of psychological stress on physiological homeostasis and disease susceptibility" by Ms. Sushri Priyadarshini, is based on aboard theme which is very difficult to justify for a PhD thesis work. This thesis has been written largely based on the theoretical analysis of the available data with a very few numbers of experiments. Beside the general introduction and review of literature the first chapter deals with the development of methodologies for systemic analysis of the physiological parameters and gene networking. The second chapter deals with the prediction of gene association in physiological processes namely Immunity, Metabolism, Aging etc. In the third chapter she has reported a few experiments which were done to validate some of the gene association using adipogenesis system. The fourth chapter is entirely based on bio-informatics analysis for mining and analysis of physiological stress data. In my view the fourth chapter is not needed for this thesis, instead of that the candidate could have validated some of the gene networking using other differentiation system.

However, the candidate has put substantial effort to correlate stress, genes expression and disease biology both by theoretical analysis and experimental validation. She has attempted to develop gene network association with physiological stress in the context of immunity and metabolism. Furthermore, the study is also extended to large number of disease which helps them to identify the disease genes and its correlation with physiological functions. Their data related to nitric oxide biosynthesis in the context of metabolism could be very important to elucidate disease biology signaling pathway. Some of the data analysis could also be helpful to understand the liver malfunction related stress. The validation of the network using adipogenesis creating an artificial stress environment is an appreciable approach. However, the quality of the differentiation system and data quantification could be further improved. The functional network of adipogenic transcription factor PPARγ2 shown by the author is an interesting finding. As I wrote in the introductory paragraph that the last chapter of the thesis need not be added, as it has not yielded any substantial new information.

The subject of the thesis is so broad it is quite difficult to introduce as a whole. However, the author has given the best effort. The data has been organized quite systhematically. The author / candidate have published couple of papers based on this work. Therefore, I recommend that Ms. Sushri Priyadarshini may be awarded PhD upon successful completion of viva examination.

Name of the Examiner: Prof. Tapas Kumar Kundu

Signature and Date May 2, 2016

Homi Bhabha National Institute

Ph.D. Thesis Evaluation Report

1. **Name of the Constituent Institution:** National Inst. of Sci. Education & Research.
2. **Name of the Student:** Mrs. Sushri Priyadarshini
3. **Enrolment No.:**
4. **Title of the Thesis:** Effects of psychological stress on psychological homeostasis and disease susceptibility.
5. **Name of the Board of Studies:** Life sciences.

Recommendations

Tick one of the following:

1. The thesis in its present form is commended for the award of the Ph.D. Degree. ☑
2. The thesis is commended for the award of the Ph.D. degree. However, my suggestions for improving the thesis may be considered at the time of the viva voce examination and if the viva voce board deems it appropriate, the same may be incorporated in the thesis based on the discussions during the viva voce examination. The revised thesis need not be sent to me. ☐
3. The thesis should be revised as per the suggestions enclosed. I would like to see the revised thesis incorporating my suggestions before I give further recommendations. ☐
4. The thesis is not acceptable for the award of the Ph.D. degree. ☐

Date:

(Signature):

Name of Examiner: Prof. Shashidhara

And affiliation

Prof. Shashidhara
Developmental Biology Division &
Dean, Research & Faculty
Indian Institute of Science
Education & Research
Dr. Homi Bhabha Road, Pune - 411 008. India

Please give your detailed report in the attached sheet. You may use additional sheets, if required.

Version approved during the meeting of Deans held during 29-30 Nov 2013

d

1. **Name of the Student:** Mrs. Sushri Priyadarshini

2. **Title of the Thesis:** Effects of Pyschological stress on psychological homeostasis and disease susceptibility.

DETAILED REPORT

attached

Name of Examiner: Prof. Shashidhara.

Signature and Date:

Prof. L. S. Shashidhara
Chair, Biology Division &
Dean, Research & Faculty
Indian Institute of Science
Education & Research
Dr. Homi Bhabha Road, Pune - 411 008 India

e

THESIS: EFFECTS OF PSYCHOLOGICAL STRESS ON PHYSIOLOGICAL HOMEOSTASIS AND DISEASE SUSCEPTIBILITY

BY: SUSHRI PRIYADARSHINI (Enrolment No: LIFE07201004006)
NATIONAL INSTITUTE OF SCIENCE EDUCATION AND RESEARCH, BHUBANESWAR

The study was aimed at investigating the metabolic implications of the psychological stress, which may suggest the disease susceptibility. In achieving this, the present study has utilized in-silico approach to elucidate the pathways involved in stress related health consequences such as impaired metabolism, immunity and reproduction etc. Literature mining approaches have proved to be very effective in providing a high-throughput, low-cost alternative to extracting meaningful information. In addition to existing gene-networking programs, new technologies were developed to corroborate the data available from in vivo and in vitro studies. The observations from the computational data were tested by inducing the psychological stress in vitro using the stress indicators such as cortisol and serotonin in co-culture ofmacrophage-adipose tissue. These results provided new insights into some of the mechanisms that might be involved in the stress-induced immunity and metabolism. The study tried to use both the approaches, in-silico and in vitro to correlate the findings. These studies highlight the role of two serotonin receptors HTR2c and HTR2a in the modulation of stress response in coordination of macrophage function. However, it has been routinely seen that the some of the most striking findings based on either in-silico or its in vitro validations don't match with those in the in vivo findings, especially when these observations are related to the central to peripheral communications, given the complexity of underlying mechanisms. The most visible lacuna in the study is in not making any efforts to validate the findings using in vivo model system. I am concerned that studies of this kind are susceptible to subjective errors. From this point of view, even the title of the thesis is bit of a misnomer. The title gives an impression that the work may involve a good deal of hard core experimental biology, but that is not the case. This indeed limits the usefulness of the study.

Nonetheless, the study highlights different possible pathways, which connects the psychological stress with the peripheral organ systems and may offer a platform for future studies. In view of the conceptual strength of the work, I recommend that the degree of Ph.D. may be awarded to the candidate.

Prof. L. S. Shashidhara
Chair, Biology Division &
Dean, Research & Faculty
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Education & Research