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Coronary Artery Bypass Grafting

Synaptic Pruning in Alzheimer's Disease

Highlights

Relationship of Clinical Manifestations

Treatment of Upper and Lower Respiratory

Discovering Thoughts, Inventing Future

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Synaptic Pruning in Alzheimer's Disease: Role of the Complement System

By Frederic H. Brucato MA & Daniel E. Benjamin PhD

Introduction- Alzheimer's disease (AD) continues to threaten aged individuals and health care systems around the world. Human beings have been trying to postpone, reduce, or eliminate the primary risk factor for AD, aging, throughout history. Despite this, there is currently only symptomatic treatment for AD and this treatment is limited to only a handful of FDA approved AD drugs.

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Synaptic Pruning in Alzheimer's Disease: Role of the Complement System

Frederic H. Brucato MA [α] & Daniel E. Benjamin PhD [σ]

I. Introduction

Alzheimer's disease (AD) continues to threaten aged individuals and health care systems around the world. Human beings have been trying to postpone, reduce, or eliminate the primary risk factor for AD, aging, throughout history. Despite this, there is currently only symptomatic treatment for AD and this treatment is limited to only a handful of FDA approved AD drugs.

This review will cover:

- AD epidemiology
- Current FDA approved drugs (treat symptomology)
- Mild Cognitive Impairment
- The transition process from MCI to AD
- Genetic and Biologic Markers in AD
- New targets
- The role of neuroinflammation in AD
- Factors and systems that influence inflammation including the complement system
- Complement system's direct involvement in AD including a role in Beta-Amyloid and Tau Pathology
- Complement inhibition in AD modulation and prevention

One in ten people older than 65 currently has AD induced dementia. Recent estimates are that AD will grow to greater than 16 million individuals by the year 2050 [1][2]. There are many risk factors for AD, but clearly, age is the strongest predictor. In 2017 approximately 6.1 Americans had AD or a form of mild cognitive impairment (MCI) that is likely to progress to AD. Also, in 2017, based on amyloidosis, neurodegeneration, or both, 46.7 million Americans exhibited preclinical signs of AD [2]. According to ClinicalTrials.gov, in 2019, there were 2231 trials for Alzheimer's disease that were either recruiting, under way, terminated, or completed. Unfortunately, we still lack drugs that can modify the course of Alzheimer's disease [3].

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The only FDA approved drugs; donepezil, galanthamine, rivastigmine (acetylcholinesterase inhibitors) memantine (N-MDA antagonist), and donepezil/memantine (acetylcholinesterase inhibitor combined with N-MDA antagonist) demonstrate varying efficacy only with symptomatic management. There are recent advances and potential breakthroughs that need more validation but may become significant [4]. A recently discovered APOE double mutation may yield insight into the mechanism of AD. However, this promise remains years away. [5] The current standards of care rely on acetylcholinesterase inhibitors, N-MDA blockers, or a combination of the two. In the 1980's, a loss of cholinergic neurons in the Nucleus Basalis of Meynert region of Alzheimer's brains gave rise to the cholinergic hypothesis. Subsequently, an accumulation of beta-amyloid protein (Aβ), especially in the hippocampus, gave rise to the beta-amyloid hypothesis. This suggested the accumulation of Aβ in the brain was the primary cause of AD. The tau hypothesis followed, which presumed that intracellular accumulations of tau, creating spindle fibers, also contributed to AD [6]. Now the picture is more complicated, but beta -amyloid and tau remain important aspects of AD. The pressure to develop-disease modifying drugs and cures for AD drugs continues to increase [7].

In order to develop effective disease modifying drugs, it is important to better understand: 1) Alzheimer's disease mechanisms such as generation and clearance of beta amyloid, p-tau, the role of APOE4, synaptic maintenance and synaptic elimination or pruning as well as and many other mechanisms 2) biomarkers that identify, predict and /or track progression of AD 3) biological systems that play a role in day-to-day homeostasis and health but also play a role in Alzheimer's disease and 4) the relationship between Mild Cognitive Impairment (MCI) and AD. What causes the progression of MCI to AD, and what prevents the progression? This is a fundamental question we will try to address in this review. The cost of Alzheimer's drug discovery and drug development is substantial. Total national cost of caring for those with Alzheimer's and other dementias is estimated at $277 billion (not including unpaid caregiving) in 2018, of which $186 billion is the cost to Medicare and Medicaid; out-of-pocket costs represent $60 billion of the total payments, while other costs total $30 billion. [8].

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Drugs that have been approved for a different indication may be repurposed for Alzheimer's disease. are highly attractive. These may include medications that are structurally or functionally related to compounds that already have passed phase I safety trials.

II. Complement System in Alzheimer's Disease

There has been much recent interest in the complement system's role in AD. The complement system facilitates the immune system' response to destroy and remove foreign pathogens. It also appears to influence beta-amyloid, tau, and APOE4 interaction in AD [9] [10]). (For complement system review see Fritzinger and Benjamin (2016) [11].

Complement system activation is a precise process, controlled by regulatory proteins found in both plasma and at host cells' surfaces. C3 protein plays a major role in complement activation and control of immune responses. Deficiencies of C3 and so-called early and late complement proteins contribute to the emergence of recurrent bacterial, viral, and fungal infections. Importantly, mannose-binding lectin occurs at low levels. This protein plays a protective role in the early stages of infection as well as controlling inflammation. C3 deficiency is a common cause of human immunodeficiency, observed in microbial infections and autoimmune diseases such as rheumatoid arthritis. However, excessive activation of complement proteins has now been linked to Alzheimer's disease [12], autoimmune diseases, schizophrenia, atypical hemolytic-uremic syndrome, angioedema, macular degeneration, and Crohn's disease [13].

In the case of multiple sclerosis, inflammation is tightly linked with neurodegeneration, and it is the accumulating neurodegeneration that underlies increasing neurological disability in progressive multiple sclerosis (MS). Complement expression can be evaluated by immunocytochemistry and, in situ hybridization causes expression of the transcript for C1qA in neurons and the activation fragment and opsonin C3b-labelled neurons and glia in the MS cortical and deep grey matter. A recent study by Watkins et al. (2016) [14] demonstrated the density of immunostained cells positive for the classical complement pathway protein C1q and the alternative complement pathway activation fragment Bb was significantly increased in cortical grey matter lesions compared to control grey matter. Cells immunostained for the membrane attack complex (MAC) were elevated in cortical lesions, indicating complement activation to completion. Classical (C1-inhibitor) and alternative (factor H) pathway regulator-positive cells were unchanged between MS and controls. Complement anaphylatoxin receptor-bearing microglia in the MS cortex were closely opposed to cortical neurons [14].

Complement immune positive neuron morphology reflects cell stress/damage, suggesting significant neurodegeneration in cortical grey matter lesions. Thus, complement appears activated in MS cortical grey matter lesions where increased complement receptor-positive microglia were found.

The finding that complement proteins are abundant and can play pathological roles in neurological conditions offers potential for therapeutic intervention. Accordingly, frequent studies have explored unique activation pathways, proteases, receptors, complexes, and natural inhibitors of complement to mitigate pathology in acute neurotrauma and chronic neurodegenerative diseases. Brennan et al. (2016) reviewed recent studies that discussed the mechanisms of complement activation in the central nervous system (CNS), and the effects of complement inhibition in cerebral ischemic-reperfusion injury, traumatic brain injury, spinal cord injury, Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease and Huntington's disease [15]. The authors of this particular review provide perspectives on how promising complement-targeted therapeutics could become part of novel and effective future treatment options [15]. In the rat, a single intracerebroventricular injection of neuraminidase from Clostridium perfringens induces ependymal detachment and death. The neuraminidase study implicates critical involvement of the complement system. In this study, complement activation, triggered by neuraminidase, was analyzed by Western blot. Primary cultures of ependymal cells and explants of the septal ventricular wall were assessed in vitro. In these models, ependymal cells were exposed to neuraminidase in the presence or absence of complement, and their viability was assessed by observing cilia or by trypan blue staining. The role of complement in neuraminidase induced ependymal damage was analyzed in vivo in two rat models of complement blockade: systemic inhibition of a C5 blocking antibody and testing in C6-deficient rats [15].

Injecting rats intracerebroventricularly with neuraminidase causes complement membrane attack complex (MAC) to immunolocalize on the ependymal surface [16]. C3 activation fragments were found in serum and cerebrospinal fluid of rats treated with neuraminidase, suggesting that neuraminidase itself activates complement. In ventricular wall explants and isolated ependymal cells, treatment with neuraminidase alone induced ependymal cell death; however, the addition of complement caused increased cell death and disorganization of the ependymal epithelium. Granados-Durán and colleagues (2016) [16] treated rats with anti-C5 or used C6-deficient rats, with intracerebroventricular injection of neuraminidase that resulted in reduced ependymal alterations, compared to

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non-treated or control rats. Immunohistochemistry confirmed the absence of membrane attack complex on the ependymal surfaces of neuraminidase-exposed rats treated with anti-C5 or deficient in C6.

The authors concluded these results demonstrate that the complement system contributes to ependymal damage and death caused by neuraminidase. However, neuraminidase alone can induce moderate ependymal damage without the aid of complement [15] [16].

It also appears that mitochondrial function and neuroinflammation are related. Neuroinflammation causes over-activation of microglia, which in turn causes increases in pro-inflammatory cytokines, processes that are hallmarks of AD [17] [18] [12]. Complement's role in normal brain development and neuropathology has not been traditionally appreciated. Complement protects the host against infection. Thus, improved function was not always predicted. Complement has been implicated in depression, epilepsy, demyelination and dementia. Complement's role in inflammation is complicated, with respect to these diseases. Activation of complement pathways may actually accentuate development of AD, bringing into focus the possibility that complement inhibition may be a viable approach to AD treatment [9] [12]. Complement's role in modulating synapse density in AD may also be a critical part of disease progression. C3 deficient mice apparently are unable to remove synapses from damaged neurons as efficiently as control mice. Reducing C3 in mice increased numbers of synapses, and improved cognitive performance according to Berg et al. (2012) [19]. While this result is somewhat counterintuitive, because C3 deficient mice don't clear or eliminate damaged neurons, it is supported by the fact that the known age-associated synaptic loss in hippocampus is also reduced in C3 deficient mice. Reducing age-associated synaptic loss is associated with better learning and memory. The implication clearly becomes complement may work against robust synaptic health in aging and may play a critical role in AD. To take this thought one step further, complement inhibition could be a viable strategy for aging and AD. Axotomized spinal motoneurons lacking C3 caused reduced removal of synaptic terminals, suggesting an important role for C3 in AD [19].

In addition, mice lacking C3 showed altered cognitive performance and synaptic function in the hippocampus. [20]. Similarly, mice lacking C3 do not show typical age-related hippocampal decline [21].

Complement C7 appears to have a role as a novel gene in AD. In a recent study by Zhang et al., (2019) [22], whole-exome sequencing of Han Chinese patients with familial and/or early-onset Alzheimer's disease was conducted. The exome was independently validated, imaged and characterized. Investigators identified an exome-wide significant rare missense variant rs3792646 (p.K420Q) in the C7 gene. Investigators validated the association in different cohorts and a combined sample (1615 cases and 2832 controls). The risk allele was associated with reduced hippocampal volume and impaired working memory performance younger adults. This risk allele may be associated with early onset AD. Overexpression of p.K420Q altered cell viability, activation of the immune system and affected β -amyloid processing. The mutant p.K420Q inhibited excitatory synaptic transmission in pyramidal cells, in an electrophysiogical assay. This result further supports the idea that C7 is a novel risk gene in AD in the Han Chinese population [22].

Accumulated beta-amyloid peptides in AD brains activate the classical C pathway by binding to a collagen-like domain (CLF) within C1q. This pathway is activated by synthetic analogues of beta-amyloid peptides, beta 1-42 and beta 1-40, bound to C1q. Beta 1-42 bound more effectively to C1q than beta 1-40. C pathway activation impacted beta1-42 more so than did beta 1-40. This C-activating capacity appears correlated with the assembly of the beta 1-42 into aggregates and/or macromolecular fibrils. While these studies are not recent, they are important to mention because they helped establish the connection between beta amyloid, inflammation and complement, especially the classical C pathway [23].

Beta-amyloid peptide is cleared from periphery by a complement-mediated mechanism that appears to be deficient in Alzheimer's disease. The mechanism should be enhanced by beta-amyloid antibodies that form immune complexes (ICs) with Aβ and therefore may be relevant to current beta-amyloid immunotherapy approaches. Targeting peripheral mechanisms that may facilitate beta-amyloid clearance has the potential as immunotherapy to treat Alzheimer's disease [24].

Alzheimer's disease appears to be associated with brain inflammation. Activated microglia are associated with brain lesions, which in turn, may also be involved with brain inflammation. Anti-inflammatory treatment may protect against AD, possibly through beta-amyloid mediated activation of the complement system. The activated complement system has anti-inflammatory properties [25] and is highly involved in immune system homeostasis [26].

It is known that complement protein C5a binds to and inhibits the receptor C5aR1. C5a inhibits pathology and AD cognitive deficits in AD mouse models. However, to be sure C5a acts via C5aR1 inhibition, C5aR1 deficient mice were generated, and compared to wild type mice plaque load and behavioral assessments such as novel object recognition (NOR), hpc dependent and independent versions and object location memory (OLM), hpc dependent. [27]

It is known that C5aR1 is expressed primarily on myeloid lineage cells, secondarily on brain and endothelial cells. Thus, gene expression was compared at 2, 5 ,7, and 10 months, across each genotype. [27]

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As stated, to accomplish this, C5aR1 knockout mice were crossed to the Arctic AD mouse. Hernandez et al. (2017) [27] found C5aR1 deficient mice did not show behavior deficits at 10 months, although amyloid plaque load was not altered. Of interest, there were no CCR2+ monocytes/macrophages near the plaques in the Arctic brain with or without C5aR1. To underscore this finding, the Arctic C5aR1KO mice showed a reduction of hippocampal neuron complexity and improved behavioral deficit. [27]

RNA-sequence analysis showed inflammation related genes were differentially expressed and expression was increased in the Arctic mice relative to wild type. Expression was decreased in the Arctic/C5aR1KO relative to Arctic. In addition, phagosomal-lysosomal gene expression was increased in the Arctic mice relative to wild-type but this increase was even more prominent in the Arctic/C5aR1KO mice. In the Artic mice, aging was associated with reduced neuronal hippocampal complexity. This effect at 10 months was correlated to the observed behavioral deficit. The reduction of neuronal complexity in hippocampus and the behavioral deficit were both rescued in Arctic/C5aR1KO.

Neurofibrillary tangles aggregated from hyperphosphorylated tau protein cause significant pathology in AD. Complement C3 (or C3a) is linked to AD pathology. An important question is whether C3a or the C3a receptor is specifically tied to tau phosphorylation. In a recent study by Hu et al. (2019) [28], investigators found that exposing SH-SY5Y cells to okadaic acid (OA) decreased cell viability and induced tau hyperphosphorylation. The C3a receptor antagonist SB290157 blocked these effects. In addition, SB290157 blocked the action of glycogen synthase kinase 3β (GSK3β) but did not affect protein phosphatase 2A C subunit (PP2Ac) and cyclin-dependent kinases 5 (CDK5). The authors concluded findings indicate the unique role C3a receptor plays in regulating tau phosphorylation via GSK3β signaling pathways and highlight C3a receptor as a potential target for treating AD [28].

Complement pathway overactivation can lead to neuronal damage in various neurological diseases. Although AD is characterized by β-amyloid plaques and tau tangles, previous work examining complement has largely focused on amyloidosis models. Wu et al. (2019) [29] find glial cells show increased expression of classical complement components and the central component C3 in mouse models of amyloidosis (PS2APP). More pronounced is tauopathy (TauP301S). Blocking complement function by deleting C3 rescues plaque-associated synapse loss in PS2APP mice and reduces neuron loss and brain atrophy in TauP301S mice. These changes were confirmed by improved neurophysiological and behavioral measurements. The authors state, C3 protein is elevated in AD patient brains, including at synapses. Processing of C3 is increased in AD patient CSF and correlate with tau. These results demonstrate that complement activation contributes to neurodegeneration caused by tau pathology and suggest that blocking C3 function might be protective in AD and other tauopathies [29].

Synapse loss and Tau pathology are hallmarks of Alzheimer's disease (AD) and other tauopathies, but how Tau pathology causes synapse loss is unclear. This study used unbiased proteomic analysis of postsynaptic densities (PSDs) in Tau- P301S transgenic mice to identify Tau-dependent alterations in synapses prior to overt neurodegeneration. Multiple proteins and pathways were altered in Tau- P301S PSDs, including depletion of a set of GTPase-regulatory proteins that leads to actin cytoskeletal defects and loss of dendritic spines. A striking accumulation of complement C1q in the PSDs of Tau-P301S mice and AD patients was observed. At synapses, C1q perisynaptic membranes accumulated in correlation with phospho-Tau and was associated with augmented microglial engulfment of synapses and decline of synapse density. A C1q-blocking antibody inhibited microglial synapse removal in cultured neurons and in Tau-P301S mice, rescuing synapse density. Thus, inhibiting complement-mediated synapse removal by microglia could be a potential therapeutic target for Tau-associated neurodegeneration [30].

III. Genetic and Biological Markers in Alzheimer's Disease

Evidence indicates the APPSwDI/Nos2-/- (CVN-AD) mouse model replicates multiple AD pathologies [31]. Badea et al. (2016) identified multivariate biomarkers that appear to predict cognitive decline. One of these biomarkers is the fornix. In vivo and ex vivo magnetic resonance imaging (MRI) reveals CVN-AD mice replicate the hippocampal atrophy (6%), characteristic of human AD. It has been shown the fornix is 23% smaller in these mice. This is important anatomically because the fornix connects the septum, hippocampus, and hypothalamus. Ultrastructural analysis has shown the fornix has reduced axonal density (47% fewer), axonal degeneration (13% larger axons), and abnormal myelination (1.5% smaller g-ratios) in these mice. CD68 staining showed that white matter pathology might not be the cause, instead could be secondary to neuronal degeneration. Alternatively, the authors state it could be due to direct microglial attack. Thus, the fornix provides multiple biomarkers to characterize circuit disruption in a mouse model of Alzheimer's disease [31]. Deposition of tau and beta-amyloid in the brain yields biomarkers that may be valuable. The core cerebrospinal fluid (CSF) AD biomarkers amyloid peptide 1–42 (Aβ 1–42), total tau (t-tau) and phosphorylated tau 181 (p-tau 181) show good

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diagnostic sensitivity and specificity. [32] Regardless, more biomarkers that can help preclinical diagnosis or facilitate tracking disease progression are needed. Activation of the complement system, occurs at very early stages in the AD brain. Therefore, CSF levels of complement proteins could be linked to cognitive and structural changes in AD and may provide diagnostic and prognostic value.[32] xMAP® technology has been used to measure complement 3 (C3) and factor H (FH) in the CSF of human controls (CN), mild cognitive impairment (MCI) and AD subjects of the AD Neuroimaging Initiative (ADNI). [32] The association between CSF biomarkers and different outcome measures were analyzed using Cox proportional hazard models (conversion from MCI to AD), logistic regression models (classification of clinical groups) and mixed-effects models adjusted for age, gender, education, t-tau/Beta-Amyloid1–42 and APOE 4 presence (baseline and longitudinal association between biomarkers and cognitive scores). Although no association was found between the complement proteins and clinical diagnosis or cognitive measures in this particular study, lower levels of C3 and FH were associated with faster cognitive decline in MCI subjects as measured by the AD Assessment Scale-cognitive subscale (ADAS-Cog) test according to study authors. FH levels were associated with larger lateral ventricular volume ($p = 0.024$), indicating potential brain atrophy. Toledo et al. (2014) conclude C3 and FH are not good diagnostic biomarkers of AD but might have modest potential as prognostic biomarkers and therapeutic targets in cognitively impaired patients. Low levels of cerebrospinal fluid complement 3 and factor H predict faster cognitive decline in mild cognitive impairment [32]. Patients diagnosed with MCI may exhibit significant behavioral and psychological signs and symptoms (BPS), symptoms also frequently observed in patients with Alzheimer's disease (AD). A recent study by Pocnet et al (2015) [33] evaluated the extent and variability of BPS in MCI vs AD, with the intent of providing an additional marker that may predict conversion from MCI to AD. Global cognitive performance, BPS, and ADL were assessed using validated clinical methods at baseline and at two-year follow-up in 46 MCI patients, 54 AD subjects and 64 controls. The BPS variability over the follow-up period was more pronounced in the MCI group than in patients with AD: some BPS improved, others occur developed or worsened, while others still remain unchanged.[33] Changes in BPS were associated with rapid deterioration of the global cognitive level in MCI patients. In particular, an increase of euphoria, eating disorders, and aberrant motor behavior, as well as worsened sleep quality, predicted a decline in cognitive functioning. Results from this study confirm MCI patients have a higher variability of BPS over time compared to AD patients. In addition, there is evidence of associations between specific BPS and cognitive decline in the MCI group associated with a potential risk of conversion for individuals with amnestic MCI to AD [33].

Another study, which evaluated potential novel protein biomarkers for MCI progression to AD, found that Chromogranin A, secretogranin II, neurexin 3, and neuropentraxin 1 were elevated in MCI patients and MCI patients progressing towards AD. Duits and colleagues (2018) [34] concluded that these proteins which are involved in vesicular transport and synaptic stability may participate in early phases of the AD pathophysiological cascade [34]. Cognitive and functional decline in beta-amyloid positive preclinical AD patients has been compared to prodromal AD subjects (beta-amyloid positive, MCI) patients. These subjects were compared to MCI patients with no existing Beta-amyloid status. Patients were followed for an average of 4 years, and a maximum of 10 years. Preclinical AD subjects showed steeper declines in brain metabolism than beta-amyloid negative progressors. Insel et al. (2017) [35] found preclinical AD subjects also showed elevated rates of white matter hyperintensity and increased CSF phosphorylated tau levels at baseline. In this particular study, Aβ-negative progressors displayed greater baseline frequency of depressive symptoms. [35].

Evidence of blood-based biomarkers for cognitive decline in aging, (MCI) and (AD) has been sparse. Cumulative evidence suggests that apolipoproteins, complement system, and transthyretin are involved in AD pathogenesis by sequestration of beta amyloid. However, no clinical study assesses the utility of "sequester proteins" in risk assessment and/or diagnosis of MCI and AD.

Serum levels of sequester proteins and their clinical potential in cognitive decline assessment were analyzed by a recent longitudinal and cross-sectional study by Uchida and colleagues [36]. A combination of apolipoprotein A1, complement C3, and transthyretin appear to be involved in AD possibly by sequestration of beta-amyloid. These proteins also appear to differentiate MCI subjects from healthy controls. The authors conclude a set of sequester proteins could be blood-based biomarkers for assessment of early stages of cognitive decline [36].

