Responses to reviewers:

We thank the reviewers for their patient efforts in find merit in our garbled ideas and prose: we tried to crystallize over 30 years of work on the topic. They helped in refining our presentation to provide clarity.

Reviewer 1:

Major Comments:

1. Are there controversies in this field? What are the most recent and important achievements in the field? In my opinion, answers to these questions should be emphasized. Perhaps, in some cases, novelty of the recent achievements should be highlighted by indicating the year of publication in the text of the manuscript.

a. **Response**: All three aspects have been highlighted in the revised manuscript, which has in part been completely re-written to incorporate the suggestions made

2. The discussion section is modest.

a. **Response**: The revised and re-written manuscript rectifies the deficiency

3. Abstract: not properly written.

a. **Response**: The abstract has been completely re-written

4. Conclusion: The section devoted to the explanation of the results suffers from the same problems revealed so far. Your storyline in the results section (and conclusion) is hard to follow. Moreover, the conclusions reached are really far from what one can infer from the empirical results.

a. **Response**: The inadequacies are addressed in the completely re-written portions of the revised manuscript

5. The discussion should be rather organized around arguments avoiding simply describing details without providing much meaning.

a. **Response**: Corrected in the re-written portion of the revision

6. Spacing, punctuation marks, grammar, and spelling errors should be reviewed thoroughly. I found so many typos throughout the manuscript. 7. English is modest. Therefore, the authors need to improve their writing style. In addition, the whole manuscript needs to be checked by native English speakers.

a. **Response** The deficiencies have been rectified and vetted by a native English speaker

Reviewer 2:

Manuscript ID: 87202 - World Journal of Experimental Medicine Manuscript Title: Emerging significance of butyrylcholinesterase Dear authors, In order to contribute to the improvement of the manuscript, some suggestions are proposed according to the guideline for minireview:

1. The title, authorship, institution, ORCID number, corresponding author, core tip, abstract and key words are missing;

a. **Response**: The information was submitted in the editorial manager; it was a lapse on our part not to have included them in the manuscript. The error has been rectified

2. The subject of mini-reviews is interesting and relevant, but most of the information is compiled in recent reviews.

a. **Response**: The focus of the review was on the potential benefits of studying individuals with loss-of-function genes. Therefore, detailed information was provided related

to this. Original studies are available in the comprehensive reviews to which the reviewer correctly referred

3. In addition, the manuscript has several formatting errors: lack of spacing or excessive spacing between words, lack of standardization in the form of writing or abbreviations, lack of formatting in general, suggesting a lack of care with the work.

a. **Response**: These deficiencies have been addressed and repaired in the revised manuscript

4. The only topic that is actually more innovative is the association of BuChE with cognition, but it was very little explored.

a **Response**: The section on BuChE has been expanded and re-written to incorporate the suggestion that has been given

Responses to the Editor:

(1) Two tables have been added

(2) Supplementary material shows family trees of individuals with variant form of the BuChE. The data were presented at 12th International Meeting on Cholinesterases-Sixth International Conference on Paraoxonases at Elche (Alicante, Spain) in 2015

(3) New and highly relevant references have been obtained according to the guidance given by the Editor

Revisions made in response to the reviewers comments and suggestion

Name of Journal: World Journal of Experimental Medicine

Manuscript Type: MINIREVIEWS

Emerging significance of butyrylcholinesterase

Sridhar GR, Lakshmi G

Sridhar GR, Endocrine and Diabetes Centre, Visakhapatnam 530002, India

Lakshmi G, Department of Medicine, Gayatri Vidya Parishad Institute of Healthcare & Medical Technology, Visakhapatnam, Andhra Pradesh, India ORCID No: 0000-00021368745X

Author contributions: Both authors contributed equally to the writing of the manuscript

Supported by: None

Corresponding author: Sridhar GR, MD DM FRCP, Endocrine and Diabetes Centre, 15-12-15 Krishnanagar, Visakhapatnam 530 002, India sridharvizag@gmail.com ORCID NO: 0000-0002-7446-1251