Previous studies have shown that beta amyloid peptide (AB) is cleared by a complement-mediated process from the peripheral circulation, and that this process is deficient in Alzheimer's disease. The process may be enhanced by beta-amyloid antibodies that form immune complexes (ICs) with beta amyloid. In turn, providing improvements to current beta amyloid immunotherapy approaches. Recent studies demonstrated complement-mediated capture of beta amyloid-antibody immune complexes compared with beta-amyloid alone in both erythrocytes and THP1-derived macrophages. [24] Beta amyloid antibodies dramatically increased complement activation and

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opsonization of beta amyloid followed by enhanced beta amyloid capture by human erythrocytes and macrophages. The present study strongly suggests that peripheral mechanisms are relevant to beta amyloid immunotherapy. Findings are also consistent with enhanced peripheral clearance of intravenously administered beta-amyloid antibody immune complexes in nonhuman primates. [24].

IV. MCI Conversion to AD

Alzheimer's disease (AD) is characterized by the deposition of tau and amyloid in the brain. While core cerebrospinal fluid (CSF) AD biomarkers beta amyloid peptide 1–42, total tau (t-tau) and phosphorylated tau 181 (p-tau 181) show good diagnostic sensitivity and specificity, these biomarkers alone don't adequately address preclinical diagnosis or disease progression. Complement system-initiated inflammation occurs at very early stages in the AD brain. Therefore, complement proteins found in CSF, could be linked to cognitive and structural changes in AD and may have diagnostic and prognostic value.

As stated previously, Toledo et al. (2014) determined compliment factors 3 & H may not be suitable markers for identifying AD, but these factors may potentially predict rapidity of MCI individuals cognitive decline [37].

MCI represents an early stage of developing cognitive impairment, however there is some consensus that not all MCI patients necessarily progress to AD. Patients diagnosed with MCI do not meet the criteria for dementia as their cognitive abilities and activity levels exceed those of demented individuals. Minor changes in instrumental activities of daily living (ADL) may occur. In some cases, they may exhibit significant behavioral and psychological signs and symptoms (BPS), also frequently observed in patients with Alzheimer's disease (AD). In this study, investigators evaluated the extent to which specific BPS are associated with cognitive decline in participants with MCI or AD. 164 participants were categorized; 46 patients with amnestic (single or multi-domain) MCI, 54 patients with AD, and 64 control participants without cognitive disorders. Global cognitive performance, BPS, and ADL were assessed using validated clinical methods at baseline and at two-year follow-up. The BPS variability over the follow-up period was more pronounced in the MCI group than in patients with AD: some BPS improve, others occur newly or worsen, while others still remain unchanged. Moreover, specific changes in BPS were associated with a rapid deterioration of the global cognitive level in MCI patients. In particular, an increase of euphoria, eating disorders, and aberrant motor behavior, as well as worsened sleep quality, predicted a decline in cognitive functioning.

Findings confirm a higher variability of BPS over time in the MCI group than in AD patients. Pocnet and colleagues [33] state this could be due to differences in baselines as some in the MCI group may have been only marginally impaired. Results provide evidence of associations between specific BPS and cognitive decline in the MCI group that might suggest a risk of conversion of individuals with amnestic MCI to AD [33].

Identification of specific tests providing a high certainty for stable MCI and factors that precipitate instability of MCI could provide greater sensitivity towards detecting and following progression of AD [38].

Ellendt and colleagues (2016) tested 130 participants annually using a test battery that included measures of memory, language, executive functions, intelligence and dementia screening tests. Exclusion criteria at baseline included severe cognitive deficits such as diagnosis of dementia, psychiatric or neurological disease. Regression and Receiver Operating Characteristic (ROC) curve analysis was used to identify potential predictors for stability or instability of MCI-diagnosis. Age, IQ and APOE status were evaluated using of test performance tests and group membership. MCI (49%) was observed at baseline with a reversion rate of 18% after two years. Stability of MCI was related to (VLMT: delayed recall, CERAD: recall drawings, CERAD: Boston Naming Test, Benton Visual Retention Test: number of mistakes). Conversion to MCI is associated with language functions. Reversion to 'normal' was primarily predicted by single domain impairment. There was no significant influence of variables such as demographic, medical or genetic. The results of this study underscore the role of repeated measurements of functional neuropsychological predictors and the need for better diagnostic reliability. In cases of high uncertainty, close monitoring over time is mandatory to more closely estimate outcome. [38]

In another study attempting to define the relationship between MCI and AD, investigators developed a multivariate model for predicting MCI-to-dementia progression at the individual patient level. [39] Using baseline data from 259 MCI patients and a probabilistic pattern classification approach, Korolev et. al. (2016) trained a classifier to distinguish between patients who did or did not progress to AD-type dementia during over a three-year period. More than 750 variables across four data sources were evaluated as potential progression predictors. Data included risk factors, cognitive and functional assessments, structural magnetic resonance imaging (MRI) data, and plasma proteomic data. Predictive utility was cross validated.

Cognitive and functional markers most strongly predicted progression while plasma proteomic markers did not predict as well [39]. The best performing model predicted at 80% using a combination of cognitive/functional markers and morphometric MRI measures. Predictors of progression included scores on the Alzheimer's Disease Assessment Scale, Rey Auditory Verbal Learning Test, and Functional Activities

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Questionnaire, as well as volume/cortical thickness of three brain regions (left hippocampus, middle temporal gyrus, and inferior parietal cortex). The study authors state calibration analysis revealed that the model is capable of generating probabilistic predictions that reliably reflect the actual risk of progression. Finally, the authors found that the predictive accuracy of the model varied with patient demographic, genetic, and clinical characteristics and could be further improved by taking into account the confidence of the predictions. In this case, we see the development of an accurate prognostic model for predicting MCI-to-dementia progression over a three-year period. The model utilizes available, cost-effective, non-invasive markers and can be used to improve patient selection in clinical trials and identify high-risk MCI patients for early treatment [39].

Microarray screening in human dentate gyrus, using entorhinal cortex expression levels, has been used to differentiate age-related memory loss from AD. Using this technique, Kandle and colleagues (2013) have shown an aged related decline in the histone acetylation regulatory molecule RbAp48. This deficiency occurs in both human and mice that age normally [40]. This provides more evidence that MCI may not be simply early stage AD and may not convert to AD in all cases, even if given enough time.

Morgan et. al (2019) reviewed 53 plasma proteins obtained from control, MCI and AD groups [41]. Ten of these showed significant differences between groups. Using pairwise comparisons of AD vs CTL, they found increased C4 and eotaxin-1, decreased sCR1, C5, and CRP and for MCI vs CTL they found increased FH, C3, and MCP-1, decreased C5 and MIP-1b. For the AD vs MCI comparison, they found increased eotaxin-1 and MIP-1b, decreased FI, C3, CRP, MCP-1. These findings increase the knowledge about potentially useful biomarkers that may predict conversion to MCI and AD.

Another recent study by Helgadottir et al. (2019) evaluated CD55 and its upstream transcription factors in the temporal cortex of a Late Onset Alzheimer's disease (LOAD) patient compared to an early onset (EOAD) patient [42]. To date, sequencing has focused primarily on germline mutations. Improved technology has created opportunities to study somatic mutations in brain tissue that shows pathology. This current study used ultra-deep sequencing on brain and blood from early-onset AD (EOAD) and late-onset AD (LOAD) patients and non-AD individuals (n = 16). 2.86 Mb of genomic areas that have been associated with AD, were targeted. This included 28 genes and upstream and downstream regulatory areas. Bioinformatics filtering identified 11 somatic single nucleotide variants in temporal cortex of AD patients. In contrast, there were none in the controls. In a LOAD patient, one variant was present at 0.4% allele frequency in temporal cortex. This variant was predicted to affect transcription factor binding sites upstream of the CD55 gene, contributing to AD pathogenesis by affecting the complement system. These results suggest that future studies targeting larger portions of the genome may increase understanding for the molecular basis of both EOAD and LOAD [42].

Another recent study by Han and colleagues (2018) tying the complement system to AD was designed to identify and characterize novel AD drug target genes. This study employed a combinatorial approach for the first time to discover AD drug targets. Investigators did this by considering ontology inference and network analysis. Potential AD drug target genes were discovered by integrating information from multiple popular databases (TTD, Drug Bank, Pharm GKB, AlzGene, and BioGRID). Enrichment analyses of the identified drug targets genes based on nine well-known pathway-related databases were conducted.

Eighteen potential drug target genes were identified, and thirteen of them had been reported to be closely associated with AD. Enrichment analyses of these identified drug target genes, based on nine pathway-related databases, revealed that four of those identified drug target genes are involved in the classical complement pathway and the process of presenting antigens [43].

Results suggested the combinatorial approach, and the remaining five new targets could enrich our understanding of AD pathogenesis and drug discovery. Moreover, this study supported validity of the combinatorial approach integrating ontology inference with network analysis in the discovery of novel drug target for neurological diseases [43].

V. Microglia, Astrocytes and Mitochondria

Cyclophilin D (CypD) is a mitochondria-specific cyclophilin that plays a pivotal role in the formation of the mitochondrial permeability transition pore (mPTP). The formation and opening of the mPTP disrupts mitochondrial homeostasis, causes mitochondrial dysfunction and eventually leads to cell death. Several recent studies have found that CypD promotes the formation of the mPTP upon binding to Aβ peptides inside brain mitochondria, suggesting that neuronal CypD has a potential to be a promising therapeutic target for Alzheimer's disease [44].

In this study, researchers generated an energy-based pharmacophore model by using the crystal structure of CypD-cyclosporine A (CsA) complex and performed virtual screening of the ChemDiv database, which yielded forty-five potential hit compounds with novel scaffolds. Investigators tested compounds using mitochondrial functional assays in neuronal cells and identified fifteen compounds with excellent protective effects against Aβ-induced mitochondrial dysfunction. To validate whether these effects were derived from

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binding to CypD, surface plasmon resonance (SPR)-based direct binding assays with selected compounds were done. Investigators discovered compound 29 was found to have the equilibrium dissociation constants (KD) value of 88.2 nM. This binding affinity value and biological activity corresponds to the predicted binding mode. The authors conclude that this study offers new insights into the rational design of small molecule CypD inhibitors and provides a promising lead for future therapeutic development [44].

In addition to amyloid-beta plaque and tau neurofibrillary tangle deposition, neuroinflammation is considered a key feature of Alzheimer's disease pathology. Inflammation in Alzheimer's disease is characterized by the presence of reactive astrocytes and activated microglia surrounding amyloid plaques, implicating their role in disease pathogenesis. Microglia in the healthy adult mouse depends on colony-stimulating factor 1 receptor (CSF1R) signaling for survival, and pharmacological inhibition of this receptor results in rapid elimination of nearly all of the microglia in the central nervous system. In this study by Spangenberg & colleagues (2016) [45], investigators wished to determine if chronically activated microglia in the Alzheimer's disease brain are also dependent on CSF1R signaling, and if so, how microglial cells contribute to disease pathogenesis. Ten-month-old 5xfAD mice were treated with a selective CSF1R inhibitor for 1 month. This resulted in elimination of approximately 80% of microglia. Chronic microglial elimination did not alter amyloid-beta levels or plaque load; however, elimination did reduce dendritic spine loss and prevent neuronal loss in 5xfAD mice, as well as reduce overall neuroinflammation. Importantly, behavioral testing revealed improvements in contextual memory. Collectively, these results demonstrate that microglia contribute to neuronal loss, as well as memory impairments in 5xfAD mice, but do not mediate or protect from amyloid pathology [45]. Activated microglia are classified into two specific states: classically activated (M1) and alternatively activated (M2) subtypes. Polarization of M1/M2 phenotype plays an important role in Alzheimer's disease (AD). However, the mechanisms regulating this process remain unclear. In this study, investigators tried to determine the role of milk fat globule epidermal growth factor 8 (MFG-E8). MFG-E8 is a unique protein which can bind to microglia and regulate its inflammatory responses. It is speculated that MFG-E8 may play a role in the balance of microglial polarization. Here, fibril amyloid beta-42 was used in vitro to stimulate mouse primary microglial cultures. Study authors found M1 marker expression, along with retained M2 marker production. It was determined that MFG-E8 pretreatment reversed the increased trend of M1 markers and the decreased expression of M2 markers, which were induced by Aβ 42. Moreover, MFG-E8 effects could be effectively blocked by an MFG E8 antibody. Further analysis on the signaling pathways showed that NF-"B upregulation and Akt downregulation in microglial cultures were observed after Aβ 42 incubation. The alteration of these pathways could also be reversed by MFG-E8 [45]. Next, investigators evaluated the effects of NF-"B and PI3K-Akt on M1/M2 alteration using their specific inhibitors. Pyrrolidine dithiocarbamate, a NF-"B inhibitor, inhibited M1 marker expression; moreover, LY294002, an Akt inhibitor, enhanced M1 marker expression. It appears MFG-E8 plays a regulatory role of microglia M1/M2 alteration providing a basis for understanding the potential role of microglia activation in AD [46].

Recent genetic evidence from Czirr et al. (2017) [47] supports a link between microglia and the complement system in Alzheimer's disease (AD). Here, investigators uncovered a novel role for the microglial complement receptor 3 (CR3) in the regulation of soluble beta-amyloid clearance, independent of phagocytosis. Unexpectedly, ablation of CR3 in human amyloid precursor protein-transgenic mice results in decreased, rather than increased, beta-amyloid accumulation. In line with these findings, cultured microglia lacking CR3 are more efficient than wild type at degrading extracellular beta amyloid by secreting enzymatic factors, including tissue plasminogen activator. Furthermore, in-vivo microdialysis showed a small molecule modulator of CR3 reduces soluble Aβ levels and Aβ half-life in brain interstitial fluid (ISF), These results suggest that CR3 limits beta amyloid clearance from the (ISF) illustrating a novel role for CR3 and microglia in brain Aβ metabolism and defining a potential new therapeutic target in AD [47].

Neuroinflammation is clearly associated with AD pathology, however its role in disease progression is unclear. The authors that review this topic state evidence suggests that Aβ complexes interact with microglial and astrocytic pattern recognition receptors that initiate immunity. This process involves secretion of pro-inflammatory cytokines, chemokines and generation of reactive oxygen species that, in excess, drive a dysregulated immune response that contributes to neurodegeneration. A neuroinflammatory response involving microglial activity, enhanced astrocyte reactivity and elevated pro-inflammatory cytokine and chemokine load has long been implicated in AD and proposed to facilitate neurodegeneration. [48].

Inflammatory components related to AD neuroinflammation include brain microglia and astrocytes, the complement system, as well as cytokines and chemokines. Cytokines play a key role in inflammatory and anti-inflammatory processes in AD. An important factor initiating the inflammatory process is the overexpression of interleukin (IL)-1. Other important cytokines in neuroinflammation are IL-6 and tumor necrosis factor (TNF)-α. By contrast, other cytokines such as IL-1 receptor antagonist (IL-1ra), IL-4, IL-10, and

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transforming growth factor (TGF)-β can suppress both proinflammatory cytokine production and their action, subsequently protecting the brain. Treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) decreases AD risk. according to basic research findings. Unfortunately, clinical trials of NSAIDs in AD patients have not provided much insight. Proinflammatory responses may be attenuated by polyphenols. Polyphenol supplementation may provide an alternative to treating Aβ [49].

Microglia modulate synaptogenesis and help repair damage from injury, by restoring neuronal connections. They also release cytokines, which in turn modulate synaptic transmission and potentially restore damaged dendritic spines. This suggests that the microglia could play a prominent role in learning and memory [50]. Thus, the compliment system appears to play many roles in the healthy and diseased brain. Recent evidence shows the complement system is involved with much more than just inflammation but appears to be is involved with regulation of synapse population, during development, normal aging and disease states such as Alzheimer's disease and schizophrenia. It has been shown that complement is directly involved with synapse tagging and elimination or synaptic pruning. In a mouse model of glaucoma, Stevens et al. (2007) demonstrated the complement protein C1q, is expressed by postnatal neurons in response to immature astrocytes [51 52].

The complement cascade provides protection from infection as well as destructive inflammation, as stated above. Complement participates in elimination of neuronal synapses which is essential for proper development. However, elimination of synapses can be detrimental during aging and disease. C1q, required for several of these complement-mediated activities. It is present in the neuropil, microglia, and a subset of interneurons in the brain. [53]

To identify the source(s) of C1q in the brain, investigators selectively inactivated the C1qa gene in the microglia or Thy-1+ neurons in both wild type mice and a mouse model of Alzheimer's disease (AD). C1q synthesis was assessed by immunohistochemistry, QPCR, and western blot analysis.

While C1q expression in the brain was unaffected after inactivation of C1qa in Thy-1+ neurons, the brains of C1qaFL/FL: Cx3cr1CreERT2 mice in which C1qa was ablated in microglia lacked C1q with the exception of limited C1q in subsets of interneurons. The study authors stated "this loss of C1q was surprising since it occurred even in the absence of tamoxifen by 1 month of age". This demonstrated that Cre activity is tamoxifen-independent in microglia in Cx3cr1CreERT2/WganJ mice. C1q expression in C1qaFL/FL: Cx3cr1CreERT2/WganJ mice continued to decline through aging and in AD model mice. No difference in C1q was detected in the liver or kidney from C1qaFL/FL: Cx3cr1CreERT2/WganJ mice relative to controls, and C1qaFL/FL: Cx3cr1CreERT2/WganJ mice had minimal, reduction in plasma C1q.

It was concluded that microglia, but not neurons or peripheral sources, are the dominant source of C1q in the brain [53]. While demonstrating that the Cx3cr1CreERT2/WganJ mice cannot be used for adult-induced deletion of microglia genes the model described enables further investigation of physiological roles of C1q in the brain and identification of therapeutic targets for the selective control of complement-mediated activities contributing to neurodegenerative disorders [53].

A prominent attribute of AD pathogenesis is neuroinflammation. Over-expression of complement proteins co-localizes with neurofibrillary tangles, thereby indicating that a complement system may be involved in neuroinflammation. The authors of the current study suggest this demonstrates, using a microglial cell line, complement activation influences neuroinflammation.

The authors determined the expression levels of the pro-inflammatory factor's TNF-α, IL-1β, and IL-6 and explored whether the neuroinflammatory response, caused by AB 42 treatment of BV-2 cells, was mediated by JAK/ STAT3 signaling [54].

C5a had an enhanced effect on the neural cell viability of BV-2 cells treated with Aβ42. In addition, C5a increased the Aβ-induced neuro-inflammatory response, and these effects were blocked by the C5aR antagonist, PMX205. The neuroinflammatory responses induced by Aβ and C5a were mediated through JAK/STAT3 signaling. By blocking this pathway with an antagonist, AG490, the expression of TNF-α, IL-1β, and IL-6 was also blocked. Thus, the complement protein C5a could exaggerate the Aβ-induced neuroinflammatory response in microglia. The study authors conclude C5aR may be a potential therapeutic tool for AD treatment.[54]

The neurogenic process, consisting of the proliferation, differentiation and maturation of neural stem cells (NSC), is regulated via epigenetic mechanisms by controlling the expression of specific sets of genes. This topic is reviewed by Li et al. (2016), [55]. They reiterate the pathology of AD, due to impairments in epigenetic mechanisms, the generation of neurons from NSCs is damaged, which exacerbates the loss of neurons and the deficits in learning and memory function associated with AD. Based on neurogenesis, a number of therapeutic strategies have shown capability in promoting neuronal generation to compensate for the neurons lost in AD, thereby improving cognitive function through epigenetic modifications. This provides potential for the treatment of AD by stimulating neurogenesis using epigenetic strategies. The epigenetics of AD and adult neurogenesis may provide therapeutic strategies for AD [55].

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Alzheimer's disease markers beta-amyloid plaques and neurofibrillary tangles composed of Aβ peptides and abnormally hyperphosphorylated tau protein are tightly correlated with AD. However, synaptic loss may be a better correlate of cognitive impairment in AD than beta-amyloid or tau pathologies. Thus, one strategy for AD is to shift the balance from neurodegeneration to neuroregeneration and synaptic repair. Kazim & Iqbal (2016) state "neurotrophic factors, by virtue of their neurogenic and neurotrophic activities, have potential for the treatment of AD". But therapeutic use of recombinant neurotrophic factors is limited because of the unfavorable pharmacokinetic properties, poor blood-brain barrier (BBB) permeability, and adverse effects. Neurotrophic factor small-molecule mimetics, offer a potential strategy to improve these limitations and have shown promise in preclinical studies. Neurotrophic factor small-molecule mimetics do show promise for AD drug development [56].

The ciliary neurotrophic factor (CNTF) small-molecule peptide mimetic, Peptide 021 (P021) has also received attention as a potential AD therapeutic. P021 is a neurogenic and neurotrophic compound which enhances dentate gyrus neurogenesis and memory processes via inhibiting leukemia inhibitory factor (LIF) signaling pathway and increasing brain-derived neurotrophic factor (BDNF) expression. It inhibits tau abnormal hyperphosphorylation by enhancing BDNF mediated decrease in glycogen synthase kinase-3 (GSK-3B, major tau kinase) activity. P021 is a small molecular weight, BBB permeable compound with suitable pharmacokinetics for oral administration. It also lacks adverse effects associated with the native CNTF or BDNF molecule. P021 has shown beneficial therapeutic effect in several preclinical studies and has emerged as a promising compound for AD drug development [56].

The practical pharmacogenetics of AD is limited to acetylcholinesterase inhibitors (AChEIs) and memantine. However, pharmacogenetic procedures should be applied to novel strategies in neurotransmitter regulators, anti-Aβ treatments, anti-tau treatments, pleiotropic products, epigenetic drugs and combination therapies. Over 60% of AD patients pathologies demand additional treatments which increase the likelihood of drug-drug interactions. Lipid metabolism dysfunction is common to AD neurodegeneration. The therapeutic response to hypolipidemic compounds is influenced by the APOE and CYP genotypes. It is paramount that the development of novel compounds and the use of combination/multifactorial treatments avoids adverse drug reactions and optimizes therapeutic potential [57].

There has been little success targeting the neurodegenerative aspect of AD. This failure has created interest in neuroregeneration and neural stem cells (NSCs) regeneration. Small molecules offer much potential to manipulate NSCs, and provides therapeutic tools that may prove very useful. Classically, these molecules have been generated either by target-based or phenotypic approaches. To circumvent specific liabilities, development of nanomedicines may offer a viable alternative.

Recent examples that could accelerate development of neuroregenerative drugs against Alzheimer's disease are reviewed by Uliassi et al. (2017) [58].

Novel approaches to AD therapy also include Rho-associated protein kinase (ROCK), a serine-threonine kinase originally identified as a crucial regulator factin cytoskeleton. Recent studies have defined ROCK as a critical component of diverse neuronal signaling pathways. Inhibition of ROCK causes several biological events such as increase of neurite outgrowth, axonal regeneration, and activation of prosurvival Akt. ROCK is a promising therapeutic target for the treatment of neurodegenerative disorders including Alzheimer disease, Parkinson's disease, Huntington's disease, multiple sclerosis, and amyotrophic lateral sclerosis [59].

VI. Synaptic Density, Tagging and Pruning

Complement protein C1q is localized to synapses in the postnatal CNS and the retina [51]. Importantly, C1q and (downstream) C3 deficient mice express major deficits in CNS synaptic elimination as shown by excess retinal innervation. C1q is localized to synapses during synaptic pruning that occurs in the developing retina and brain. Further experiments by the Steven's lab (Shi et al. 2015) on aged C3 deficient (KO) mice found an additional role of C3 for synaptic maintenance. In hippocampal region CA3, the investigators found aged wild type (WT) mice had showed synaptic loss while the C3 KO animals did not have such loss. In aged WT mice, synaptic loss in CA3 was followed by neuronal loss. Electrophysiology and behavior studies reinforce the loss of CA3 synapses in aged WT mice. [60] [21].

Steven's lab found, relative to aged WT animals, aged C3 KO mice exhibited enhanced LTP, suggesting greater synaptic activity and connectivity. In addition, aged WT mice differed in learning and memory as well as behavior. WT mice were more anxious than C3 KO mice on the plus maze. Finally, CA3 KO mice demonstrated better memory in the water T-maze than aged WT mice, but only upon a test of reversal learning. C3 KO mice, in addition, showed superior context fear conditioning memory, a hippocampal dependent task, during normal aging. In this study, it appears that complement protein C3 or any downstream signaling, may be harmful to synapses in specific brain regions [21].

It is well established that synapse loss is observed in Alzheimer's disease and corresponds to

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cognitive decline. In an Alzheimer's disease mouse model that incorporates beta-amyloid injections to model the disease, C1q is increased and is associated with synapses before amyloid plaques develop, [61]. Inhibition of C1q or C3 reduces both microglia and early synapse loss. In order for beta-amyloid to create toxicity, C1q must be present. Activation errors of the complement dependent pathway and microglia may mediate synapse loss in Alzheimer's disease. Additional studies have identified other complement proteins as mediators of synaptic elimination. Complement protein C4A and C4B have also been linked to synaptic elimination or pruning [62].

Increased complement protein C4 has been observed in previous studies. C4 has two isoforms which are encoded by genes C4A and C4B. AD patients tested for these isoforms, show increased C4A and C4B copy numbers and increased C4 protein expression. This observation suggests C4A and C4B may be possible risk factors for AD [63]. In addition to mediating synapse elimination in the developing CNS, C1q has also been found to increase dramatically (300-fold) in the aged mouse and human [65].The localization of C1q to synapses observed by Steven's et al. (2007) has also been observed by Stephan et al. (2013) [64].

Inhibition of the complement pathway via viable complement inhibitors may offer a new strategy to attack Alzheimer's disease. The complement system's role in learning and memory is becoming a topic of much interest. Irradiation of the hippocampal granule cell layer, attenuates neural progenitor differentiation, presumably due to induced inflammation. To investigate the roles of C3, young C3 -/- mice were subject to irradiation and compared to wild type mice. Once recovered, the C3-/- mice showed 55% more microglia, and tended to demonstrate more proliferating cells in the GCL than WT mice. These results apparently influenced future learning capacity as adult C3-/- mice showed better place learning than WT. Further experiments by the Steven's lab on aged C3 deficient (KO) mice found an additional role of C3 for synaptic maintenance [21].

Understanding spine dynamics further increases understanding of AD cognitive impairment and dementia. Recent studies tracking both spines and synaptic markers in vivo reveal that 20% of spines lack PSD-95 and are short lived. Although they account for most spine dynamics, Berry & Nedvivi (2017) [65] state their remodeling is unlikely to impact long-term network structure. The synaptic tagging and capture (STC) hypothesis has opened new areas of research on how activity-dependent gene products may interact with potentiated synapses maintain long-lasting synaptic plasticity. One candidate in this process is Arc/arg3.1, initially assumed to participate in STC processes during LTP. Accumulating evidence indicates that Arc/arg3.1 might rather contribute in weakening of synaptic weights than in their strengthening [65].

Long-lasting forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) underlie learning and memory. Although Arc/arg3.1 was initially assumed to participate in the STC processes during LTP, accumulating evidence indicates Arc/arg3.1 might weaken rather than strengthen synaptic weights [66].

In particular, analyses of Arc/Arg3.1 protein dynamics and function in the dendrites after plasticity-inducing stimuli have revealed a novel form of inactivity-dependent redistribution of synaptic weights, known as "inverse synaptic tagging." The original synaptic tagging and inverse synaptic tagging likely co- exist and are mutually non-exclusive mechanisms, which may help coordinate the redistribution of synaptic weights and promote the enhancement and maintenance of their contrast between potentiated and non-potentiated synapses during the late phase of long-term synaptic plasticity. Arc/Arg3.1, an immediate early gene product which is captured and preferentially targeted to non-potentiated synapses, may provide insight into synaptic tagging and AD according to Okumo et al. (2017) [66].

Recently, it has been shown that the G9a/GLP complex promotes long-term potentiation (LTP) and its associative mechanisms such as synaptic tagging and capture (STC) [67]. The mechanics of this process are not understood. Regulation of G9a/GLP complex by inhibiting its catalytic activity reverses the amyloid-β oligomer-induced deficits in late-LTP and STC. The authors of this study suggest this reversal is achieved by releasing the transcription repression of the brain-derived neurotrophic factor (BDNF) gene. Catalytic inhibition of the G9a/GLP complex leads to BDNF expression upregulation in brain slices treated with oAβ. This inhibition of the G9a/GLP complex ensures the availability of BDNF that subsequently binds its receptor tyrosine kinase B (TrkB) and maintains the late-LTP. Furthermore, the capture of BDNF by weakly activated synapses re-establishes STC. Sharma et al. (2017) [67] conclude reinstatement of functional plasticity and associativity in AD-like conditions provide the first evidence for the role of G9a/GLP complex in AD. Investigators propose G9a/GLP complex as the possible target for preventing oAβ-induced plasticity deficits in hippocampal neurons [67].

Another method for modulating long-lasting forms of memory and synaptic plasticity is by suppressing microRNA-mediated translational silencing at activated synapses through translin/trax. Mice that lack translin/trax have defective synaptic tagging. An absence of translin/trax prevents post learning upregulation of the protein ARCR1C. In mice lacking translin/trax, long term memory deficits are also induced by inhibiting ARCR1 [68]. Recent reports indicate adeno-associated virus (AAV1 and AAV9) exhibit

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anterograde transsynaptic spread properties. AAV1-Cre from transduced presynaptic neurons effectively and specifically drove CRE-dependent transgene expression in selected postsynaptic neuronal targets, and thus allowed the tracing and functional manipulation of axonal projections from the latter input-defined neuronal population. Application of this tool in superior colliculus (SC) revealed that SC neuron subpopulations receiving corticocollicular projections from auditory and visual cortex specifically, drove flight and freezing, two different types of defense behavior, respectively.[69] Such anterograde transsynaptic tagging is thus useful for forward screening of distinct functional neural pathways embedded in complex brain circuits [68][69].

Recent research indicates that a novel class of signaling molecules, the inositol pyrophosphates, act as energy sensors. These signaling molecules can alter the balance between mitochondrial oxidative phosphorylation and glycolytic flux, affecting ATP at a cellular level. Neuronal inositol pyrophosphate synthesis relies on the activity of the neuron enriched inositol hexakisphosphate kinase 3 (IP6K3) enzyme. In this particular study, investigators tried to verify an involvement of inositol pyrophosphate signaling in neurodegenerative disorders, by tagging single nucleotide polymorphism (SNP) analysis of the IP6K3 gene in patients with familial and sporadic late onset Alzheimer's disease (LOAD). Two SNPs in the 5'-flanking promoter region of the IP6K3 gene were associated with sporadic LOAD. Assessing the functionality of the two polymorphisms by luciferase assay revealed that one of them (rs28607030) affects IP6K3 promoter activity. In this case the activity of the G allele increased. As the same allele may reduce disease risk, it may be related to upregulation of IP6K3 expression, consequently increasing inositol pyrophosphate synthesis. The authors of the study conclude this is the first evidence of genetic variability in the IP6K3 gene altering LOAD pathogenesis [70].

Research implicates the classical complement cascade in normal brain development and in disease. Complement proteins C1q, C3, and C4 participate in synapse elimination, tagging inappropriate synaptic connections between neurons for removal by phagocytic microglia. Neurodevelopmental disorders, such as schizophrenia and autism, are thought to be caused by an imbalance in synaptic pruning, and recent studies suggest that dysregulation of complement could promote this synaptic pruning imbalance [71].Moreover, in the mature brain, if complement is mistakenly activated to stimulate synapse loss, neurodegenerative diseases may result. Similar pathways can also be activated in response to inflammation, as in West Nile Virus infection or in lupus, where peripheral inflammation can promote microglia-mediated synapse loss. Whether synapse loss in disease is a true reactivation of developmental synaptic pruning programs remains unclear; nonetheless, complement proteins represent potential therapeutic targets for both neurodevelopmental and neurodegenerative diseases. Inhibition of the complement system, at specific neurodegenerative stages, could prove to be a viable therapy for AD and schizophrenia [71].

Microglia are glial cells in the central nervous system (CNS) that have well-known roles in neuronal immune function, responding to infections and brain injury and influencing the progress of neurodegenerative disorders. Microglia expend considerable energy continuously making contacts with pre- and postsynaptic elements of neural circuits. Pruning of synapses may be equivocal to “fine-tuning” of neural circuits. Further dysfunction of such a homeostatic role of microglia could be a primary cause of neuronal disease. As such, neuronal functions including cognition, personality, and information processing are affected by immune status. Understanding interactions between microglia and synapses, the possible cellular and molecular mechanisms that mediate such contacts could be of great value towards understanding neurodegenerative diseases such as AD and schizophrenia [72]. Amyloid protein precursor (APP) is involved in synaptic formation and function. In the human and rodent cingulate cortex, APP is preferentially located in the presynaptic active zone, indicating subsynaptic APP distribution is conserved across species and brain regions. Synaptic APP immunoreactivity decreases in aged cortical samples in deceased males (20-80 years of age). In contrast, the synaptic levels of "alpha-secretase (ADAM10) and beta-secretase (BACE1) did not significantly change. Decreased APP levels may be related to reduced allostasis of synapses in the aged brain and the greater susceptibility of neurodegenerative disorders [73]. Actin-regulating proteins are essential in regulating the shape of dendritic spines, which are sites of neuronal communication. Age related neurodegeneration is attributed to, in part, cofilin and related actin-regulating proteins. The analysis of cofilin motility in dendritic spines using fluorescence video-microscopy may help us understand synaptic functions. To date, the flow of cofilin has not been analyzed by automatic means. Dendrite Protein Analysis (Dendrite PA), a novel automated pattern recognition software may help analyze protein trafficking in neurons [74]. Using spatiotemporal information present in multichannel fluorescence videos, the Dendrite PA generates a temporal maximum intensity projection that enhances the signal-to-noise ratio of important biological structures, segments and tracks dendritic spines, estimates the density of proteins in spines, and analyzes the flux of proteins through the dendrite/spine boundary. According to On et al. (2017), the motion of a dendritic spine is used to generate spine energy images, which are then used to automatically classify the shape of

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common dendritic spines such as stubby, mushroom, or thin. By tracking dendritic spines over time and using their intensity profiles, the system can analyze the flux patterns of cofilin and other fluorescently stained proteins. The cofilin flux patterns correlate with the dynamic changes in dendritic spine shapes. Results also have shown that the activation of cofilin using genetic manipulations leads to immature spines while its inhibition results in an increase in mature spines [74].

Understanding synaptic protein turnover is not only important for determining fundamental aspects of learning and memory, but also has direct implication for understanding pathological conditions like aging, neurodegenerative diseases, and psychiatric disorders. Proteins involved in synaptic transmission and synaptic plasticity are typically concentrated at synapses of neurons and thus appear as puncta (clusters) in immunofluorescence microscopy images. Quantitative measurement of the changes in puncta density, intensity, and sizes of specific proteins provides valuable information on their function circuit development, synaptic plasticity, and synaptopathy. Puncta quantification is time and labor intensive. Recently a software tool has been described that is designed for the rapid semi-automatic detection and quantification of synaptic protein puncta from 2D immunofluorescence images generated by confocal laser scanning microscopy. The software, dubbed as SynPAnal (for Synaptic Puncta Analysis), streamlines data quantification for puncta density and average intensity, thereby increases data analysis throughput compared to a manual method. SynPAnal is stand-alone software written using the JAVA programming language, and thus is portable and platform-free. This new tool has the potential to greatly accelerate understanding of synaptic dynamics in aging and AD [75].

It is well known that chronic stress can induce maladaptive neurophysiological changes, ultimately leading to cognitive impairment. Senescence-accelerated mouse prone 8 (SAMP8) is a naturally occurring animal model that is useful for investigating the neurological mechanisms of chronic stress and Alzheimer's disease.

In this study SAMP8 mice were exposed to unpredictable chronic mild stress (UCMS) for 4 weeks. Then, these mice performed the Morris Water Maze (MWM) test to assess the effect of UCMS on learning and memory. The effects of UCMS on cognition in mice, were evaluated by measuring changes in postsynaptic density 95 (PSD95) and synaptophysin (SYN) proteins, known to be essential for synaptic plasticity.

The Morris water maze experiment revealed that the cognitive ability of the SAMP8 mice decreased with brain aging, and that chronic stress aggravated this cognitive deficit.

Decreased cognition and synaptic plasticity are related to aging, an unsurprising effect. However chronic stress aggravated this cognitive deficit while decreasing SYN and PSD95 expression in the SAMP8 mice. Neurological mechanisms of chronic stress on cognition might be associated with a decrease in hippocampal SYN and PSD95 expression, which may make the SAMP8 mice a valuable model for studying the relationship between aging, synaptic plasticity and stress [76].

Wang et al (2016) focused on how dihydrotestosterone (DHT) regulates synaptic plasticity in the hippocampus of mild cognitive impairment male senescence-accelerated mouse prone 8 (SAMP8) mice. Five-month-old SAMP8 mice were divided into control castrated and castrated-DHT groups, in which the mice were castrated and treated with physiological doses of DHT for a period of 2 months. To determine the regulatory mechanisms of DHT in the cognitive capacity, the effects of DHT on the morphology of the synapse and the expression of synaptic marker proteins in the hippocampus were investigated using immunohistochemistry, qPCR and western blot analysis. The results showed that the expression of cAMP-response element binding protein (CREB), postsynaptic density protein 95 (PSD95), synaptophysin (SYN) and developmentally regulated brain protein (Drebrin) was reduced in the castrated group compared to the control group. However, DHT promoted the expression of CREB, PSD95, SYN and Drebrin in the hippocampus of the castrated-DHT group. Thus, androgen depletion impaired the synaptic plasticity in the hippocampus of SAMP8 and accelerated the development of (AD)-like neuropathology, suggesting that a similar mechanism may underlie the increased risk for AD in men with low testosterone. In addition, DHT regulated synaptic plasticity in the hippocampus of mild cognitive impairment (MCI) SAMP8 mice and delayed the progression of disease to Alzheimer's dementia. The study authors conclude androgen-based hormone therapy is a potentially useful strategy for preventing the progression of MCI in aging men. Androgens enhance synaptic markers (SYN, PSD95, and Drebrin), activate CREB, modulate the fundamental biology of synaptic structure, and lead to the structural changes of plasticity in the hippocampus, all of which result in improved cognitive function [77].

Mitochondrial dysfunction, oxidative stress and beta-amyloid formation are believed to contribute to to neuronal and synaptic degeneration underlying cognitive decline in Alzheimer's disease (AD). The senescence-accelerated mouse-prone 8 (SAMP8) mice are well characterized aging models for mechanistic and translational research for AD. The present study by Jia et al. (2016) [77] characterized mitochondrial and synaptic alterations in SAMP8 mice relative to SAMR1 control mice. This study explored the protective effect of the small molecule peptide SS31, a cell membrane penetrant antioxidant, on mitochondrial and synaptic

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protein integrity as well as cognitive performance. Electron microscopic analysis determined mitochondrial/synaptic deterioration in 10 months old SAMP8 relative to SAMR1 mice. SAMP8 changes following 8 weeks treatment with SS31 (5 mg/kg/day, i.p.) Hippocampal lysates in SAMP8 mice relative to SAMR1 revealed elevation of beta amyloid 42, mitochondrial fission protein (DLP1, Fis1) and matrix protein cyclophilin D (CypD). In addition, lysates showed reductions of mitochondrial fusion protein (Mfn2) and synaptic (i.e., synaptophysin, postsynaptic density protein 95 and growth associated protein 43) proteins [78]. These altered protein expressions in the SAMP8 mouse brain were restored with the SS31 treatment. Moreover, the SS31 treatment rescued learning and memory deficits detected in10 month-old SAMP8 mice. Study authors conclude these findings suggest that this mitochondria-targeting antioxidant peptide may be of potential utility for AD therapy, including a possible lowering of central AB levels and protection of mitochondrial homeostasis and synaptic integrity, which may help slow down cognitive decline [78]. Neurexin1 (Nrxn1) and Neuroligin3 (Nlgn3) are cell adhesion proteins, which are important to age-related synaptic plasticity decline. However, the expression of these proteins during aging has not been thoroughly analyzed. In the study by Kumar and Thakur (2015) investigators measured the age-related changes in the expression of these proteins in cerebral cortex and hippocampus of 10-, 30-, 50-, and 80-week-old male mice. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis indicated that messenger RNA (mRNA) level of Nrxn1 and Nlgn3 significantly increased from 10 to 30 weeks and then decreased at 50 weeks in both the regions. However, in 80-week-old mice, Nrxn1 and Nlgn3 were further downregulated in cerebral cortex while Nrxn1 was downregulated and Nlgn3 was upregulated in hippocampus. These findings were corroborated by immunoblotting and immunofluorescence results. When the expression of Nrxn1 and Nlgn3 was correlated with presynaptic density marker synaptophysin, it was found that synaptophysin protein expression in cerebral cortex was high at 10 weeks and decreased gradually up to 80 weeks. In hippocampus, it decreased until 50 weeks and then increased remarkably at 80 weeks. Furthermore, Pearson's correlation analysis showed that synaptophysin had a strong association with Nrxn1 and Nlgn3 in cerebral cortex and with Nlgn3 in hippocampus. The findings showed that Nrxn1 and Nlgn3 are differentially expressed in cerebral cortex and hippocampus which might be responsible for alterations in synaptic plasticity during aging. These finding warrant continued Nrxn1, Nlgn3 research in aged cerebral cortex and hippocampus [79].

Major histocompatibility complex class I (MHCI) proteins may modulate synaptogenesis, synaptic plasticity, and memory consolidation during development. Ultrastructural analyses revealed a decrease in spine head diameter and post synaptic density (PSD) area, as well as an increase in overall synapse density, and non-perforated, small spines [80].

There is increasing evidence for the role of the major histocompatibility complex class I (MHCI), a protein complex best known for antigen presentation and immunological surveillance in the adaptive immune system, in a second function within the central nervous system (CNS). Lazarczyk et al. (2016) stated "Originally, the brain was considered to be 'immunologically privileged', with low expression of MHCI unless evoked in response to traumatic injury or functional impairment in learning and memory". We now know MHCI is expressed on neurons during development and early adulthood in brain regions including the neocortex, hippocampus, spinal motoneurons, and substantia nigra. In the developing CNS, MHCI has been shown to modulate synaptic plasticity, axonal and dendritic morphogenesis, and neuronal polarity all functions that are completely distinct from its role in the peripheral immune system [80].

In rat hippocampus, increasing neuronal expression of MHCI and the following associated proteins: such as β2-microglobulin (β2M), transporter associated with antigen processing (TAP), paired immunoglobulin-like receptor B (PirB), and killer cell lectin-like receptor (Klra; also known as Ly49) causes an association with cognitively impaired as well as cognitively intact aged rats compared to adult rats. However, MHCI expression in humans appears to be significantly increased in cognitively intact oldest-old (≥87 years of age) individuals and decreased in cognitively impaired oldest-old, relative to younger-old (≤86 years of age) cognitively intact individuals. Moreover, recent genome association studies found alleles of human leukocyte antigen A, one of the three human MHCI genes, associated with increased risk of Alzheimer's disease. Lazarczyk concludes, "depending on the species and the cognitive tasks assessed, an age-related increase in MHCI is important in regulating and preserving cognitive function and thus may be a crucial mechanism for maintaining memory function associated with successful aging". As variability in neuronal and spine morphology has been associated with memory formation and cognitive function, MHCI may regulate memory and cognition through the formation and/or elimination of synapses, similar to its developmental function. MHCI is known to modulate excitatory glutamate receptor function. Altered activity of these receptors has been linked to dendritic spine clustering and their expression at the synapse correlated with age-related cognitive decline [80].

Several neuropsychiatric disorders are associated with cognitive and social dysfunction. Post-mortem studies of patients with schizophrenia by

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Piskorowski et al. (2016) [81] have revealed specific changes in Area CA2, a hippocampal recently found to be critical for social memory formation. To examine how Area CA2 is altered in psychiatric illness, investigators used the Df(16)A+/− mouse model of the 22q1 microdeletion, a genetic risk factor for developing several neuropsychiatric disorders, including schizophrenia. Several age-dependent CA2 alterations were reported: a decrease in the density of parvalbumin-stained interneurons, a reduction in the amount of feed-forward inhibition and a change in CA2 pyramidal neuron intrinsic properties. Results show that Area CA2 is less plastic in Df(16)A+/mi ce, making it nearly impossible to evoke action potential firing in CA2 pyramidal neurons and Df(16)A+/− mice display impaired social cognition, providing a potential mechanism and a neural substrate for this impairment in psychiatric disorders [81].

Also in hippocampus, Mulholland et al (2018) [83] have recently shown that Donepezil changes dendritic spine density and morphology in alcohol exposed adolescent rats. When these alcohol exposed rats were treated with Donepezil as adults, dendritic spine alterations and epigenetic modifications were reversed. This raises the prospect that AD patients with a history of alcohol use and/or abuse may respond differently to their non-drinking AD cohorts and these differences are due to dendritic spine morphology [82].