Abstract
Butyrylcholinesterase (BuChE; EC 3.1.1.8), an enzyme structurally related to acetylcholinesterase is widely distributed in the human body. It has a role in the detoxification of chemicals such as succinylcholine, which is a muscle relaxant used in anesthetic practice. It is well known because variant forms of the enzyme with little or no hydrolytic activity exist in some communities. Exposure to succinylcholine results in prolonged apnea. Its other functions include an ability to hydrolyze acetylcholine, the cholinergic neurotransmitter in the brain when its primary hydrolytic enzyme acetylcholinesterase is absent. To assess if it has other potential roles, it was studied in relation to insulin resistance, type 2 diabetes mellitus, cognition, hepatic disorders, cardiovascular and cerebrovascular disease, and inflammatory conditions. It appears to have an influenced and be influenced to these conditions via lipid metabolism. Individuals who lack enzyme activity are otherwise healthy other than having adverse outcomes on exposure to drugs that are hydrolyzed by it. Therefore, it is a candidate for the study of loss of function mutations in humans. Longitudinal studies in these individuals can assess if they are protective against metabolic diseases, or if the enzyme can be used as a biomarker for Alzheimer's disease and response to its drug treatment.

Key words: Esterase; Acetylcholinesterase; Variant; Cholinergic; Metabolic syndrome; Cognition; Knock-out model

Sridhar GR, Lakshmi G, Emerging significance of butyrylcholinesterase

Core tip: Butyrylcholinesterase [BuChE], a hepatic enzyme, hydrolyzes succinylcholine, a muscle relaxant. Its other functions are not clearly delineated. Individuals with variant forms of the enzyme are healthy, unless they are given succinylcholine during anesthesia. The enzyme may have regulatory roles in lipid metabolism, cholinergic response and in Alzheimer's disease. People with variant forms of the enzyme are natural human knock-out model and can be followed up to study the impact of harboring variant forms of BuChE.

BUTYRYLCHOLINESTERASE AND COGNITION

Alzheimer's disease (AD) that often accompanies aging is the most common cause of cognitive decline [36]. According to the cholinergic hypothesis, degeneration of cholinergic neurons in the basal forebrain results in cognitive dysfunction in Alzheimer's disease [37]. Others that act via the cholinergic system include amyloid deposition, tau phosphorylation, neuroinflammation and vascular damage (Pozzi et al, 2022) [38]. Acetylcholine, the neurochemical transmitter in the cholinergic synapses is inactivated by AChE, and to a lesser extent by BuChE. The cholinergic system is part of the cholinergic anti-inflammatory pathway. The cholinergic hypothesis received additional support by cholinesterase inhibitor

drugs which increase acetylcholinesterase at the synaptic cleft are effective in the management of AD [39].

Apart from its role in the cholinergic hypothesis, BuChE has been implicated in the deposition of amyloid. Amyloid hypothesis proposes that abnormal folding of β amyloid protein may contribute to the pathogenesis of Alzheimer's disease [40]. Variant form of the enzyme, called K-variant (Ala567Thr (A539T) may act synergistically with others such 4 allele of apolipoprotein E and iron as a risk factor for Alzheimer's disease [41,42].

The underlying pathogenic mechanism in all these disparate conditions appears to involve dysregulation of the inflammatory response leading to adverse outcome.

KNOCK OUT ANIMAL MODELS TO ELUCIDATE FUNCTION OF GENES

In the field of functional genomics, gene-knock out models of animals can be employed to assess the function of genes. When a specific gene is artificially inactivated, the phenotypic effect gives information about the function of the inactivated gene [68] . Humans who harbor loss of function genes can provide better information than knock-out animal models [70]. The differences may relate to the existence of other regulators upstream or downstream of the gene and to environmental factors [71].

MacArthur (2012), reported that an average healthy person has 100 inactivated genes, of which 20 are homogenous [72]. Another study which used whole exome sequences from European populations (n:1432) reported that nearly 45% (n:76) of loss of function mutations were newly identified [[73]. Narasimhan et al (2016) studied the effects of rare gene knockouts in adults born of consanguineous marriage by performing exome sequencing in 3222 adults of Pakistani origin domiciled in Britain. Upon linking of sequence data to their lifelong health records, there was no relationship between those with loss of function genes and their consultation for health issues or for prescription medication use. The latter were taken as surrogate markers for their state of health [74].

Loss of function mutations can result from: (a) nonsense SNP leading to a premature stop codon, producing a truncated protein sequence (b) splicing can be affected by a SNP at a canonical splice site (c) an insertion or deletion variants located in the gene coding region that can disrupt the full- length transcript leading to frameshifts (d) loss of function mutation can arise from a loss of an initiation codon [75].

Individuals with loss of function mutations who are apparently healthy were referred to as 'experiments of nature'. Studying them could help in the search for new drug targets and in identifying or exploring if such mutated genes could have beneficial effects [76].