Synapse density is reduced in postmortem cortical tissue from schizophrenia patients, which indicates increased synapse elimination takes place. In this important study by Sellgren & Gracias (2019) [83], investigators used a reprogrammed in vitro model of microglia-mediated synapse engulfment. They demonstrated increased synapse elimination in patient-derived neural cultures and isolated synaptosomes. This excessive synaptic pruning reflects abnormalities in both microglia-like cells and synaptic structures. Schizophrenia risk-associated variants within the human complement component 4 locus are associated with increased neuronal complement deposition and synapse uptake. This observation, however, does not completely explain the increase in synapse uptake. Additionally, the antibiotic minocycline reduces microglia-mediated synapse uptake in vitro and is associated with a decrease in schizophrenia risk compared to other antibiotics in a cohort of young adults. The authors conclude preventing excessive pruning may be one strategy for delaying or preventing the onset of schizophrenia in high-risk individuals [83]. The importance of this insight cannot be understated. Synapse development, growth and elimination are dynamic processes that continue throughout life. Synaptic elimination, or pruning, is important for removing weak, damaged or unnecessary synapses from the brain. Synaptic elimination is modulated by neuronal activity. Recently, the classical complement cascade has been implicated in promoting synaptic pruning. Specifically, microglial cells recognize activated complement component 3 (C3) bound to synapses targeted for elimination, thus facilitating their removal.

The authors point out as this is a highly relevant process for adequate neuronal functioning, disruptions or exacerbations in synaptic pruning could lead to severe circuitry alterations that could underlie neuropathological alterations typical of neurological and neuropsychiatric disorders. This, as has been previously alluded to, raises the possibility that excessive synaptic elimination in AD may involve or be associated with compliment proteins. In turn, this process of complement mediated pruning could involve microglia activity. Further studies are likely to continue connecting the role of the complement cascade and C3 to AD dynamics. This raises the possibility that a complement-based therapy could be developed as a new target for AD [83] [84]. This review covered the following topics:

- AD epidemiology
- Current FDA approved drugs (treat symptomology)
- Mild Cognitive Impairment
- Conversion of MCI to AD
- Genetic and Biologic Markers in AD
- New targets
- The role of neuroinflammation in AD
- Factors and systems that influence inflammation including the complement system
- Complement system's direct involvement in AD including a role in Beta-Amyloid and Tau Pathology
- Complement inhibition in AD modulation and prevention

We began by discussing AD demographics, growth curves and current cost of the disease. While many clinical trials are underway, with many different targets, we underscored the fact that currently there are only a handful of FDA approved drugs, and none address prevention, just symptomatic treatment. We addressed MCI and conversion of MCI to AD, relevant biological and genetic markers that may predict conversion to AD and help define AD itself. These biological and genetic markers may provide new drug or treatment targets in the future. We then changed our focus to inflammation in AD and the role that the complement cascade plays in inflammation, thus setting the stage for complement's potential role in AD. It is known that complement also plays a role in beta-amyloid and tau pathology, thus increasing the potential influence of complement to AD. Finally, we discussed complement inhibition specifically and AD modulation and prevention.

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HbA1c as a Predictor of Postoperative Infection in Type 2 Diabetic Patients after Coronary Artery Bypass Grafting

By Patricia Veloso Facury Lasmar Ferreira, Wagner José Martorina & Bruno Bastos Godoi

Abstract- Coronary artery disease (CAD) is one of the leading causes of morbimortality worldwide. In patients with diabetes mellitus type 2 (DM2), CAD is more likely to be a complex disease and often requires cardiac surgery. Furthermore, perioperative blood glucose levels control is associated with the followingoutcomes: surgery success, mortality, and infection postoperative. This is a retrospective study, we have collected data form, 43 patients, between 2015 and 2017, with diabetes type 2 and who had passed through a Coronary Artery Bypass Grafting. Those with infection postoperative had a glycohemoglobin 7, 9 (SD ±1,4), and those without infection had an HbA1c 7, 25 (SD ±0,94) and a p-value from 0,039. Adding this was identified that the average of creatinine clearance in patients with the infectious disease was 59 (SD ±21, 3) and 67 (SD ±26) in those without infection, calculated a p-value from 0,039. High levels of Hb1Ac are a predictor of infection disease postoperative.

Keywords: *diabetes mellitus;coronary disease; cardiovascular surgical procedures; cross infection; glycated hemoglobin A.*

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HbA1c as a Predictor of Postoperative Infection in Type 2 Diabetic Patients after Coronary Artery Bypass Grafting

Patricia Veloso Facury Lasmar Ferreira [α], Wagner José Martorina [σ] & Bruno Bastos Godoi [ρ]

Abstract- Coronary artery disease (CAD) is one of the leading causes of morbimortality worldwide. In patients with diabetes mellitus type 2 (DM2), CAD is more likely to be a complex disease and often requires cardiac surgery. Furthermore, perioperative blood glucose levels control is associated with the followingoutcomes: surgery success, mortality, and infection postoperative. This is a retrospective study, we have collected data form, 43 patients, between 2015 and 2017, with diabetes type 2 and who had passed through a Coronary Artery Bypass Grafting. Those with infection postoperative had a glycohemoglobin 7, 9 (SD ±1,4), and those without infection had an HbA1c 7, 25 (SD ±0,94) and a p-value from 0,039. Adding this was identified that the average of creatinine clearance in patients with the infectious disease was 59 (SD ±21, 3) and 67 (SD ±26) in those without infection, calculated a p-value from 0,039. High levels of Hb1Ac are a predictor of infection disease postoperative. Thereby, patients with coronaropathy must have glucose levels, mainly HbA1c levels, and renal function evaluated before cardiac surgery, to obtain a lower risk of postoperative infectious complications.

Keywords: diabetes mellitus;coronary disease; cardiovascular surgical procedures; cross infection; glycated hemoglobin A.

I. Introduction

One of the leading causes of morbimortality worldwide is coronary artery disease (CAD), which has many risk factors in the pathophysiology of the issue, which is why the forerunning risk factor is diabetes mellitus type 2(DM2) [1].In patients with DM2, CAD is more likely to be a complex disease characterized by small, diffuse, calcified, multivessel disease and often requires coronary revascularization [2,3].

Many clinical trials[1,2,4–8] have demonstrated that postoperative myocardial infectious complications in patients with DM2 are associated with the following conditions: blood glucose control, length of stay in the hospital, or in the intensive unit care (ICU), renal failure and previous lung disease [8,9].

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The current study evaluates which variables are associated with a significant risk of hospital infection of any nature in patients with DM2. The central hypothesis is that preoperative blood glucose level, and HbA1c on in-hospital outcomes, principally associated with infections after CABG.

II. Methodology

This is a transversal study; we have collected data between 2015 and 2017 from all patients with type 2 diabetes who passed through a Coronary Artery Bypass Grafting. During this period, CABG performed in 43 patients with DM2. This number has reached because of inclusion criteria: 1) patients who would pass through a cardiac surgery, 2) patients diagnosed with DM2, 3) patients under the care of endocrinology staff. And the exclusion criteria were faulting data at the medical records of those patients. Moreover, were collected the following records: gender, age, HbA1c level, time diabetic diagnosed, insulin user (yes or not), time-elapsed in hospital and the ICU, smoking history, pulmonary disease, and infection postoperative. The Ethical Committee in Research approved this study by the BIOCOR hospital.

III. Statistical Analysis

The chi-square test used in categorical variables (for example, presence or absence of lung disease, use of insulin or not, etc.), with the p-value calculated by the person's square. In the univariate analysis, the continuous numerical variables, when in the standard distribution in the course of Gauss, were analyzed by the T-test of independent samples. And, when not parametric, they were analyzed by the Mann Whitney U test.

IV. Results

Were collected data from 43 patients with DM2 that passed through a CABG for myocardial revascularization. The average age was 65, an average of 8 years diagnosed with DM2 (standard deviation of 8,8), and from all, only 34,8% used insulin to treat and normalize glucose levels (Table 1).

Moreover, were identified an average of glycohemoglobin of 7, 4. Furthermore, 14, 6% of the

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patients had some type of infectious disease after CABG.

Table 1: Characteristics of the patients about each variable

Variables	N (%) or median (Quartile 25-75%) or average (SD)
Age (years)	65 (± 9,3)
Female	17 (39,6%)
Glycohemoglobin (%)	7,40 (±1,08)
Length of DM2 (years)	8 (±8,8)
Length of hospital stay until surgery(days)	5 (±8,8)
Drain time in UCI (days)	1
Length of stay in UCI (days)	2 (±1,5)
Creatinine clearance (mL/min)	67 (±25,5)
Presence of infectious disease after CABG	8 (14,6%)
Use of insulin before hospitalization	15 (34,8%)
Smoking	3 (7,3%)
Pulmonary disease	8 (19,5%)

Those with infection postoperative had a glycohemoglobin 7, 9 (SD ±1,4), and those without infection had an HbA1c 7,25 (SD ±0,94) and a p-value from 0,039 (graphic1).Adding this was identified that the average of creatinine clearance in those patients with the infectious disease was 59 (SD ±21,3) and 67 (SD ±26) in those without infection, calculated a p-value from 0,039 (table 2).

Table 2: Characteristics of patients into two groups: absence and presence of infectious disease postoperative and the p-value of each one

Variables	Absence of infectious disease	Presence of infectious disease	p- value
Average age (years)	65 (± 9,32)	65 (± 8,9)	0,059
Female	15 (34,8%)	2 (4,6%)	0,51
Glycohemoglobin (%)	7,25 (±0,94)	7,9 (±1,4)	0,039
Length of DM2 (years)	8 (±8,98)	10 (±9)	0,057
Length of hospital stay until surgery (days)	6,7 (1,5-8)	12,8 (4 – 25)	0,223
Drain time in UCI (days)	1	1	1
Length of stay in UCI (days)	2 (±1,5)	2 (±1,7)	0,062
Creatinine clearance (mL/min)	67 (±26)	59 (±21,3)	0,039
Use of insulin prior to hospitalization	11 (30,6%)	4 (57,1%)	0,177
Smoking	2 (5,7%)	1 (16,7%)	0,341
Pulmonary disease	28 (82,9%)	5 (71,4%)	0,482

V. Discussion

This study has shown that high levels of Hb1Ac and low levels of creatinine clearance are a predictor of infection disease post CADG.

Izadi, *et al.* (2014)[19] have demonstrated that immunosuppression induced by hyperglycemia-ketoacidosis can be considered as the precedent factor in patients with DM2 to infection, principally after CADG[1,5,13,18,19]. Comparatively, our study showed high levels of infection disease postoperative in patients with previous uncontrolled Hb1AC.

It is controversial what glycemic parameter used as a predictor of postoperative infection disease after CABG. Some authors defend tight blood glucose control as a decreasing factor of morbidity, mortality, and lower rates of infection after cardiac surgeries, principally associated with blood glucose < 200 mg/dL, and <139mg/dL if in ICU. Others defend a more straight target of blood glucose (81 to 108 mg/dL) [1,7,9,12,24]. Other researchers advocate that patients with HbA1c lower than 7% have a better outcome than patients with poorly controlled diabetes (HbA1c> 7%)[13], which also demonstrated with our results that those patients with infectious disease had an HbA1c> 7,9.

Also, patients with HbA1c lower than 7% had similar outcomes compared to those without diabetes diagnosis [16,18,25]. This can corroborate with Halkos *et al.* (2008)[16], demonstrating a significant increase in in-

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hospital mortality, renal failure, cerebrovascular accident, deep sternal wound infection, and a composite index of infection in those patients with HbA1c levels higher than 7%. So then, preoperative glycosylated hemoglobin emerges as a predictor of mortality and principally infections postoperative after CABG [16].

Another variable as a predictor of postoperative infection is the creatinine clearance (CrCl). As shown by Kassaian*et al.* (2012)[18] and Halkos *et al.* (2008)[16], CrCl, as HbA1c levels, predict infection disease postoperative [7,8,12,26,27]. We have also shown similar results. In all patients with infection after CAGD, the CrCl was lower than 59, with a p-value of 0,039.

There are potential limitations in our study that needs to mention. First, it designs retrospective research. Second, this was a single-center experience, and larger multi-center studies should confirm our findings. Third, multivariable analysis was not made, so independent associations cannot be summarized from this study.

VI. Conclusion

In summary, high levels of Hb1Ac are a predictor of infection disease post CADG. Thereby, patients with coronaropathy must be the glucose levels, and mainly HbA1c levels, evaluated before cardiac surgery. Moreover, creatinine clearance should also be evaluated to predict poor outcomes postoperative in patients with DM2.

These findings demonstrate that it is quite essential a holistic medical evaluation in patients with DM2 before cardiac surgeries, focusing on the glucose levels control, HbA1c, and renal function to obtain a lower risk of postoperative infectious complications.

Further researches must be developed to evaluate, prospectively, with a biggersample, a significant association between HbA1c and postoperative infection in patients with DM2 submitted through a CABG.

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By Braimah, Joseph Odunayo, Edike Nnamdi & Elakhe Sarah Oluwatosin

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Keywords: *fish-bone, pareto, africa, trend, pie chart.*

GJMR-F Classification: *NLMC Code: WD 300*



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Keywords: fish-bone, pareto, africa, trend, pie chart.

I. Introduction

On the 10th of January 2020, 41 cases of pneumonia due to a novel coronavirus (2019-nCoV) was reported by Chinese health officials, including seven patients with severe sickness and one death. The symptoms of the virus include fever, cough, and difficulty breathing [1]. The initial diagnosis date for a case acknowledged in China was on the 08th of December, 2019. Preliminary investigation of viral genomes from China and other countries suggests that first transmission from a zoonotic reservoir to humans might have occurred as early as late October. The initial cases reported had links to seafood and live animal market in Wuhan, China, suggesting the disease of humans from an animal source [2 and 3]. Health authorities in China have restricted transportation in and out of heavily affected cities and are continuing to check close contacts, together with health care workers, for illness. Some territories in Asia and countries across the globe are tesingt incoming travelers from Wuhan. Coronaviruses (COVID-19) are a large group of viruses. There are numerous known human coronaviruses that always cause mild respiratory disease, for instance, the common cold. However, at least twice before, coronaviruses have emerged to infect people and cause severe disease: such as severe acute respiratory syndrome (SARS) and the Middle East respiratory syndrome (MERS) [4, 5 and 6]. The cases in this epidemic tested negative for both SARS and MERS. The clinical characteristics of disease, such as the incubation period, have not been determined. On the bases of incubation period of SARS and MERS, signs of 2019-nCoV might appear from 2-14 days after contact to infected person. Human to human spread has been documented, and healthcare workers have also been infected. Similar to other coronaviruses, people may be contagious before showing any symptoms of the disease [3 and 7].

As of 9:00 AM, 14 April 2020, a total of 15,284 COVID-19 cases and 816 (CFR: 5%) deaths were reported in 52 African countries. Out of the 52 Member States that were reported cases, six of them have community transmission, 44 have local transmission, and two have imported cases. Ever since the last brief, the number of tested positive COVID-19 cases has increased by 52% (that is, 5,198 cases). The five countries in Africa with the highest increasing number of cases are South Africa (2,272; 15%), Egypt (2,190; 14%), Algeria (1,914; 13%), Morocco (1,763; 12%), and Cameroon (820; 5%). When the population is been taken into consideration, Djibouti (30.2), Mauritius (25.5), Seychelles (11.2), Tunisia (6.1), and Morocco (4.8) are reporting a large amount of cases per 100,000 populations within the continent. Fifteen countries are reporting case death rates higher than the worldwide case fatality rate of 6%. See Table 1 below for the complete list of countries in Africa reporting cases, deaths, and COVID-19 recoveries as well as transmission type being reported. Africa CDC is currently working with all affected countries and is organizing laboratory, supervision, and other response support were needed [8, 9, 10, and 11].

a) Intervention of Africa CDC

1. Africa CDC set in motion its Emergency Operations Center and Incident Management System (IMS) for the 2019-nCoV outbreak on the 27th of January, 2020.
2. Africa CDC is getting test kits for and also working with laboratories in the Member States to check specimens for novel coronavirus infection.
3. Training of 16 African laboratories took place in Senegal on 6th-7th February, 2020.
4. In partnership with WHO, Africa CDC has set up a specimen referral system. After training was

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completed, Africa CDC shared the list of laboratories with capacity to test for COVID-19 with all Member States and how to submit specimens.

5. Africa CDC is working to train and set out epidemiologists at headquarters and within the RCCs for daily event tracking and risk analysis, to be communicated with the Member States.
6. Africa CDC is also providing training and technical support to at-risk African airports.
7. Africa CDC is developing informational materials on infection prevention which are also been shared with the Member States.
8. Africa CDC is organizing weekly updates with the national public health institutes in Member States and forming working groups for high precedence areas of coronavirus control.
9. Africa CDC is working with the Member States to build infection impediment and control capacities in healthcare facilities, and with the airline division to support screening of travelers.
10. Africa CDC will continue to provide an updated and important information to the Member States as the outbreak progress.
11. Africa CDC has commenced a continent-wide network of 300 clinicians across the continent to talk about COVID-19 clinical management and is holding weekly webinars [10 and 11].

The problem is, how prompt should health management organizations interfere in order to curb the increase of this pandemic across the infected countries by intervention organizations? Secondly, if proper action is not properly taken, what will be the status of the pandemic in Africa as a continent?

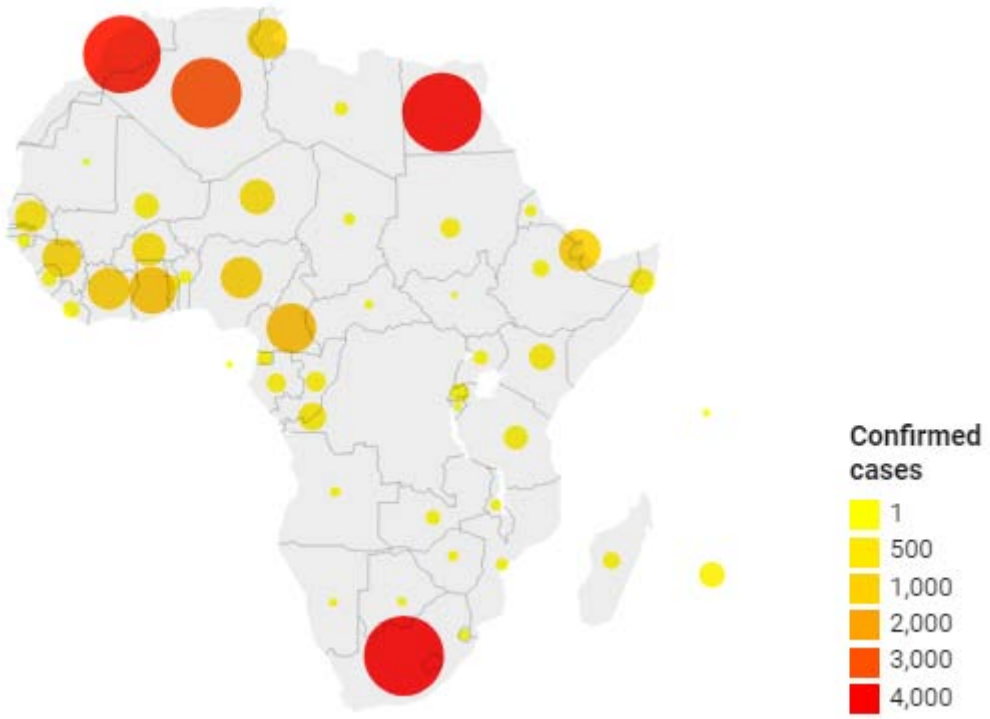
II. Materials and Methods

In the course of proffering solutions to the above-posed questions, quality control tools were adopted to monitor the pandemic. Quality health control can be apply to various aspects of health care. Timeliness in health control relates to obtaining needed care while minimizing delays in intervention on any disease outbreak. Quality health control also looks at the consumer point of view of health care needs by the government (health management organizations/ ministries).

Appropriate steps must be taken by physician and other health providing activities whenever there is an epidemic or pandemic outbreak to maintain quality health standards in any society.

To understand the problem posed by the delay in the recent global ravaging outbreak (COVID-19), it may be useful to describe the trend to mitigate the impact of the virus spreads in Africa.

The control tools used in analyzing COVID-19 data include a fish-bone diagram, Pareto chart, control chart, bar chart, trend analysis, and pie chart.



Source: Africa CDC (https://africacdc.org/covid-19)

Figure 1: COVID-19 pandemic in Africa as at 24th of April 2020

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III. Results and Analysis

The data used in this write-up were up to date published data in NCDC official website (https://africacdc.org/covid-19) as at 10:00 PM on the 24th April 2020.

Table 1: Full list of countries in Africa with COVID-19 Cases

Summary table of confirmed cases in Africa (as of 23 April 2020)			
Location	Cases	Deaths	Recoveries
South Africa	3,953	75	1,473
Egypt	3,891	287	1,004
Morocco	3,568	155	1,004
Algeria	3,007	407	1,355
Cameroon	1,334	43	668
Ghana	1,154	9	99
Ivory Coast	1,004	14	359
Djibouti	986	2	252
Tunisia	918	38	190
Nigeria	873	28	197
Guinea	862	6	170
Niger	662	22	193
Burkina Faso	609	39	389
Senegal	479	6	257
Réunion	412	0	238
Democratic Republic of the Congo	377	25	47
Mauritius	331	9	266
Somalia	328	16	8
Mayotte	326	4	125
Kenya	320	14	89
Mali	309	21	77
Tanzania	284	10	11
Republic of the Congo	186	6	16
Gabon	167	2	24
Sudan	162	13	14
Rwanda	154	0	87
Madagascar	121	0	58
Ethiopia	116	3	21
Liberia	101	8	20
Togo	88	6	56
Equatorial Guinea	84	1	7
Cape Verde	82	1	1
Zambia	76	3	37
Sierra Leone	64	1	10
Uganda	63	0	46

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Libya	60	1	15
Benin	54	1	27
Guinea-Bissau	50	0	3
Mozambique	46	0	9
Eritrea	39	0	6
Chad	33	0	8
Malawi	33	3	3
Eswatini	31	1	8
Zimbabwe	28	4	2
Angola	25	2	6
Botswana	22	1	0
Namibia	16	0	7
Central African Republic	14	0	10
Burundi	11	1	4
Seychelles	11	0	6
Gambia	10	1	2
Mauritania	7	1	6
Western Sahara	6	0	5
São Tomé and Príncipe	4	0	0
South Sudan	4	0	0
Total	27,210	1,286	8,087

Source: World Health Organzation (WHO)

a) Fish-Bone Diagram

The figure below depicts the symptoms to check out for in a COVID-19 infected person in Africa.

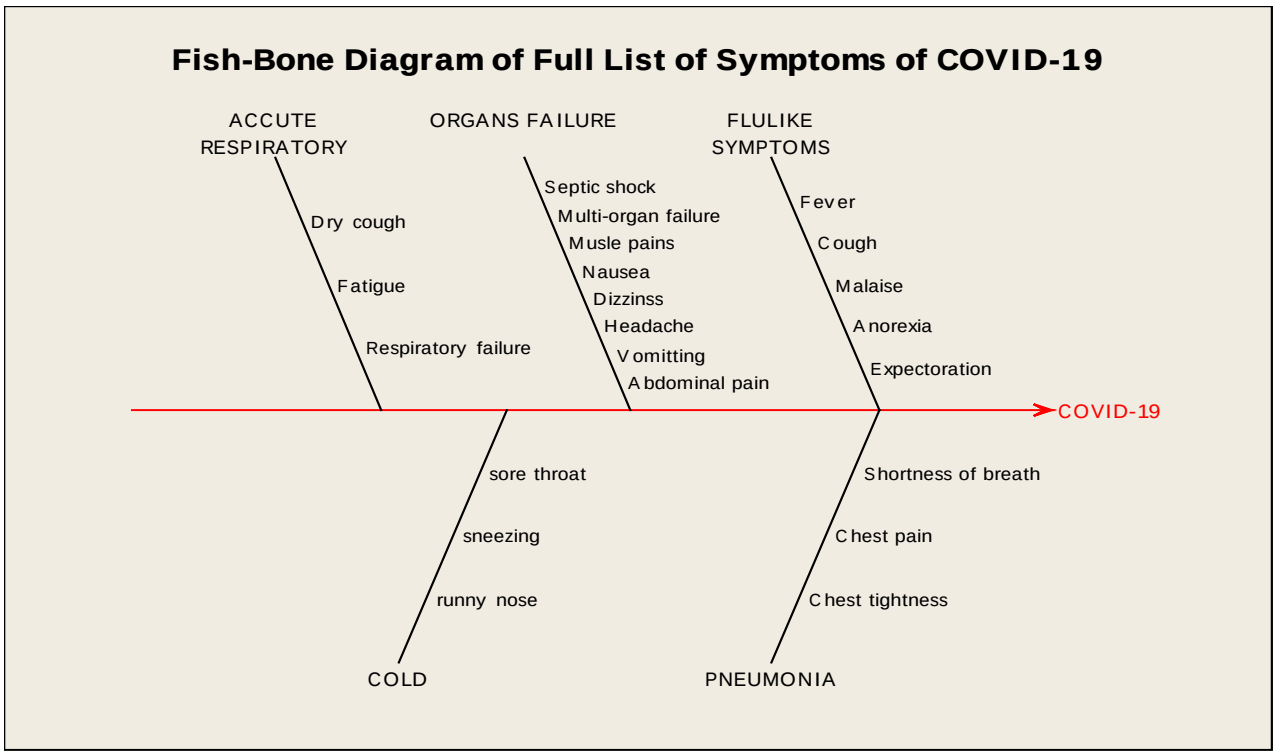


Figure 2: Fish-bone diagram of COVID-19 symptoms

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Figure 2 above is the full list of symptoms associated with a COVID-19 infected patient at both the initial and late stages of the disease in Africa.

b) Pareto Chart

To identify the Areas (Countries) where more intervention would be more needed, we would draw a horizontal line from the 80% mark on the vertical cumulative percentage axis. Where it crosses the line graph, and down to the horizontal axis is drawn.

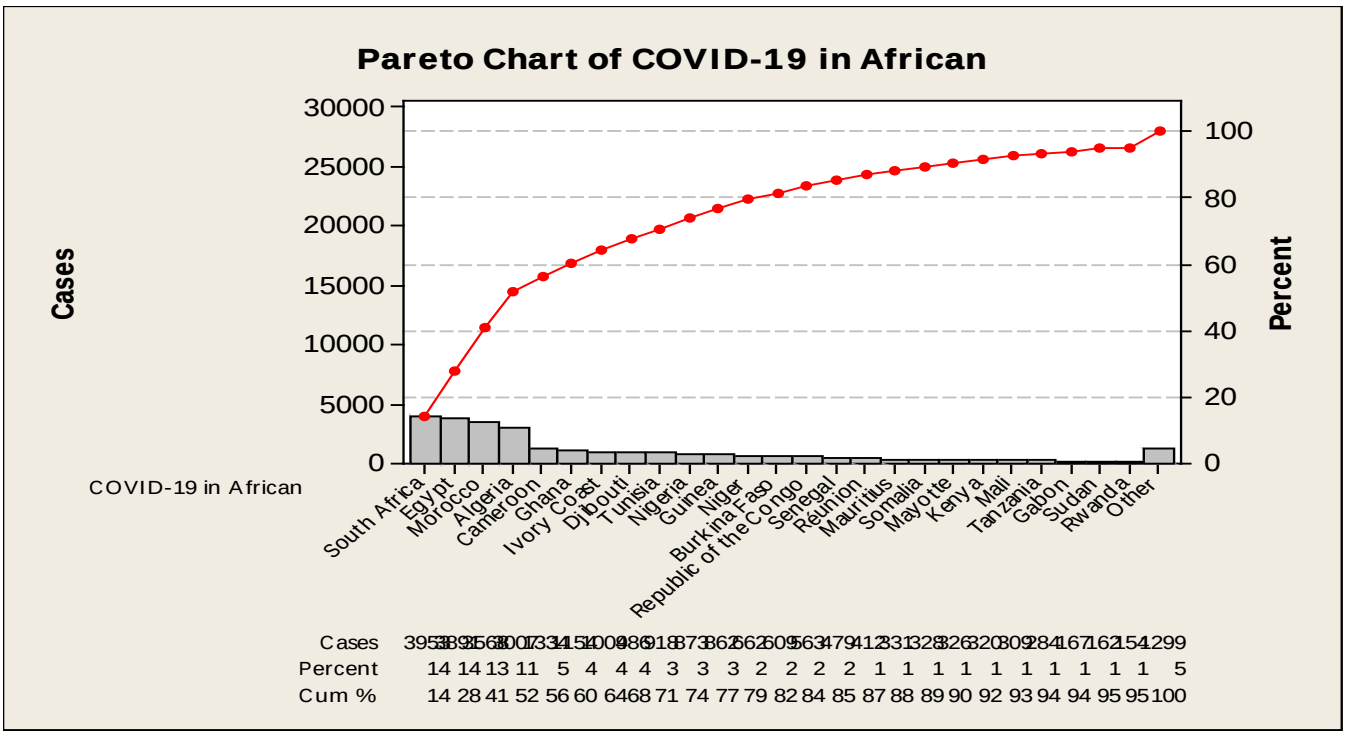


Figure 3: Pareto plot of COVID-19 infected African Countries

From the Pareto chart above, intervention efforts by World Health Organization (WHO) and other intervening bodies like Organization for African Union should give a better attention to the 14 countries to the left of the vertical line (South Africa, Egypt, Morocco, Algeria, Cameroon, Ghana, Ivory Coast, Djibouti, Tunisia, Nigeria, Guinea, Niger and Burkina Faso), known as the vital few. Therefore, the government and other intervention bodies should intervene more in these 14 countries among other African countries since they contribute 80% of the total out in the entire infected countries in the continent.

c) Trend Analysis

Figure 3 below shows the trend plot for the pattern of the outbreak of COVID-19 in Africa.

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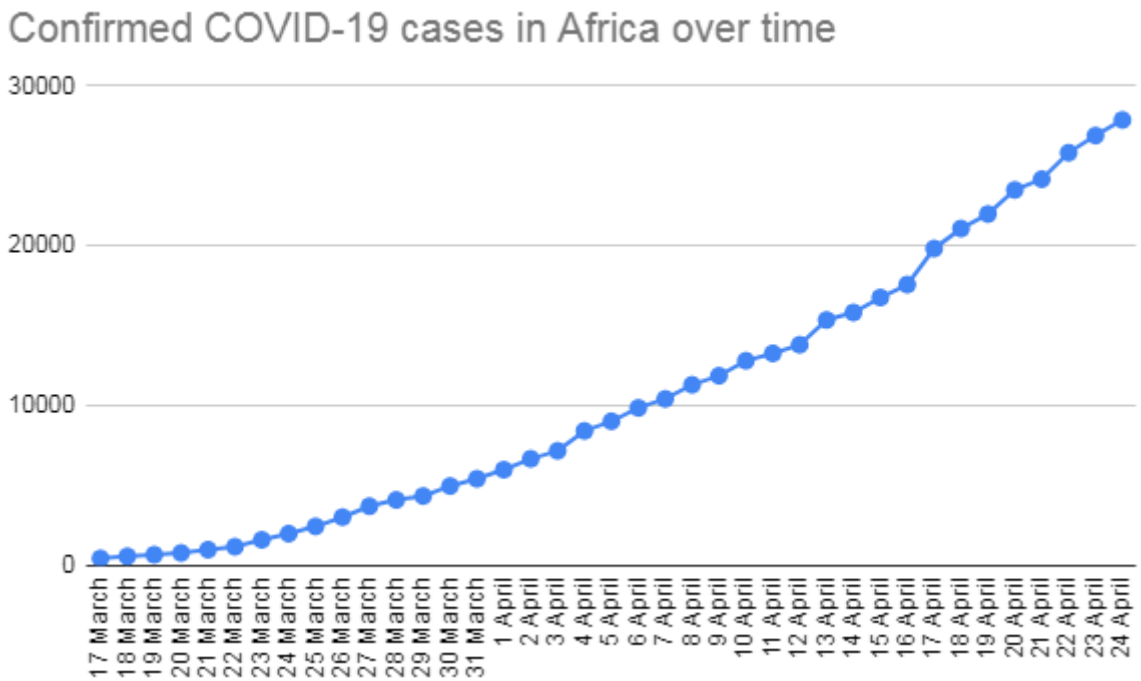


Figure 4: Trend plot of both active and daily confirmed cases is an increasing trend from 17th March till 24th of April.

Figure 4 above shows an increasing trend of laboratory-confirmed cases and an upward and downward trend of the daily confirmed cases from the 17th of March 2020. The increase in the rate of spread indicates that more effort need to be put in place to curb this pandemic spread both government and intervening bodies across Africa and the world.

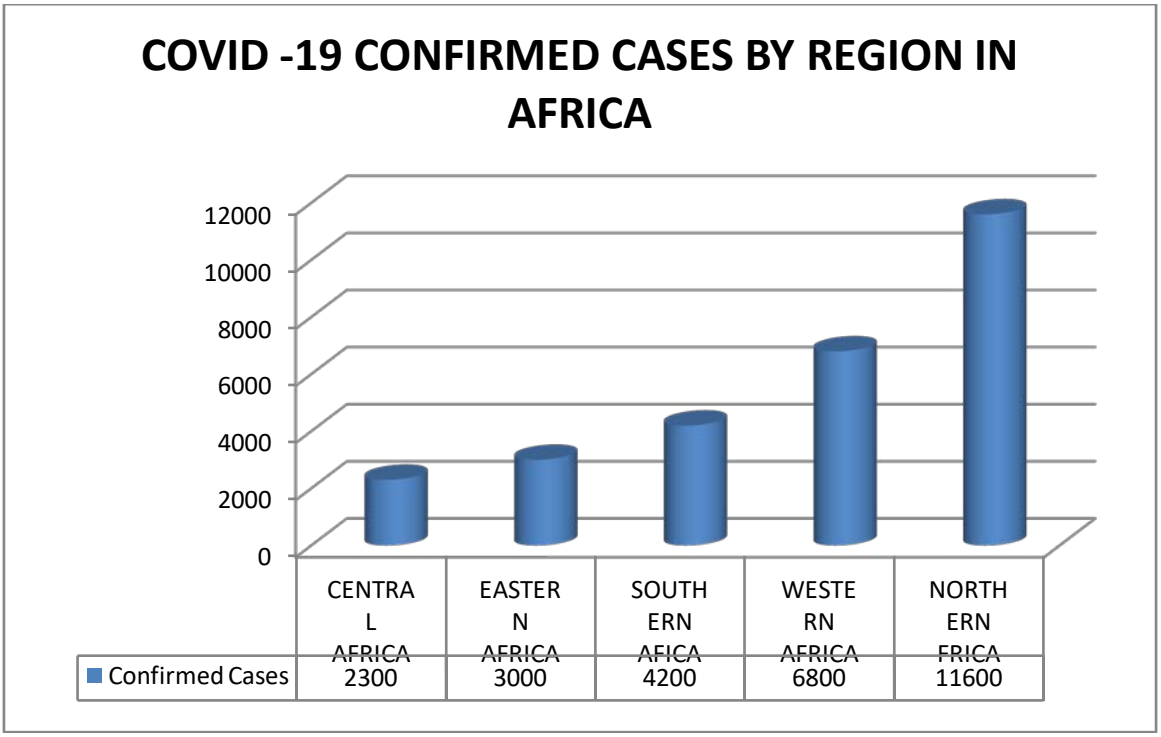


Figure 5: Bar chart of confirmed cases of COVID-19 by region outbreak in Africa

The bar chart above shows that Northern Africa has the highest COVID-19 with Western Africa the next, followed by Southern Africa, Eastern Africa, and the least being Central Africa.

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d) Cumulative Sum (CUSUM) Chart

The cumulative sum (CUSUM) control scheme is an efficient monitoring tool in detecting small shifts in the mean of a process (death rate). In particular, the Average Run Length (ARL) of CUSUM control charts shows that they are better than Shewart control charts when it is desired to detect shifts in the mean that are less two sigma or less. Let max(a,b) be the maximum of *a* and *b*. The i[th] CUSUM for an upward shift, $S_{hi}(i)$ for the i[th] observation (deaths), is defined as;

$S_{hi}(i) = \max(0, S_{hi}(i-1) + x_i - \mu_0 - k_1)$, where k_1 is the reference value for the upward CUSUM.

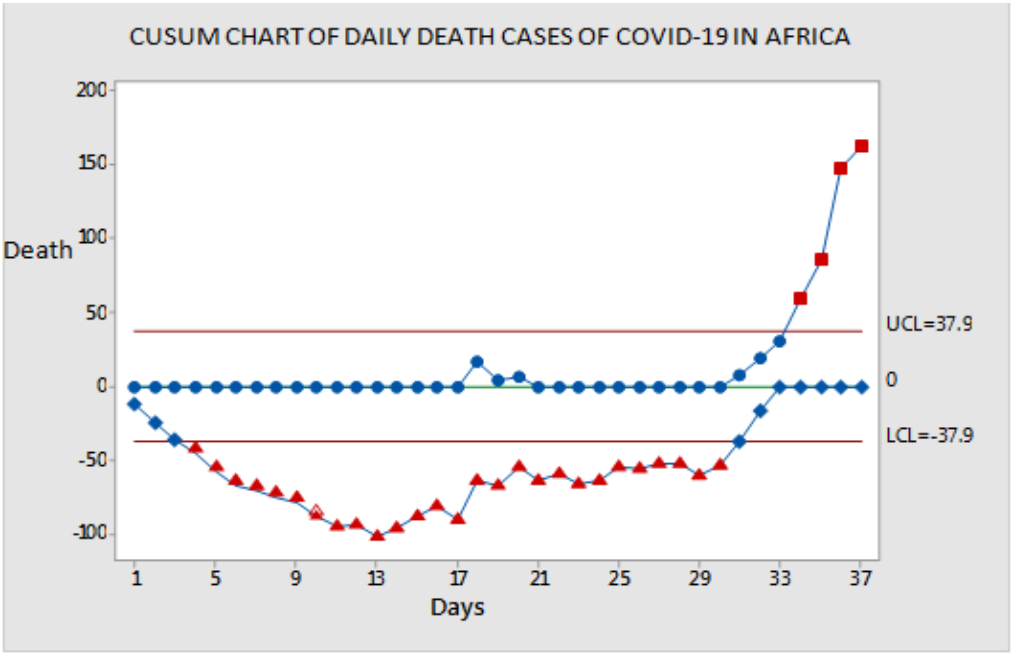


Figure 6: CUSUM chart of daily death cases of COVID-19 in entire Africa

The Figure above shows the CUSUM chart of death cases of COVID-19 between the 4th of March and 24th April, 2020. It reveals that the recorded death of COVID-19 in Africa are out of statistical control since the CUSUM points from the last four days (i.e., 21st April to 24th April) plotted above the upper control limit.

e) Pie Chart

To assess the performance of strategies put in place to monitor and curb the outbreak of this pandemic in the Africa continent, the number of laboratories confirmed cases, number of death, and number of discharged on testing negative after treatment were plotted in a pie chart using their percentages.

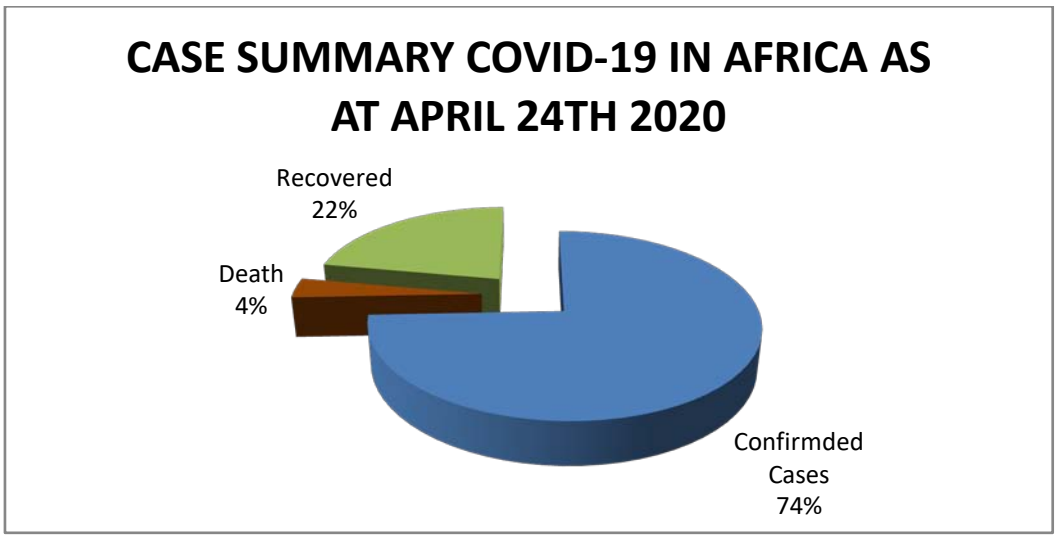


Figure 7: Pie chart of summary of COVID-19 cases in Africa

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The number of recovered and discharged cases after treatment is 22%, and the number of death is 4%. This 4% death rate shows that the pandemic control is still under control in Africa.

IV. Conclusion

This paper aimed at monitoring COVID-19 outbreak in Africa and to make out the effect of palliative measures put in place to curb the spread of the virus by national and international bodies. The study reveals that 14 countries were more vulnerable, comprising of South Africa, Egypt, Morocco, Algeria, Cameroon, Ghana, Ivory Coast, Djibouti, Tunisia, Nigeria, Guinea, Niger, and Burkina Faso. The said 14 constitute the vital few (80%) of the entire outbreak in entire Africa continent. The pandemic is also on an increasing trend, with; Northern Africa having the highest cases of COVID-19 with Western Africa the next, followed by Southern Africa, Eastern Africa, and the least being Central Africa. The study also shows that the death rate of the pandemic is already out of control since the 21st of April, 2020. Lastly, the spreading outbreak is still under control with the measures carried out by various organizations and government in curtailing the spread of the pandemic.

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Neonatal Candidemia: Clinical Presentation, Laboratory Profile, Risk Factors and Immediate Outcome in a Tertiary Hospital in Kerala- A Case-Control Study

By Dr. Jayaram Sankar. KR, Dr. Sunil Daniel & Dr. Rekha Rachel Philip

Abstract- *Objective:* To identify the clinical and laboratory profile and risk factors of bloodstream candida infection in newborns and to assess the immediate outcome of candidemia in newborns.

Design: Case - control study.

Setting: Tertiary care NICU of Govt. T.D. Medical College, Alappuzha, Kerala from 1st January 2010 to 31st December 2014.

Methods: Through consecutive sampling, we got 94 cases and 188 controls. For comparison, chi-square test was used, and for strength association Odds ratio was used. Analysis was done using SPSS V18. Binary logistic regression has been used to identify independent risk factors.

Keywords: *neonatal candidemia, silent hypoxemia, sub threshold feed tolerance.*

GJMR-F Classification: *NLMC Code: WS 205*

NEONATALCANDIDEMIACLINICALPRESENTATIONLABORATORYPROFILERISKFACTORSANDIMMEDIATEOUTCOMEINATERTIARYHOSPITALINKERALAACASECONTROLSTUDY

Strictly as per the compliance and regulations of:

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Results: In our study the prevalence rate of neonatal candidemia was 3.89%. Silent hypoxemia (OR – 50.54, CI – 6.66, 383.41) and sub-threshold feed tolerance (OR – 3.38, CI – 2.81, 4.07) were found to be two symptoms of neonatal candidemia with high specificity. The mortality rate among neonates with candidemia was 18.1%, and in controls was 8.5% . The mean hospital stay of cases were 25.17 days, and of controls were 10.46 days.

Conclusion: Management of maternal vaginal candidiasis, judicious use of antibiotics, maintaining euglycemia, and early enteral feeding may reduce the risk for neonatal candidemia. Duration of hospital stay and mortality were high in neonates with candidemia. Silent hypoxemia and sub-threshold feed tolerance are two specific symptoms to suspect neonatal candidemia.

Keywords: neonatal candidemia, silent hypoxemia, sub threshold feed tolerance.

I. Introduction

Neonatal sepsis is frequently due to organisms colonizing the skin and mucosal surfaces, such as Coagulase-negative Staphylococci and Candida (1). Blood stream infection (BSI) due to Candida species in the neonatal intensive care unit (NICU) is less frequent than that due to Gram-positive or Gram-negative bacteria, but it has higher morbidity and mortality rates (2). Candida is the third most common etiologic agent in late-onset neonatal sepsis and is responsible for 8 to 15% of hospital-acquired infections (3). Preterm infants have high Candida colonization rates compared to term infants, and it is well established that colonization with Candida is inversely proportional to gestational age (4, 5). Colonization precedes invasive Candida infection, and the number of colonization sites and density of skin colonization with Candida correlate with candidemia (6-8). Even though Candida albicans is the most prevalent yeast pathogen, BSIs caused by Candida non-albicans, particularly Candida parapsilosis complex and Candida glabrata complex has been increased in recent years (9, 10). Newborns who survived from invasive candidiasis frequently have a long-term neurological impairment, including cerebral palsy, blindness, hearing impairment, cognitive deficits, and periventricular leukomalacia (9). Candida infections are responsible for an 'attributable mortality' of 18–25%, significant morbidity, and healthcare costs (11). The incidence and associated mortality due to candidemia can be influenced by several factors, including the population at risk, healthcare facility standards, Candida spp. involved and anti-fungal resistance (12).

Clinical presentation of candidemia resembles sepsis syndrome and to establish a clinical diagnosis of candidemia is difficult. (13). Here, in this study, we are trying to identify the clinical and laboratory profile of neonatal candidemia and the risk factors associated with it.

Ethical consideration: Permission to conduct this study was obtained from the institutional research committee and ethical committee of Government T. D Medical College, Alappuzha.