Study of natural human knockouts and the correlation of genotype-phenotype is a field in its infancy which can provide insights into population genetics, and into the evolution of genes [69,71].

Butyrylcholinesterase and its variants qualify as natural human knockouts, because, other than prolonged apnea following exposure to succinylcholine, individuals harboring BuChE variants are apparently healthy [77].

SIGNIFICANCE OF STUDYING BUTYRYLCHOLINESTERASE VARIANTS

Unlike other gene knock-out animal models, variants of BuChE have a high prevalence in isolated ethnic groups, mainly south Indian from Vysya community, and certain Eskimos in western Alaska [34]. Li et al (2008) developed an animal BuChE gene knock-out model to test drug toxicity [78]. The model had a normal phenotype unless challenged with drug administration. The nicotinic receptor function was normal while muscarinic receptor function was altered, while. KO model showed altered cognitive functions. The effect of (R)-bambuterol, a specific and reversible inhibitor of BuChE suggested it may be used in the treatment of early cognitive decline [79].

BUTYRYLCHOLINESTERASE VARIANTS IN HUMANS

Lockridge et al (2016) reviewed the naturally occurring genetic variants of BuChE[80]. Thirty four loss of function mutations were identified; all of them were tolerated, viz, having a nonfunctional gene was compatible with life. Humans harboring silent BuchE gene are healthy and fertile [77]. Lando et al (2003) reported that among healthy blood donors (n:2609), 59 had low plasma BuChE activity[80].

In the Netherlands Organisation for Applied Research (TNO) Prins Mauritis Laboratory and CDC, the frequency of BuChE mutations was nine out of 121,000 alleles. Some of the mutants showed complete absence of enzyme activity [81].

The commonest missent mutation is called the K-variant (Ala567Thr (AS39T), which is associated with 30% lower BuChE plasma activity compared to native BuChE. It results from an unknown mutation in a regulatory region [80]. Other variants are less common except in communities such as south Indian Vysyas or Eskimos. Where genotyping is not possible, estimating dibucaine and fluoride number could serve as a surrogate. Family studies in the south Indian state of Andhra Pradesh showed that various phenotypic forms of BuChE deficiency in inbred families (Data presented as oral presentation at 12th International Meeting on Cholinesterases-Sixth International Conference on Paraoxonases at Elche (Alicante, Spain) in 2015 (Supplement 1).

BuChe activity increases with progression of Alzheimer's disease and may eventually replace the function of AChE [82]. Individuals with BuCHE-K variant could have deleterious outcomes when donepezil is given to patients with mild cognitive impairment. It can therefore serve as a pharmacogenetic marker in the choice of drugs for cognitive impairment [82].

Reasons why non-functional proteins persisted were attributed to inbreeding, founder effect or their retention having no adverse effect. They could even confer evolutionary advantage, although the advantage if any, is currently unknown. Based on its ability to modulate lipid

metabolism, it could perhaps lower the risk of cardiovascular disease despite high-fat diets [34].

Studies on human loss of function genes which were published did not identify variants of BuChE [72,73,74]. It is likely that specific endogamous ethnic groups where consanguineous marriages are prevalent and who have a high prevalence of variant BuChE enzyme were not sampled. To screen communities for genetic diseases where consanguinity if prevalent, advanced genetic testing using multigene panels and whole exome sequencing are being considered [83,84]. Studies on variant BuChE do not require such complex technologies. When genomic testing is not possible, even phenotyping using fluoride and dibucaine number can be a first stage screening test[80].

CONCLUSION

Butyrylcholinesterase is an enzyme that plays a role in detoxification of noxious agents. The fact that it has been retained across evolution suggests it could play other physiological roles as well. Studies in a variety of conditions including metabolic disorders, Alzheimer's disease, coronary artery disease, cerebrovascular and infections and inflammatory states suggest that it regulates the inflammatory responses. Isolated populations harbor dysfunctional forms of the enzymes. They are healthy, until exposed to drugs or toxins such as succinylcholine when they have an aberrant response. These natural human knock-out models for butyrylcholinesterase can be followed up to understand their life trajectory and susceptibility or protection against diseases. [Table 2]