II. Methods

This study was a retrospective case-control study. Sex matched cases and controls were selected through consecutive sampling by file review of newborns of NICU of Govt. T.D. Medical College from 1st January 2010 to 31ST December 2014.Cases were identified through the review of a meticulously maintained NICU logbook in which relevant details

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(including the IP number, name, age, sex, date of admission, discharge diagnosis, date of discharge/death, and status at discharge) of all admitted cases were entered. We also reviewed the blood culture record of all cases and controls for final case confirmation from Microbiology Laboratory. All babies in whom blood culture yielded candida were considered as cases. Two newborns of the same-sex as that of the case and admitted within one week prior or after the case but negative for candida blood culture were considered for controls. New born babies who were on antibiotics before sampling for blood culture and whose case books incomplete or not available were excluded.

Case records of all these newborns were retrieved from the medical record library. After applying exclusion criteria, cases and controls were finalized. Details of risk factors (maternal, neonatal, and nosocomial), clinical features, investigation results, and outcome of all cases and controls were recorded in the proforma and analyzed.

III. Definitions

Candidemia: The blood culture yields candida species organisms.

Silent hypoxemia: Low Spo2 with no tachypnea/ bradypnea/apnoea/ respiratory distress/ hypothermia and with no evidence of shock.

Feed intolerance: The enteral milk feeding has to be stopped due to presence of one or more of the following- gastric residuals >50% of previous feed, vomiting, abdominal distension, visible bowel loops, and blood in the stool (occult/ gross).

Sub-threshold feed tolerance: The gastric residue is 20-50% of previous feed consecutively for at least three times without features of feed intolerance.

For statistical analysis, continuous variables were summarized using mean and standard deviation and categorical variables using frequencies and percentages. To identify risk factors of candida infection, Chi-square test was used, and for the strength of association odds ratio was used. Binary logistic regression was performed using the enter method to identify independent predictors of neonatal candidemia and mortality associated with candidemia. All analysis was performed using SPSS V18.

IV. Results

From1st January 2000 to 31st December 2014, there were 2754 admissions to the newborn nursery. Out of this, 107(3.9%) had candidemia. Seven babies who were started on fluconazole before blood culture and 6 with incomplete case records were excluded from the study. Finally, 94 cases and 188 controls were included in this study. 92(97.9%) cases were Candida nonalbicans, and the rest were candida albicans. Cases and controls were comparable concerning sex, route of delivery, and gravidity. The frequency of Gestational diabetes mellitus, Gestational hypertension, PROM, and 3rdtrimester UTI was also comparable (Table-1).

Table-1: Baseline Characteristics of Cases and Controls

VARIABLE	CASE-94 (100%)	CONTROL-188 (100%)	P value
Sex (M)	57(60.6)	114(60.6)	.518
Route of delivery (vaginal)	42(44.7)	100(53.2)	.207
PrimiGravida	51(54.3)	114(60.6)	.309
GDM	3(3.2)	6(3.2)	1.000
GHT	14(14.9)	19(10.1)	.244
PROM	20(21.3)	23(12.2)	.054
3rd trimester UTI	4(4.3)	7(3.7)	1.000

There was a significant difference in the mean age of admission between cases and controls. Symptoms &signs such as lethargy (67%), respiratory distress (67%), apnoea (35.1%), silent hypoxia (21.3%), seizure(14.9%), feed intolerance (33%), sub-threshold feed tolerance(16%), weak cry (86.2%), prolonged CFT (48.9%), mucosal candida infection (5.3%), abdominal distension (54.3%) and hepato-splenomegaly (22.3%) was significantly higher in cases (table-2). Newborn babies with candida BSI showed a significantly higher proportion of thrombocytopenia (75.5%), positive C-reactive protein (47.9%), and abnormal CSF (9.6%). Urine fungal hyphae (14.9%) were isolated only from neonates with candidemia. The mean WBC count was significantly lower in cases (10858) compared to controls (14191) (table-2).

Table-2: Comparison of clinical and laboratory profile of cases and controls

VARIABLE	CASE-94 (100%)	CONTROL-188 (100%)	P value	OR	95% CI
Age on admission (Mean & SD)	1.10 (0.39)	1.74(1.19)	<0.001		
Lethargy	63(67)	37(19.7)	<0.001	8.29	4.74, 14.52
Resp distress	63(67)	75(39.9)	<0.001	3.06	1.82, 5.15

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Apnoea	33(35.1)	18(9.6)	<0.001	5.11	2.68, 9.73
Silent hypoxemia	20(21.3)	1(.5)	<0.001	50.54	6.66, 383.41
Seizures	14(14.9)	9(4.8)	0.005	3.418	1.44, 8.37
Feed intolerance	31(33)	2(1.1)	<0.001	45.76	10.65, 196.7
Sub threshold feed tolerance	15(16)	0(0)	<0.001	3.38	2.81, 4.07
Weak Cry & activity	81(86.2)	61(32.4)	<0.001	12.97	6.7, 25.1
Prolonged CFT	46(48.9)	23(12.2)	<0.001	6.88	3.8, 12.5
Mucosal candida	5(5.3)	0(0)	.004	3.1	2.6, 3.7
Abdominal distension	51(54.3)	13(6.9)	<0.001	15.97	7.98, 31.97
HSM	21(22.3)	6(3.2)	<0.001	8.73	3.39, 22.5
Thrombocytopenia	71(75.5)	31(16.5)	<0.001	15.6	8.5, 28.7
Positive CRP	45(47.9)	35(18.6)	<0.001	4.02	2.3, 6.9
Abnormal CSF	9(9.6)	2(1.1)	.001	9.85	2.08, 46.56
Urine fungal hyphae	14(14.9)	0(0)	<0.001	3.35	2.78, 4.03
Total WBC count (Mean & SD)	10858(7088)	14191(5444)	<0.001		

When the maternal risk factors associated with the development of candidiasis were analyzed, statistical significance was seen for maternal vaginal candidiasis at the time of labor and for mothers who had received antenatal steroids. Among the neonatal risk factors, prematurity, LBW, birth asphyxia, resuscitation which need bag and mask/ ET, delayed enteral feed >24 hours, and neonatal hyperglycemia was significantly associated with neonatal candidemia. Nosocomial risk factors significantly associated with neonatal candidemia were amino acid infusion, steroids, more than two antibiotics, central venous access, endo tracheal intubation, assisted ventilation, oxygen, intravenous immunoglobulin, aminophylline, caffeineand surfactant. (Table-3)

Table-3: Comparison of risk factors associated with systemic candidiasis among cases and controls

VARIABLES	CASES=94	CONTROLS= 188	P value	OR	95% C I
Preterm	80(81.5)	77(41)	<0.001	8.24	4.4, 15.6
LBW	84(89.4)	85(45.2)	<0.001	10.2	4.98, 20.8
Very preterm (<28wks)	34 (36.2)	19 (10.1)	<.001	5.04	2.7,9.5
Extremely LBW (<1.0kg)	12 (12.8)	5(2.7)	0.001	5.4	1.8,15.7
Ante natal steroid	33(35.1)	17(9.0)	<0.001	5.44	2.83, 10.47
Maternal vaginal candidiasis	18(19.1)	5(2.75)	<0.001	8.668	3.106, 24.2
Birth asphyxia	31(33)	31(16.5)	0.002	2.49	1.4, 4.44
Active Resuscitation	24(25.5)	23(12.2)	0.006	2.5	1.3, 4.6
Delayed enteral feed (>24hrs)	73(77.7)	57(30.3)	<0.001	7.99	4.49, 14.22
Neonatal Hyperglycemia before C&S	69(73.4)	30(16)	<0.001	14.5	8, 26.5
Amino acid infusion	78(83)	32(17)	<0.001	23.77	12.3, 45.93
Steroid for baby	12(12.8)	5(2.7)	0.002	5.36	1.83, 15.7
Ranitidine	7(7.4)	6(3.2)	0.134	2.44	.796, 7.48
More than 2 antibiotics used	78(83)	113(60.1)	<0.001	3.24	1.8, 5.97
CV catheter	13(13.8)	3(1.6)	<0.001	9.9	2.75, 35.7
Endo-tracheal intubation	33(35.1)	16(8.5)	<0.001	5.81	2.99, 11.3
Assisted ventilation	38(40.4)	26(13.8)	<0.001	4.23	2.36, 7.58
Oxygen	74(78.7)	69(36.7)	<0.001	6.4	3.6, 11.4
Abdominal surgery	3(3.2)	3(1.6)	0.404	2.03	0.4, 10.3
IVIG	11(11.7)	6(3.2)	0.007	4.02	1.44, 11.24
Aminophylline	6(6.4)	0(0)	0.001	3.14	2.64, 3.73
Caffeine	54(57.4)	20(10.6)	<0.001	11.34	6.1, 21.0
Surfactant	30(31.9)	15(8.0)	<0.001	5.41	2.7, 10.7

As many of these risk factors are interdependent, inorder to identify the independent risk factors of candidemia, we did binary logistic regression. The independent risk factors were; more than two antibiotics, amino acid infusion, neonatal hyperglycemia before blood culture, and maternal vaginal candidiasis (table-4).

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Table-4: Independent risk factors of neonatal candidiasis

Variable	Adjusted odds ratio (95% CI)	P value
Maternal candidiasis	12.7 (2.5, 65.5)	.002
Amino acid infusion	5.5 (1.6,18.7)	.006
More than 2 antibiotics used	5.05 (1.8, 13.9)	.002
Hyperglycemia	10.9 (4.2,28.1)	<.001

The mortality rate among admitted newborns with candidemia and without candidemia was 18.1% and 8.5%,respectively. This finding was statistically significant. The mean duration of hospital stay among cases was also significantly higher. (table-5)

Table-5: Comparison of immediate outcome between cases and controls

VARIABLE	CASE-94 (100%)	CONTROL-188 (100%)	P value	OR	95% CI
Death	17(18.1)	16(8.5)	.018	2.37	1.14, 4.94
Duration (days) of hospital stay (mean & SD)	25.17(12)	10.46(11.6)	<0.001		

On univariate analysis, we found that the following were predictors for mortality in neonatal candidiasis; vaginal delivery, preterm <32 weeks GA, birth weight <1.5 kg, lethargy, respiratory distress, apnoea, prolonged CFT, skin mottling, endo tracheal intubation, assisted ventilation, administration of caffeine or surfactant ,and abdominal surgery. (Table-6)

Table -6: Risk factors of mortality in neonatal candidemia

VARIABLES	Death=17(100%)	Discharged=77(100%)	P value	OR	95% C I
Route of delivery-vaginal	13(76.5)	29(37.7)	0.004	5.38	1.6, 18.1
<32 weeks gestation	12(70.6)	22 (28.6)	0.001	6.0	1.9, 19.0
ELBW & VLBW	14 (82.4)	31 (40.3)	0.002	6.9	1.8, 26.1
Lethargy	15(88.2)	48(62.3)	0.040	4.5	.97, 21.3
Respiratory distress	15(88.2)	48(62.3)	0.040	4.5	.97, 21.3
Apnoea	13 (76.5)	20 (26)	<0.001	9.3	2.7, 31.7
Prolonged CFT	13 (76.5)	33 (42.9)	0.012	4.33	1.3, 14.5
Skin mottling	6 (35.3)	5 (6.5)	0.001	7.86	2.0, 30.2
Endotracheal Intubation	14 (82.4)	19 (24.7)	<0.001	14.2	3.7, 55
Assisted ventilation/ CPAP	16 (94.1)	22(28.6)	<0.001	40	5, 320
Abdominal surgery	2 (11.8)	1 (1.3)	0.026	10.1	.86, 119
Caffeine	14 (82.4)	40 (51.9)	0.022	4.3	1.1, 16.2
Surfactant	10 (58.8)	20 (26)	0.009	4.1	1.37, 12.1
Active resuscitation	8 (47.1)	16 (20.8)	0.025	3.39	1.12, 10.1

Multivariate regression analysis found out only three independent predictors of mortality. These were apnoea, abdominal surgery, and active resuscitation.(Table-7)

Table-7: independent risk factors for mortality in neonatal candidemia

Variable	Adjusted odds ratio (95% CI)	P value
Apnoea	36.3(1.81, 74)	.020
Abdominal surgery	332 (1.3, 831)	.039
Active Resuscitation	14 (1.1, 186)	.041

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V. Discussion

We report a prevalence of 3.89%for neonatalcandidemia during the study period. This prevalence rate is comparable to the prevalence reported from developed countries.[14- 16]

Our study found a significant difference in the age of admission between cases and controls contrary to a study from China (17).This may be due to the difference in indications for admission among the hospitals.

The clinical features associated with neonatal candidemia reported in our study namely lethargy, respiratory distress, apnoea, silent hypoxia, seizure, feed intolerance, sub-threshold feed tolerance, weak cry, prolonged CFT, mucosal candida infection, abdominal distension, and hepatosplenomegaly were similar to that reported in the literature (18,19).The two new signs we identified were silent hypoxemia (specificity- 99.46% and sensitivity-21%) and sub-threshold feed tolerance (specificity- 100% and sensitivity-15.9%),which is not reported in other studies. In some of the cases, these early signs were the reason for suspecting candidiasis.

We found that neonatal candidemia was significantly associated with thrombocytopenia, positive CRP, a relatively low total WBC count, and abnormal CSF, which is consistent with reports elsewhere.

Most of the significant riskfactors for neonatal candidemia we obtained were documented in the literature (18, 19, 22, 23). Many of these factors are interdependent. Regression analysis revealed only four independent risk factors that are significantly associated with neonatal candidemia. Though the administration of ranitidine and abdominal surgery are reported to be significant risk factors, we could not find this association.

The death rate of neonates with candidemia was significantly higher than neonates without candidemia (18.1% vs. 8.5%). Other studies also report a higher death rate among systemic candidiasis compared to other inpatients of the NICU (24).The duration of hospitalization was also high for cases.

The independent risk factors of mortality in neonatal candidemia were apnoea, abdominal surgery, and invasive resuscitation. The increased mortality in the babies who underwent abdominal surgery may be linked to large inoculum size of candida organisms, which is already colonized in GIT and use of broad-spectrum antibiotics after surgery, which may facilitate its growth. The anaerobic environment during apnoea and before resuscitation may facilitate adhesion, tissue invasion, and disruption of host immune function by candida through increased production of secretory aspartyl proteinases (SAP), which may increase the severity of the infection.

Our study has few limitations. We could not perform species differentiation among the candida organisms and antifungal susceptibility. Assessments of complications using USG abdomen & CT of the head could not be performed in many cases because of logistic problems.

In conclusion, in developing countries, Candidemia is a common cause of BSI among neonates. Candida nonalbicansis the predominant type identified. Two new signs (silent hypoxemia and sub-threshold feed tolerance) identified were more specific for neonatal candidemia. Management of maternal vaginal candidiasis during pregnancy, judicious use of antibiotics in newborns, maintaining euglycemia in the newborn period, early enteral feed, and avoiding amino acid infusion may help in the reduction of neonatal candidiasis. Early identification of this problem, especially among babies who had apnoea, active resuscitation, and abdominal surgery and managing them, may help to reduce mortality.

What is already known: Clinical diagnosis of neonatal candidemia is difficult What this study adds: Silent hypoxemia and sub-threshold feed tolerance are two most specific features of neonatal candidiasis.

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Ozonated Air Therapy for the Treatment of Upper and Lower Respiratory Tract Infections as a Potential Therapy for Prevention and Treatment of COVID19 Infection

By Sagar A. Jawale

Abstract- *Introduction:* In India and worldwide, there are millions of cases of acute respiratory infections annually killing hundreds of thousand people. It also has billions of dollars of losses worldwide. There are frequent outbreaks of deadly infections such as severe acute respiratory syndrome (SARS) in 2003, caused by a novel coronavirus (SARS-CoV), the novel swine-origin influenza A (H1N1) virus in Mexico in March 2009, Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 and the current pandemic with Novel Corona Virus -Covid19. I did my research to find a common effective, safe, and cheap therapynamed as Ozonated air inhalation therapy (OAIT) for respiratory infections.

Materials and methods: In the last one year, I treated 21 patients (group A) with upper and lower respiratory tract infections (URTI and LRTI) with 0.1 ppm Ozonated air inhalation therapy (OAIT). OAIT was given as a monotherapy. In the same time period, 36 patients (group B) were given conventional treatment in the form of antibiotics, anti-histaminic and analgesics kept as control.

Keywords: *ozonated air inhalation therapy (OAIT), OAIT for respiratory infections, OAIT for COVID 19.*

GJMR-F Classification: *NLMC Code: WD 300*



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Results: In group A, for URTI, the average time of recovery of most symptoms was 26.7 hours compared to 123 hours for group B. The average time of recovery for LRTI in group A was 38.2 hours compared to 171.9 hours, as in group B. Both variables came as statistically significant. ($P < 0.05$) The therapy group A patients, had a few and mild side effects. Three patients (14.2%) had mild cough, five patients (23.8%) had a mild headache and only one patient (4.76%) had throat irritation.

Discussion: Ozonated air inhalation therapy (OAIT) is described for the first time in the medical literature. It involves breathing Ozonated air of 0.1 parts per million (ppm) concentration by a mask for 15 minutes in adults and 5 minutes for children. Ozone is a safe gas that kills all bacteria, viruses, fungi, and molds in 60 seconds in concentration of 0.04 to 0.1 ppm, whereas the toxicity for small animals is 3 to 12 ppm. In an in vitro study, application of ozone significantly decreased the absolute count of microorganisms. In another study, a single topical application by nebulization of a low dose ozone was given to all potentially pathogenic bacterial strains with known resistance to antimicrobial agents. Their growth was completely inhibited by Ozone nebulization. Ozonated water was found to be an efficient bactericidal agent against biofilms after as little as 30 seconds of exposure. In a study for deactivating viruses (17) Ozone deactivated the following 12 viruses such as influenza, strain H3N2, HSV (herpes simplex virus type 1, rhinovirus types 1A and 14 Adenovirus types 3 and 11, mouse coronavirus, Sindbis virus (SINV), yellow fever virus (YFV), vesicular stomatitis virus (VSV), poliovirus. In a study, Systemic Ozone Therapy was given by rectal Insufflation for Immunoglobulin A deficiency proved that Ozone therapy increased levels of IgA immunoglobulin. Ozone is a potent modulator of the immune system; it creates mild oxidative stress, which makes the immune system produce a large number of Interferons, agents that attack micro-organisms, and kills them. It also increases tumor necrosis factor and interleukin-2. Ozone disrupts the integrity of the bacterial cell envelope by a process of oxidation of the phospholipids and lipoproteins, and thus kills bacteria, viruses, fungi, yeast, and protozoa. Ozone therapy stimulates the oxygen metabolism, causes an increase in the red blood cell glycolysis rate, and also activates the Krebs cycle, and increases the production of ATP. The OAIT is safe as it utilizes an Ozonized air of 0.1 ppm for 15 minutes. It is far below the toxicity level set by the FDA and OSHA. Ozone is used in medicine since a long time, particularly by naturopathic and homeopathic doctors in European countries for the treatment of various viral and bacterial infections, knee arthritis treatment by Intra-articular Ozone therapy, for slipped vertebral disc and periodontal diseases. There are numerous publications on medical Ozone therapy in indexed international journals. The OAIT is very cheap. The machine for treatment costs only USD 200 and the cost per therapy sessions is just few cents. The therapy is going to be effective against the vast majority of infectious respiratory illnesses such as Influenza, novel swine-origin influenza A (H1N1), novel coronavirus (SARS-CoV), (23) Middle East respiratory syndrome coronavirus (MERS-CoV), (23) and the current COVID19 viral infections. It will also be effective against pulmonary tuberculosis and its multi drug-resistant variant. The therapy can be immediately tried on new unknown species of micro-organisms before anything is known about them until a specific vaccine or treatment is developed.

Conclusions: OAIT is a safe, effective, cheap therapy that is readily available to the masses, particularly at the time of epidemics for upper and lower respiratory infections. We need more research and a larger number of patients to know more about it. The therapy has the potential to save many patients worldwide from a variety of respiratory infections.

Keywords: ozonated air inhalation therapy (OAIT), OAIT for respiratory infections, OAIT for COVID 19.

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I. Introduction

In India and internationally, the acute upper respiratory tract infections (URTI) and lower respiratory tract infections (LRTI) are extremely frequent. In India, about 26.3 million cases of acute respiratory infections (ARI) were reported in 2011, with an incidence of about 2,173 cases per one hundred thousand population. ARI contributes to 15-30% of all under-five deaths in India.(1)

In Greenland, the incidence of upper and lower respiratory tract infections was 1.6 and 0.9 episodes per 100 days at risk, respectively. Up to 65% of the episodes of ARI caused activity restriction; 40% led to contact with the health center. (2)

Respiratory infections have a tremendous financial loss in India, and the world over. In the united states alone, upper and lower respiratory tract infections are estimated to be responsible for approximately $15 billion in direct treatment costs. Physician charges account for about one half, and hospital care accounts for approximately one-quarter of these costs. (3)

Respiratory tract infections have a lot of mortality as well. A study (4) was conducted in the year 2013, lower respiratory tract infections caused more than 2·6 million deaths worldwide. It makes them the fifth leading cause of death overall and the leading infectious cause of death in children younger than five years.

A lot of respiratory tract infections are caused by viruses. Although there are antiviral drugs not for all viruses. They are very costly and have severe side effects, hence they are not recommended for a lot of infections. It means we have to leave a lot of viral diseases to be taken care of just by the patient's immune system.

In 2003, there was worldwide panic and chaos due to the outbreak of severe acute respiratory syndrome (SARS), caused by a novel coronavirus (SARS-CoV). SARS had claimed the lives of 774 among 8,098 affected cases scattered in 29 countries on all five continents.

The outbreak of human infection due to the novel swine-origin influenza A (H1N1) virus began in Mexico in March 2009. (7)From April 12, 2009 to April 10, 2010, us center for disease control estimated that there were 60.8 million cases (range: 43.3-89.3 million), 274,304 hospitalizations (range: 195,086-402,719), and 12,469 deaths (range: 8868-18,306) in the United States due to the (H1N1) pdm09 virus.

The Middle East respiratory syndrome coronavirus (MERS-CoV) (8) is a lethal zoonotic pathogen that was first identified in humans in Saudi Arabia and Jordan in 2012. Between April 2012 and December 2019, 2499 laboratory-confirmed cases of MERS-CoV infection, including 858 deaths (34·3% mortality) were reported from 27 countries to WHO, the majority of which were declared by Saudi Arabia (2106 cases, 780 deaths)

Currently, the world is facing a pandemic with Covid19 (9), the Novel Corona Virus with over million cases infected worldwide, and 54,226 deaths. It has mortality ranging from 2-10 %.

In the year 2018, according to WHO, a total of 1.5 million people died from TB (including 251 000 people with HIV). Tuberculosis is one of the top 10 causes of death worldwide, and the leading cause of death from a single infectious agent (above HIV/AIDS). Estimated 10 million people fell ill with tuberculosis(TB) worldwide in the year 2018 which includes, 5.7 million men, 3.2 million women, and 1.1 million children. The OAIT can be used as an adjuvant therapy in pulmonary tuberculosis. Multidrug-resistant TB (MDR-TB) has become a public health crisis and a threat to the health security. According to the WHO, there were 4,84,000 new cases of tuberculosis with resistance to rifampicin, which is the most effective first-line drug for treatment of tuberculosis. Out of the 4,84,000 cases, 78% had MDR-TB.

All these viruses keep mutating and changing their antigenicity which makes it very difficult to create a vaccine. Due to frequently changing viral strains, the vaccine is ineffective on the newly mutated virus. The treatment is equally challenging. Even today, there is no specific and guaranteed treatment to these viruses. It means such new outbreaks with new viruses will keep happening in the future, putting human life at risk. We need a general treatment option for the respiratory mico-organisms so that we get time before a specific vaccine and treatment is found. It will reduce the morbidity and mortality of respiratory tract infections.

I did my research to find an effective, safe, and cheap solution to these micro-organisms. Since we are dealing with respiratory tract infections, the new therapy to be invented should be in the form of an inhalant so that it acts on the bronchial tree as well as the alveoli of the lungs. Although a lot of anti-microbial agents such as alcohol, iodine, formalin, heatkill the mico-organisms in a petri dish, they are too toxic to be used in the human body. I also wanted my new therapy to be free of antibiotic resistance.

The name of my therapy for the above-mentioned problem is Ozonated air inhalation therapy (OAIT).Ozone is a molecule that contains three atoms of oxygen and thus has the formula O3. It is a pale blue gas with a pungent smell. Ozone was first discovered in 1839 by German scientist Christian Friedrich Schonbein.

II. Materials and Methods

A written permission from the Institutional ethical committee set at Jalgaon medical association (IMA) is taken for the study. In the last one year, I treated 21

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patients (group A) with upper and lower respiratory tract infections (URTI and LRTI) with 0.1 ppm Ozonated air inhalation therapy (OAIT). The trial was taken in my Jawale Institute of Pediatric Surgery. The OAIT was given as a monotherapy to group A without any antibiotics, antivirals, antihistaminic and analgesics. In the same time period, 36 patients (group B) were given conventional treatment in the form of antibiotics, anti-histaminic, and analgesics. Critical patients were excluded in both groups. Age range was six months to 76 years in group A and eleven months to 78 years in group B. All patients have been treated on OPD basis. Patients were distributed in both groups in a random manner. Detailed history and throat examination of all the patients were performed on every visit. Every patient's complete hemogram was done before and after 15 days of the therapy. Every patient's X-ray was done before therapy and after 15 days who had positive X- ray findings. The duration of therapy was 15 minutes for adults, and 5 minutes for children. The variables to be studied on both groups were history, clinical examination findings, complete hemograms, X- rays and time taken for total resolution of symptoms. Patients were classified into URTI and LRTI based on the above parameters. The Ozone machine used for the therapy is a modified form of air purifier that produces Ozone gas of 15 mg/ hour. It means 0.25 mg Ozone per minute. The output of the machine is calibrated with an air Ozone meter to 0.1 ppm Ozonated air. The patient breaths Ozonated air by connecting a disposable Oxygen mask with its tube to the output of the machine.

In group A, fourteen patients had URTI (66.66%) and seven patients had LRTI (33.33%). Eighteen patients had the fever (85.7%), nine had rhinorrhea (42.8%), thirteen patients had a cough (61.9%) and fourteen had a sore throat (66.66%), and three had purulent sputum (14.2%). X ray chest showed mild infiltration in three patients of LRTI in group A. All seven patients who had LRTI showed increased WBC count above normal limits. In group B, twenty patients had URTI (55.55%), and sixteen patients had LRTI (44.44%). %). Twenty-eight patients had a fever (77.7%), thirteen had rhinorrhea (36.1%), eighteen patients had a cough (50%) and twenty- one had a sore throat (58.33%) and eight had purulent sputum (22.22%). X ray chest showed mild infiltration in six patients of LRTI in group A. All fourteen patients who had LRTI showed increased WBC count above normal limits. In group A, all fourteen patients who had URTI required the treatment only once. The remaining seven patients who had LRTI required the treatment on an average three times with a gap of 24 hours.

III. Results

The patients were followed up daily for first the first seven days and then weekly for a month and monthly thereafter. The longest follow up was one year and shortest of three months. In group A, for URTI, the average time of recovery of most symptoms was 26.7 hours compared to 123 hours for group B. This difference came statistically significant ($P < 0.05$). The average time of recovery for LRTI in group A was 76.8 hours compared to 171.9 hours as in group B. This difference also came as statistically significant. ($P < 0.05$)In group A, Xray chest done after fifteen days showed total disappearance of infiltration on X ray chest in all three patients and in group B, three patients had total resolution of infiltration on Xray chest and another three had reduced infiltration. The raised WBC count in seven patients in group A came within normal limits in one-week time. Only four out of the fourteen patients had WBC count in normal limits. The remaining ten patients had a WBC count in normal limits only after twenty-one days. The therapy group A patients had a few and mild side effects. Three patients (14.2%) had a mild cough which disappeared after three hours without treatment. Five patients (23.8%) had a mild headache which disappeared in six hours. Only one patient (4.76%) had a throat irritation that disappeared after twelve hours without treatment.

IV. Discussion

Ozonated air inhalation therapy (OAIT) is described for the first time in the medical literature as a treatment of upper and lower respiratory tract infections.It involves breathing Ozonated air of 0.1 parts per million (ppm) concentration by a mask for 15 minutes in adults and 5 minutes for children. The therapy can be repeated with a 12-hour gap if necessary. In group A patients, it was given as a monotherapy to see its effectiveness. Hence, no critically ill patients were included in group A. Group B patients were kept as control who received conventional treatment for their symptoms. The recovery was dramatically fast in group A than B. OAIT was effective in URTI with just a single application. Whereas in LRTI, it required an average of three applications with a gap of 12-hours.

Rhinoviruses account for 25 to 30 percent of URIs; respiratory syncytial viruses (RSV), parainfluenza and influenza viruses, human metapneumovirus, and adenoviruses for 25 to 35 percent; corona viruses for 10 percent; and unidentified viruses for the remainder. (10) Streptococcus pneumoniae and Hemophilus influenzae were the most detected bacteria with 14.2% (20/141) followed by Klebsiella pneumoniae, 9.2% (13/141), Staphylococcus aureus, 7.1% (10/141), and Moraxella catarrhalis, 4.3% (6/141). Bacterial coinfection accounted for 23% (14/61) with Hemophilus influenzae being implicated in 19.7% (12/61). (11)

Ozone is a safe gas that kills all bacteria, viruses, fungi, and molds in 60 seconds (12).The

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concentration of ozone which kills bacteria, has been variously reported to be 0.04 to 0.1 ppm (14), whereas the toxicity for small animals is 3 to 12 ppm (Stockinger 1959). Hence 0.1 ppm is the concentration of Ozone in the air used in the therapy.

In an in vitro study (13) the antibacterial effect of ozone was tested on the suspension of three different bacteria inoculated in prepared canals of extracted human teeth. Application of ozone significantly decreased the absolute count of microorganisms (89.3%), as well as the count of each type of bacteria separately (Staphylococcus aureus 94.0%; Staphylococcus epidermidis 88.6% and Enterococcus faecalis 79.7%).

A single topical application of a low dose Ozone by nebulization was applied to the bacterial colonies in a study. It completely inhibited the growth of all potentially pathogenic bacterial strains with known resistance to antimicrobial agents. (15) The bacteria inhibited were Escherichia coli, oxacillin-susceptible Staphylococcus aureus, vancomycin-resistant Enterococcus faecalis, oxacillin-resistant Staphylococcus aureus, carbapenem-resistant Acinetobacter Baumannii, extended-spectrum beta-lactamase-producing Klebsiella pneumoniae, Acinetobacter Baumannii susceptible only to carbapenems, and Pseudomonas aeruginosa susceptible to imipenem and meropenem. All isolates were completely inhibited by the Ozone Oxygen mixture. Growth occurred in the other 2 groups which were not exposed to the Ozone Oxygen mixture.

In a study, Ozonated water was found to be an efficient bactericidal agent (16) against biofilms after as little as 30 seconds of exposure. The study proved that ozonated water effectively destroyed Staphylococcus aureus, and Pseudomonas aeruginosa bacterial biofilms in vitro.

In a study for deactivating viruses (17) Ozone deactivated the following 12 viruses:- influenza, strain H3N2, human isolate (from BC Centre for Disease Control), propagated in MDCK cells; HSV (herpes simplex virus type 1, BC-CDC), placed in Vero cells; rhinovirus types 1A and 14 (RV 1A and RV 14, from ATCC), placed in H-1 cells; Adenovirus types 3 and 11 (ATCC), in A549 cells; mouse coronavirus (MCV, from Dr. Pierre Talbot) in DBT cells. Sindbis virus (SINV), yellow fever virus (YFV), vesicular stomatitis virus (VSV), poliovirus (PV, vaccine strain), vaccinia virus (VV), all ATCC strains, were grown in Vero cells. All the stock viruses were prepared as clarified cell-free supernatants, with titers ranging from 106 to 109 pfu (plaque-forming units) per ML.

In a study, Systemic Ozone Therapy was given by rectal Insufflation for Immunoglobulin A deficiency (18). It proved that Ozone therapy is a safe, and minimally invasive treatment modality for the treatment of IgA deficiency, as it produced antioxidant and immunomodulatory effects.

Ozone is a powerful modulator of the immune system (19). Inside the blood, it creates mild oxidative stress which makes the immune system produce a large number of Interferons, agents that attack micro-organisms and kills them. Ozone increases the production of interferon and the tumor necrosis factor and interleukin-2 when administered at a concentration of between 30 and 55 μg/cc. A cascade of immunological reactions occurs after the production of interleukin-2 [19] Ozone is a twin brother of Oxygen, hence can even enter a cell and cross blood- brain barrier. Thus, the Ozone generated in minute quantities in the blood can kill tissue infection.

A study was performed to clarify the immunomodulating properties of Ozone (19) by stimulation by Ozone on 1)isolated peripheral human blood mononuclear cells (PBMC) from normal donors with either Ozone or Ozonatedserum 2) the range (in terms of O3 concentrations) of the therapeutic window 3)the stimulatory and toxic effects and 4) the pattern, of both proinflammatory and immunosuppressive cytokine production up to 86 hours after exposure to O3.Results show that ozone can act as a weak inducer of cytokines producing IL-6,IL-4, TNF-α, IFN-γ, IL-2, and IL-10. Most importantly, there is a significant relationship between cytokine production and ozone concentration. Analysis of the proliferation index shows that progressively increasing O3 concentrations inhibit IP and therefore appear cytotoxic. Ozone therapy, as a sole treatment, is shown in a case report (18) to quickly and completely resolve a rapidly advancing case of tick bite cellulitis.

Ozone disrupts the integrity of the bacterial cell envelope by a process of oxidation of the phospholipids and lipoproteins, and thus kills bacteria, viruses, fungi, yeast, and protozoa. In fungi, Ozone inhibits cell growth at certain stages. Ozone deactivates the viruses by damaging the viral capsid and upsets the reproductive cycle by disrupting the virus-to-cell contact with peroxidation. The cells have a weak enzyme coating which make them vulnerable to invasion by viruses. It also makes them susceptible to oxidation and elimination from the body, which then replaces them with healthy cells. [21] The best part of the story is micro-organisms could not develop resistance to Ozone as its action is a direct physical damage to the cell wall.

Ozone therapy stimulates the oxygen metabolism. It leads to an increase in the red blood cell glycolysis rate. This leads to the stimulation of 2,3-diphosphoglycerate which increases the amount of oxygen released to the tissues. Ozone activates the Krebs cycle by enhancing oxidative carboxylation of pyruvate. This increases the production of ATP. Ozone causes a significant reduction in NADH which oxidizes cytochrome C. There is an increased production of enzymes which act as free radical scavengers and cell-wall protectors. Such enzymes are, glutathione peroxidase, catalase and superoxide dismutase. Ozone

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also leads to the production of prostacyclin, which is a vasodilator. [21]

The argument for the safety of OAIT is as follows. The machine used for the therapy is a modified form of Ozone air purifier that produces Ozone gas from atmospheric Oxygen. Since atmospheric Oxygen is only 21% of total air, hence Ozone production is limited (0.1 ppm) and can- not reach toxic levels by any chance. As already stated, Ozone has a great level of safety margin. It kills all bacteria, viruses, fungi, and molds at a low concentration of 0.04 ppm which is extremely safe for humans. The Food and Drug Administration (FDA) allows ozone output of indoor medical devices to be 0.05 ppm or below. The Occupational Safety and Health Administration (OSHA) has set a safety limit of Ozone for the workers. It states that the workers should not be exposed to an average concentration of Ozone more than 0.10 ppm for 8 hours. OAIT exposes patient to 0.1 ppm Ozone only for 15 minutes.0.1ppm is an extremely small concentration of Ozone and means just one part in ten million parts of air.US FDA has approved Ozone gas for the processing of a variety of dairy, food and poultry products in year 2001. (24)A lot of European countries add Ozone in water as a disinfectant treatment. The concentrations used are about ten ppm. After drinking Ozonated water, the gas is also absorbed in the blood. Yet no side effects are reported in the medical literature about drinking Ozonated water. French people are drinking Ozonated water for more than 100 years now without any problem.

The ancient Vedic plant holy basil, is also known as Tulsi (Ocimum Tenuiflorum), produces oxygen for 20 hours and Ozone for 4 hours. We need modern research to prove this claim. That is why every household was advised to have it and worship it. The Ozone produced by the plant has saved millions of Indians of deadly respiratory infections in the era when there were no antibiotics and antivirals. Even today, everyone can have these plants in the garden and gallery of houses for the said reason.

Ozone is used in medicine for a long time, particularly by naturopathic and homeopathic doctors in European countries. The common indications are a variety of viral and bacterial infections, Intraarticular Ozone therapy for knee arthritis, for slipped vertebral disc and for periodontal diseases (22). There are numerous publications on medical ozone therapy in indexed international journals.

Ozone is a natural part of the atmosphere with the concentration of ten ppm in the upper stratosphere. The natural amount of ozone in the lower atmosphere is generally around 0.04 ppm. That amount is not harmful to human health. It means we are breathing 0.04 ppm Ozone since time immemorial and is perfectly normal. It appears to be nature's plan for keeping micro-organisms in check to avoid epidemics.

But, due to environmental pollution, the Ozone in the lower and upper atmosphere has dramatically reduced creating an Ozone hole. I measured Ozone concentration with an air Ozone meter in the air around my city in many places and it is nearly zero due to pollution. That is why we have to produce Ozone artificially indoors and outdoors to protect ourselves from deadly microbial infections. One such way is to use indoor Ozone air purifiers which produce Ozone in 0.05 ppm concentration. If an air Ozone air purifier is put in the room of an infected person, his infection is not going to spread. I prefer to keep one in my consulting room that keeps me safe from the micro-organisms brought by the patients. If you keep it on 24/7, viruses can-not enter your house and office. We need Ozone air purifiers in every public place like railway waiting rooms, railway bougies, Buses, cars, airport, aero plane cabins, school, hospitals, offices, doctor's consulting rooms, etc. By this policy, we are going to prevent a lot of infectious respiratory illness and epidemics. By use of mass-scale Ozone air purifiers, we are also doing service to humanity as it will help healing the Ozone hole faster.

One reason for apathy towards Ozone therapy is that Ozone is a naturally occurring gas and can- not be patented. It is very cheap and no money can be earned by selling it. But, such a commercial attitude of the corporations is hurting the interest of patients.

OAIT is very cheap. The machine for therapy costs only USD 200 and the cost per therapy sessions is just few cents. That is why, it can be delivered on a mass scale in epidemics. The therapy is affordable even to developing countries; Hence, it will have a vast positive impact on global healthcare.

The therapy is going to be effective against a vast majority of infectious respiratory illnesses such as Influenza, novel swine-origin influenza A (H1N1), novel coronavirus (SARS-CoV), (23)Middle East respiratory syndrome coronavirus (MERS-CoV), (23) and the current COVID19 viral infections. It will also be effective against pulmonary tuberculosis and its multi drug-resistant variant.

The influenza group of viruses have an incubation period of 2 to 4 days. Some of the strains have incubation period of 7-14 days. During this period, the person remains a carrier and spreads the infection. The OAIT can be given to such carriers to eradicate the infection, and the spread of the disease can be limited. All the contacts of deadly infections such as H1N1, MERS-CoV and Covid 19 can be subjected to a prophylactic OAIT to eradicate the organism in the incubation period itself.

By giving OAIT early to every patient of URTI, we can reduce the morbidity as well as mortality as the disease will not progress further, which leads to a lot of complications.

The therapy can be immediately tried on new unknown species of micro-organisms before anything is

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known about them until a specific vaccine or treatment is developed. The therapy will save many lives as many people lose their lives till specific treatment or vaccines are invented.

I do not claim that OAIT will be equal or superior to the specific vaccine or specific drug treatment. But the therapy is safe, cheap, and easily available with great compliance. A large part of the world population can only afford and have access to such a therapy particularly in desperate situations such as epidemics.

I also agree that this article is only the beginning of this type of research. A vast number of studies and clinical trials will be necessary on variety of micro-organisms in the future to perfect the therapy.

V. Conclusions

OAIT is a safe, effective, cheap therapy that is easily available to the masses particularly at the time of epidemics for the upper and lower respiratory infections. We need more research and a larger number of patients to know more about it. The therapy has the potential to save many patients worldwide from a variety of respiratory infections. The treatment can be used as an immediate measure for new infections with unknown micro-organisms even before a specific vaccine and treatment is developed.

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Relationship of Clinical Manifestations of Heart Connective Tissue Dysplasis with Indicators of Hydroxyprolin Level and Mineral Imbalance

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Abstract- Connective tissue dysplasia is a unique ontogenetic anomaly in the development of the body, which is one of the complex, far from studied questions of modern medicine, and is the morphological basis of functional changes in cardiac activity. We studied 115 children of preschool and school age with connective tissue dysplasia and small heart development abnormalities. A high frequency of occurrence of external phenotypic markers was revealed for the syndrome of dysplasia of the connective tissue of the heart and stigma of embryogenesis. An increase in the average level of hydroxyproline in blood serum in children with small abnormalities of the development of the heart in combination with cardiovascular pathology and patterns characterizing the relationship of the clinical manifestations of the disease and mineral imbalance has been established.

Keywords: connective tissue dysplasia, small abnormalities of the development of the heart, hydroxyproline, trace elements, children.

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Relationship of Clinical Manifestations of Heart Connective Tissue Dysplasis with Indicators of Hydroxyprolin Level and Mineral Imbalance

Feruza Mahmudjanovna Akhrarova

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Keywords: connective tissue dysplasia, small abnormalities of the development of the heart, hydroxyproline, trace elements, children.

I. Introduction

Despite the great interest in recent years in the connective tissue dysplasia syndrome, many issues regarding the formation of cardiovascular pathology in children with cardiac manifestations of connective tissue dysplasia remain to date poorly understood. The development of many heart abnormalities is based on dysplasia of the connective tissue of the heart. Connective tissue dysplasia is a unique ontogenetic anomaly of the body's development, which is one of the complex, far from studied questions of modern medicine [10, 12]. These anomalies are the morphological basis of functional changes in cardiac activity, and with organic lesions of the heart can aggravate their prognosis [4, 8].

One of the most comprehensive definitions of connective tissue dysplasia is a genetically determined disorder in the development of connective tissue, characterized by defects in the fibrous structures and the main substance of the connective tissue, leading to a disorder of homeostasis at the tissue, organ and organism levels, in the form of various morphological and functional disorders, visceral and locomotor organs with progressive the course and determining features of associated pathology, as well as pharmacokinetics and pharmacodynamics of drugs [5, 7].

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Small abnormalities of the development of the heart is one of the manifestations of connective tissue dysplasia, so they can be combined with other signs of it. Small abnormalities in the development of the heart in children are a fairly common condition. According to various authors, small abnormalities of heart development occur from 2.2 to 10% of cases, in children with pathology of the cardiovascular system - in 10–25% of cases (up to 68.9%, depending on the contingent of subjects) [2, 9].

The aim of the study is to study the relationship of the clinical manifestations of dysplasia of the connective tissue of the heart with indicators of the level of hydroxyproline and an imbalance of trace elements.

II. Materials and Methods

We studied 115 preschool and school-age children who received inpatient treatment in the departments of cardiac rheumatology of the Central Clinical Hospital No. 4 and the Tashkent Medical Institute, Tashkent Of these, 95 children with connective tissue dysplasia and small abnormalities in the development of the heart and 20 practically healthy children of a similar age who made up the control group. Of the 95 children with connective tissue dysplasia, 55 comprised group I — with cardiovascular pathology against the background of small abnormalities of the heart and 40 children — group II without cardiovascular pathology against the background of small abnormalities of the heart.

The external and internal phenotypic characters, the age-sex structure, the nature of complaints, as well as the characteristics of the markers of connective tissue metabolism were studied.

III. Results and Discussion

The study of the age category in the studied groups of children with small abnormalities of heart development showed that the majority of patients were teenagers from 8 to 12 and 12-16 years old, in the group of practically healthy children were mostly young children and adolescents 12-16 years old (Fig. 1).

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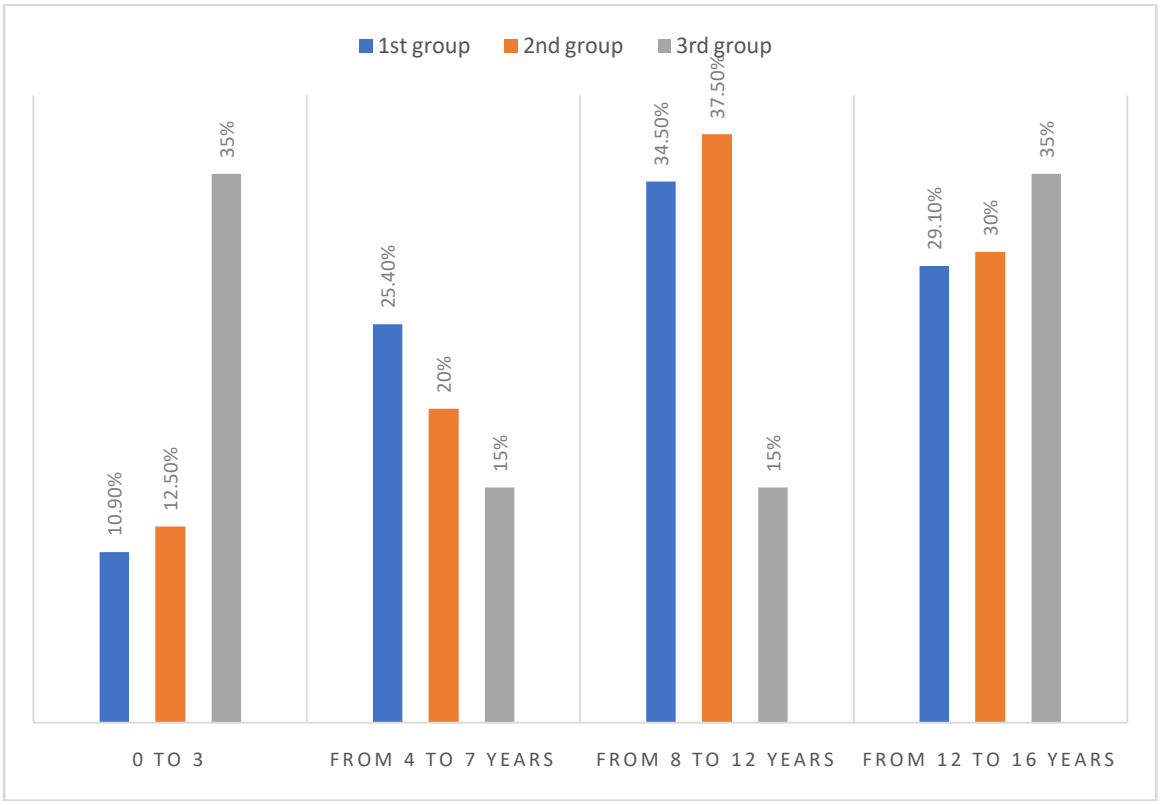


Fig. 1: Age categories of the examined patients

When analyzing gender differences among the examined children with small abnormalities of the heart, in combination with cardiovascular pathology and without cardiovascular pathology, the prevalence of boys was revealed - 60% and 52.5% (Fig. 2).

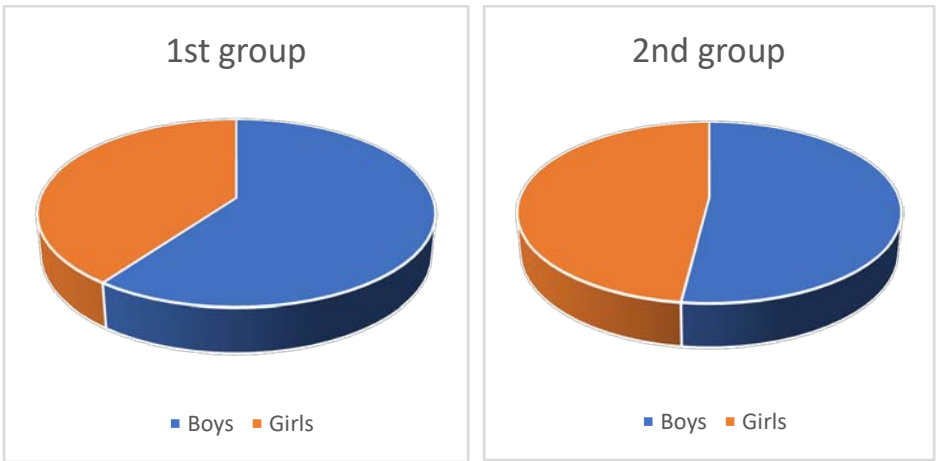


Fig. 2: The distribution of the examined patients depending

The clinical picture in children with small abnormalities in the development of the heart is quite diverse. Its manifestations often begin in adolescence. An analysis of complaints in patients with small abnormalities of the heart shows that significantly more often complaints were presented by children with small abnormalities of the heart, weighed down by cardiovascular pathology. The leading cardiovascular pathology in the children examined by us with minor cardiac abnormalities was arrhythmic syndrome (Table 1).

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Table 1: Characterization of the frequency of complaints in patients with minor abnormalities of the heart

Complaints	I-group (n = 55)	II –group (n = 40)
Heartfailure	39 (70,9%)	14 (35,0%)
Cardialgia	21 (38,2%)*	9 (22,5%)
Heartbeats	40 (72,7%)*	17 (42,5%)
Headache	29 (52,7%)**	9 (22,5%)
Dizziness	16 (29,1%)	8 (20,0%)
Decreasedappetite	23 (41,8%)	13 (32,5%)
Fatigability	50 (90,9%)*	21 (52,5%)
Apathy	19 (34,5%)**	5 (12,5%)
Thefeelingof "chilliness"	21 (38,1%)*	11 (27,5%)
Coolingbrushes	17 (30,9%)	10 (25,0%)

*Note: * - reliability (p <0.01) between group I and II of patients with connective tissue dysplasia, ** - reliability (p <0.05) between group I and II of patients with connective tissue dysplasia*

Children with a combination of mitral valve prolapse and abnormal left ventricular complaints more often complained of “aching” pains in the heart area, a feeling of palpitations, cephalgia and dizziness, fatigue, a feeling of “coldness” and cooling hands, at room temperature. Dizziness appeared when the body position changed (from the wedge to orthosis) and with a sharp turn of the head in 2/3 of the children. Among other complaints, 15.8% of the general population of children with small abnormalities of heart development also had dyspeptic disorders in the form of abdominal pain, not always associated with eating, heartburn, heaviness in the right hypochondrium, fast satiety, constipation.

Pallor of the skin was observed in 41.0% of children with mitral valve prolapse, in 32.0% with abnormal left ventricular passages and 47.4% with a combination of mitral valve prolapse and abnormal left ventricular passages.

To identify the informative value of phenotypic signs in the diagnosis of cardiovascular pathology, we scored external and visceral signs of systemic involvement of connective tissue in children (Table 2).

Table 2: Scoring of external and visceral signs of systemic involvement of connective tissue in children

Signs	Total points in group I	Total points in group II
Osteoarticular		
Funnelchestdeformity	98	84
Keeledchestdeformity	30	22,5
Dolichostenomelia	40	17,5
Scoliosis	96	56
Kyphosis	64	28
joint hypermobility	100	72
Halluxvalgus	30	30
Arachnodactyly	15	0
Otherchestdeformity	16,5	18
Flatfeet	50	35
Ectodermal (skin, teeth):		
Hyperpigmentation of the skin over the spinous processes of the vertebrae	56	54
Increasedskinextensibility	58,5	42
Ecchymoses, petechiae, nosebleeds	69	57
Atrophicstriae	24	4,5
"Corns" on the back surface of the feet	21	15
Visiblevenousnetwork	16	15
Teethingabnormalities	14	18
Muscular:		
Musclehypotension	82,5	60
Visceralsymptoms		
Osteopeniasevere / moderate	100	58
Mitral valve prolapse (all types) / other minor abnormalities of the heart	100	62
Other minor abnormalities of the heart	42	31,5
Biliary dyskinesia against the background of an abnormality of the gallbladder	57	39
Refluxdisease	32	25

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An analysis of the obtained data shows that the most characteristic phenotypic signs in children with small abnormalities of the heart and cardiovascular pathology are: funnel chest deformity, keeled chest deformity, dolichostenomelia, scoliosis, kyphosis, hypermobility of joints, muscle hypotension, osteopenia, mitral valve prolapse all types) / other small abnormalities of the development of the heart.

The main component of connective tissue is collagen, accounting for more than 30% of the total mass of body proteins, with 50% of it being in bone and tendon-muscle tissue [5]. The amino acid composition of collagen has been well studied. However, specific markers of this protein are proline and hydroxyproline [7]. As a result of the breakdown of collagen, peptides are excreted in the urine or cleaved by specific enzymes to amino acids. Hydroxyproline is an amino acid that is part of collagen, a protein of bone and connective tissue, which is an indicator of their metabolic rate, released from peptides is found mainly in blood and urine, and part of it is oxidized in the liver [1,7]. Its increase is observed in diseases associated with the breakdown of connective tissue. The appearance of hydroxyproline in blood serum and urine is the result of catabolic processes in the connective tissue and may reflect the degree of activity of this process [6].

A study of the serum hydroxyproline content showed that the level of free hydroxyproline in the blood in patients with small abnormalities of the heart and cardiovascular pathology was significantly ($P < 0.05$) higher compared with patients with small abnormalities of the heart without cardiovascular pathology and amounted to - 29, 4 ± 2.4 μmol / L and 20.2 ± 1.5 μmol / L, respectively. The indicators of hydroxyproline in healthy children amounted to 16.1 ± 1.2 μmol / L (table. 3).

Table 3: The serum hydroxyproline content in children in the studied groups (M ± m; μmol / L)

Groupofpatients	The average level of hydroxyproline
Small abnormalities of the development of the heart with cardiovascular pathology	29,4 ± 2,4*
Small abnormalities of the development of the heart without cardiovascular pathology	20,2 ± 1,5
Healthychildren	16,1 ± 1,2

*Note: * - reliability between indicators of the compared groups (P <0.05).*

The content of trace elements of selenium, copper, manganese and magnesium in blood serum was studied in 30 examined children, 12 of them with small abnormalities of the heart and cardiovascular pathology, 8 with small abnormalities of the heart without cardiovascular pathology and 10 practically healthy children. It was found that the microelement profile in children with small abnormalities of the heart, complicated by cardiovascular pathology, compared with children with small abnormalities of the heart without cardiovascular pathology, is characterized by a decrease in the concentration of selenium (Se) ($p > 0.01$), copper (Cu) ($p > 0.01$), manganese (Mn) ($p > 0.01$) and magnesium (Mg) ($p < 0.05$) in blood serum (Table 4).

Table 4: Indicators of trace elements in the blood in the studied groups

(mcg / g)	Se	Cu	Mn	Mg
Children with small abnormalities of the development of the heart and CVP (I-group) (n = 12)	0,052±0,015	0,493±0,076	0,0056±0,0009	0,508±0,092
Children with minor abnormalities of the heart without CVP (group II) (n = 8)	0,067±0,014	0,592±0,071	0,0073±0,0016	0,585±0,083
Controlgroup (III –group) (n = 10)	0,178±0,055	0,918±0,172	0,033±0,035	0,845±0,062
P 1:2	> 0,01	> 0,01	> 0,01	<0,05
P 1:3	< 0,05	< 0,05	> 0,01	< 0,05
P 2:3	> 0,01	< 0,05	< 0,05	> 0,01

Note: P is the reliability between the indicators of the examined groups of children.

Analyzing the content of selenium in groups of patients with small abnormalities of the development of the heart and cardiovascular pathology, and the control group, a significant deficiency of this microelement was revealed ($p < 0.05$). With a low selenium content in mothers during pregnancy, infant mortality increases

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and the number of children with various deformities increases. The relationship between the deficit of Se and Cu is noted.

With copper deficiency, disorders of the synthesis of connective tissue, functional disorders of the nervous system, impaired liver function, decreased immunobiological reactivity, damage to the eyes, blood-forming organs, allergic contact dermatitis, and bone formation disorders are noted. It is known that both inadequate and excessive intake of copper in the body can lead to a violation of vital functions, especially during pregnancy. By analyzing the copper content in the compared groups, a comparative copper deficiency was revealed in both groups of children with small heart development abnormalities, with relatively stable rates being normal. In children with small abnormalities of the heart, weighed down by cardiovascular pathology, a significant ($p < 0.05$) decrease in the level of copper was revealed in comparison with the control group.

The importance of magnesium in the development of connective tissue disorders, in the treatment and rehabilitation of patients with connective tissue dysplasia is described in a number of works [3, 11]. Today it is known that magnesium ions are involved in the processes of connective tissue metabolism, control the normal functioning of cardiomyocytes at all levels of subcellular structures, and are involved in the regulation of myocardial contractile function. At the same time, intracellular magnesium deficiency increases the activity of the sinus node, which shortens the time for atrioventricular conduction, reduces absolute refractoriness and lengthens the relative refractoriness, which can lead to the development of various rhythm disturbances. An analysis of the content and dynamics of the levels of magnesium and manganese shows that they are similar to the first two trace elements and can serve as a kind of indicator of the course of the main process involving the immune system in the pathological process, which indicates the depletion of protective resources and leads to the development or aggravation of the pathological process.

IV. Conclusion

1. The analysis of clinical and phenotypic manifestations of connective tissue dysplasia syndrome in children with small cardiac abnormalities revealed that children with minor cardiac abnormalities burdened by cardiovascular pathology presented significantly more often complaints;
2. A high frequency of occurrence of external phenotypic markers of dysplasia of the connective tissue of the heart and stigma of embryogenesis was revealed. The relationship between the profiles of the external stigma of connective tissue dysplasia and small heart abnormalities has been established;
3. There was an increase in the average level of hydroxyproline in blood serum in children with small cardiac abnormalities in combination with cardiovascular pathology, compared with healthy children;
4. The regularities characterizing the relationship of the clinical manifestations of diseases and mineral imbalance are established. Some pathogenetic lines of the development of the pathological process in children with dyslementoses were determined. It is proved that the state of elemental status is an important informative criterion for assessing the severity of the underlying disease.

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- The names of first main headings (Heading 1) must be in Roman font, capital letters, and font size of 10.
- The names of second main headings (Heading 2) must not include numbers and must be in italics with a font size of 10.

Structure and Format of Manuscript

The recommended size of an original research paper is under 15,000 words and review papers under 7,000 words. Research articles should be less than 10,000 words. Research papers are usually longer than review papers. Review papers are reports of significant research (typically less than 7,000 words, including tables, figures, and references)

A research paper must include:

a) A title which should be relevant to the theme of the paper.
b) A summary, known as an abstract (less than 150 words), containing the major results and conclusions.
c) Up to 10 keywords that precisely identify the paper's subject, purpose, and focus.
d) An introduction, giving fundamental background objectives.
e) Resources and techniques with sufficient complete experimental details (wherever possible by reference) to permit repetition, sources of information must be given, and numerical methods must be specified by reference.
f) Results which should be presented concisely by well-designed tables and figures.
g) Suitable statistical data should also be given.
h) All data must have been gathered with attention to numerical detail in the planning stage.

Design has been recognized to be essential to experiments for a considerable time, and the editor has decided that any paper that appears not to have adequate numerical treatments of the data will be returned unrefereed.

i) Discussion should cover implications and consequences and not just recapitulate the results; conclusions should also be summarized.
j) There should be brief acknowledgments.
k) There ought to be references in the conventional format. Global Journals recommends APA format.

Authors should carefully consider the preparation of papers to ensure that they communicate effectively. Papers are much more likely to be accepted if they are carefully designed and laid out, contain few or no errors, are summarizing, and follow instructions. They will also be published with much fewer delays than those that require much technical and editorial correction.

The Editorial Board reserves the right to make literary corrections and suggestions to improve brevity.



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Format Structure

It is necessary that authors take care in submitting a manuscript that is written in simple language and adheres to published guidelines.

All manuscripts submitted to Global Journals should include:

Title

The title page must carry an informative title that reflects the content, a running title (less than 45 characters together with spaces), names of the authors and co-authors, and the place(s) where the work was carried out.

Author details

The full postal address of any related author(s) must be specified.

Abstract

The abstract is the foundation of the research paper. It should be clear and concise and must contain the objective of the paper and inferences drawn. It is advised to not include big mathematical equations or complicated jargon.

Many researchers searching for information online will use search engines such as Google, Yahoo or others. By optimizing your paper for search engines, you will amplify the chance of someone finding it. In turn, this will make it more likely to be viewed and cited in further works. Global Journals has compiled these guidelines to facilitate you to maximize the web-friendliness of the most public part of your paper.

Keywords

A major lynchpin of research work for the writing of research papers is the keyword search, which one will employ to find both library and internet resources. Up to eleven keywords or very brief phrases have to be given to help data retrieval, mining, and indexing.

One must be persistent and creative in using keywords. An effective keyword search requires a strategy: planning of a list of possible keywords and phrases to try.

Choice of the main keywords is the first tool of writing a research paper. Research paper writing is an art. Keyword search should be as strategic as possible.

One should start brainstorming lists of potential keywords before even beginning searching. Think about the most important concepts related to research work. Ask, “What words would a source have to include to be truly valuable in a research paper?” Then consider synonyms for the important words.

It may take the discovery of only one important paper to steer in the right keyword direction because, in most databases, the keywords under which a research paper is abstracted are listed with the paper.

Numerical Methods

Numerical methods used should be transparent and, where appropriate, supported by references.

Abbreviations

Authors must list all the abbreviations used in the paper at the end of the paper or in a separate table before using them.

Formulas and equations

Authors are advised to submit any mathematical equation using either MathJax, KaTeX, or LaTeX, or in a very high-quality image.

Tables, Figures, and Figure Legends

Tables: Tables should be cautiously designed, uncrowned, and include only essential data. Each must have an Arabic number, e.g., Table 4, a self-explanatory caption, and be on a separate sheet. Authors must submit tables in an editable format and not as images. References to these tables (if any) must be mentioned accurately.

Figures

Figures are supposed to be submitted as separate files. Always include a citation in the text for each figure using Arabic numbers, e.g., Fig. 4. Artwork must be submitted online in vector electronic form or by emailing it.

Preparation of Eletronic Figures for Publication

Although low-quality images are sufficient for review purposes, print publication requires high-quality images to prevent the final product being blurred or fuzzy. Submit (possibly by e-mail) EPS (line art) or TIFF (halftone/ photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Avoid using pixel-oriented software. Scans (TIFF only) should have a resolution of at least 350 dpi (halftone) or 700 to 1100 dpi (line drawings). Please give the data for figures in black and white or submit a Color Work Agreement form. EPS files must be saved with fonts embedded (and with a TIFF preview, if possible).

For scanned images, the scanning resolution at final image size ought to be as follows to ensure good reproduction: line art: >650 dpi; halftones (including gel photographs): >350 dpi; figures containing both halftone and line images: >650 dpi.

Color charges: Authors are advised to pay the full cost for the reproduction of their color artwork. Hence, please note that if there is color artwork in your manuscript when it is accepted for publication, we would require you to complete and return a Color Work Agreement form before your paper can be published. Also, you can email your editor to remove the color fee after acceptance of the paper.

Tips for writing a good quality Medical Research Paper

1. Choosing the topic: In most cases, the topic is selected by the interests of the author, but it can also be suggested by the guides. You can have several topics, and then judge which you are most comfortable with. This may be done by asking several questions of yourself, like "Will I be able to carry out a search in this area? Will I find all necessary resources to accomplish the search? Will I be able to find all information in this field area?" If the answer to this type of question is "yes," then you ought to choose that topic. In most cases, you may have to conduct surveys and visit several places. Also, you might have to do a lot of work to find all the rises and falls of the various data on that subject. Sometimes, detailed information plays a vital role, instead of short information. Evaluators are human: The first thing to remember is that evaluators are also human beings. They are not only meant for rejecting a paper. They are here to evaluate your paper. So present your best aspect.

2. Think like evaluators: If you are in confusion or getting demotivated because your paper may not be accepted by the evaluators, then think, and try to evaluate your paper like an evaluator. Try to understand what an evaluator wants in your research paper, and you will automatically have your answer. Make blueprints of paper: The outline is the plan or framework that will help you to arrange your thoughts. It will make your paper logical. But remember that all points of your outline must be related to the topic you have chosen.

3. Ask your guides: If you are having any difficulty with your research, then do not hesitate to share your difficulty with your guide (if you have one). They will surely help you out and resolve your doubts. If you can't clarify what exactly you require for your work, then ask your supervisor to help you with an alternative. He or she might also provide you with a list of essential readings.

4. Use of computer is recommended: As you are doing research in the field of medical research then this point is quite obvious. Use right software: Always use good quality software packages. If you are not capable of judging good software, then you can lose the quality of your paper unknowingly. There are various programs available to help you which you can get through the internet.

5. Use the internet for help: An excellent start for your paper is using Google. It is a wondrous search engine, where you can have your doubts resolved. You may also read some answers for the frequent question of how to write your research paper or find a model research paper. You can download books from the internet. If you have all the required books, place importance on reading, selecting, and analyzing the specified information. Then sketch out your research paper. Use big pictures: You may use encyclopedias like Wikipedia to get pictures with the best resolution. At Global Journals, you should strictly follow here.



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6. Bookmarks are useful: When you read any book or magazine, you generally use bookmarks, right? It is a good habit which helps to not lose your continuity. You should always use bookmarks while searching on the internet also, which will make your search easier.

7. Revise what you wrote: When you write anything, always read it, summarize it, and then finalize it.

8. Make every effort: Make every effort to mention what you are going to write in your paper. That means always have a good start. Try to mention everything in the introduction—what is the need for a particular research paper. Polish your work with good writing skills and always give an evaluator what he wants. Make backups: When you are going to do any important thing like making a research paper, you should always have backup copies of it either on your computer or on paper. This protects you from losing any portion of your important data.

9. Produce good diagrams of your own: Always try to include good charts or diagrams in your paper to improve quality. Using several unnecessary diagrams will degrade the quality of your paper by creating a hodgepodge. So always try to include diagrams which were made by you to improve the readability of your paper. Use of direct quotes: When you do research relevant to literature, history, or current affairs, then use of quotes becomes essential, but if the study is relevant to science, use of quotes is not preferable.

10. Use proper verb tense: Use proper verb tenses in your paper. Use past tense to present those events that have happened. Use present tense to indicate events that are going on. Use future tense to indicate events that will happen in the future. Use of wrong tenses will confuse the evaluator. Avoid sentences that are incomplete.

11. Pick a good study spot: Always try to pick a spot for your research which is quiet. Not every spot is good for studying.

12. Know what you know: Always try to know what you know by making objectives, otherwise you will be confused and unable to achieve your target.

13. Use good grammar: Always use good grammar and words that will have a positive impact on the evaluator; use of good vocabulary does not mean using tough words which the evaluator has to find in a dictionary. Do not fragment sentences. Eliminate one-word sentences. Do not ever use a big word when a smaller one would suffice.

Verbs have to be in agreement with their subjects. In a research paper, do not start sentences with conjunctions or finish them with prepositions. When writing formally, it is advisable to never split an infinitive because someone will (wrongly) complain. Avoid clichés like a disease. Always shun irritating alliteration. Use language which is simple and straightforward. Put together a neat summary.

14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.



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Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.



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Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

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Administration Rules to Be Strictly Followed before Submitting Your Research Paper to Global Journals Inc.

Please read the following rules and regulations carefully before submitting your research paper to Global Journals Inc. to avoid rejection.

Segment draft and final research paper: You have to strictly follow the template of a research paper, failing which your paper may get rejected. You are expected to write each part of the paper wholly on your own. The peer reviewers need to identify your own perspective of the concepts in your own terms. Please do not extract straight from any other source, and do not rephrase someone else's analysis. Do not allow anyone else to proofread your manuscript.

Written material: You may discuss this with your guides and key sources. Do not copy anyone else's paper, even if this is only imitation, otherwise it will be rejected on the grounds of plagiarism, which is illegal. Various methods to avoid plagiarism are strictly applied by us to every paper, and, if found guilty, you may be blacklisted, which could affect your career adversely. To guard yourself and others from possible illegal use, please do not permit anyone to use or even read your paper and file.

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Please note that following table is only a Grading of "Paper Compilation" and not on "Performed/Stated Research" whose grading solely depends on Individual Assigned Peer Reviewer and Editorial Board Member. These can be available only on request and after decision of Paper. This report will be the property of Global Journals.

Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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