Table 1: **Potential roles of butyrylcholinesterase**

(1) Known functions:
    a. Metabolism of drugs and toxins (eg, succinylcholine, carbamates, glucoalkaloids)
    b. Affected by dietary fats
    c. Influences the expression of metabolic syndrome, via action on lipids
(2) Associations and predictors of outcomes in disease states
    a. Nutritional status
    b. Hepatic disorders
    c. Cardiovascular disease
        i. Acute coronary syndrome
        ii. Acute myocardial infarction
    d. Injuries to brain and cerebrovascular and disease
        i. Ischemic brain stroke
        ii. Traumatic brain injury
    e. Alzheimer's disease
        i. Predisposition
        ii. Onset
        iii. Response to anticholinesterase medications
    f. Pre-eclampsia
    g. Inflammatory and infections
        i. Sepsis
        ii. HIV infection
        iii. Hansen's disease
    h. Other conditions
        i. Wilsons disease
        ii. Chronic obstructive pulmonary disease

Table 2:

Potential areas for studies in subjects with variant forms of butyrylcholinesterrase and therapeutic potential of the enzyme

a. Follow up of asymptomatic individuals for any protective or predictive role in cardiovascular disease and Alzheimer's disease
b. Production from transgenic sources as a pharmacologic agent
c. Potential drug target

Responses to the second round of review:

Please revise according to the expert's opinion of the second review and supplement Answering reviewers' documents:

(1) The results and discussion section is very weak and no emphasis is given on the discussion of the results like why certain effects are coming in to existence and what could be the possible reason behind them?
    a. Response: The available evidence and explanations were summarized as an additional text. Comprehensive knowledge about the functions of the protein has not yet been generated. The proposal made in the manuscript is to initiate studies for filling the gaps in knowledge, particularly among individuals with variant forms of the protein. They serve as 'human knock-out models'
        i. 'Butyrylcholinesterase (BChE) serves a critical role in the hydrolysis of esters. Unlike acetylcholinesterase (AChE) with which it shares structural and functional properties, BChE acts on a broader number of substrates, but has lower catalytic efficiency on acetylcholine [85]. It's roles extend into cardiovascular health; recent clinical studies suggest a correlation between increased plasma BChE activity and longevity in patients with severe cardiovascular disease [16]

            BChE also serves as a prognostic marker for liver and non-liver diseases, protein-energy malnutrition, and obesity by reflecting the availability of amino acidic substrates [86] . It is also involved in neurodegenerative disorders, particularly Alzheimer's disease (AD). BChE not only co-regulates cholinergic transmission by hydrolyzing acetylcholine alongside AChE, but potentially interferes with the course of AD. Inhibitors of BChE are therefore used in the treatment of AD and other disorders of cognition by ameliorating cholinergic deficiency [87]

            Recent findings show that BChE's regulates substrates such as cocaine and ghrelin. Recombinant BChE mutants and viral gene therapy are being developed against cocaine addiction, and in exploring the understanding of BChE's role in obesity [88]. (Table 2).

As a therapeutic agent, Phase I trials showed the safety of pure BChE, thereby giving an option in preventing nerve agent toxicity. Gene therapy using vectors that allow long-term expression of BChE after a single injection is being explored [89].

Animal studies have illustrated that pretreatment with BChE can prevent adverse effects from lethal doses of nerve agents like soman, sarin, and VX [90]. Other technologies employed to synthesise BChE include glycosylation and PEGylation that can enhance its pharmacokinetics [88].
BChE's multi-faceted nature, as a pharmacological target and tool, deepens our understanding of biological pathways in health and disease. Further phenotype-genotype studies will throw light on its potential effects. In this context, subjects with variant forms of BChE serve as critical comparators in such studies.'

(2) 'Audio core tip: Please offer the audio core tip, the requirement are as follows: In order to attract readers to read your full-text article, we request that the first author make an audio file describing your final core tip. This audio file will be published online, along with your article. Please submit audio files according to the following specifications…'
    a. Response: The audio file has been modified to meet the specifications
(3) Regarding the figures: Please provide the decomposable figure of figures, whose parts are all movable and editable, organize them into a PowerPoint file, and submit as "Manuscript No. -Figures.ppt" on the system, we need to edit the words in the figures. All submitted figures, including the text contained within the figures, must be editable.
    a. Response: The changes have been incorporated
(4) Please provide the text in your figure(s) in text boxes. Also, please add "Copyright © The Author(s) 2023" below the image.------4
    a. Response: This has been done
(5) Please add text editable Tables 1 and 2 at the end of the article.------
    a. Response: The text editable Tables were added at the end of the article
(6) 'Please complete all the revisions based on the version of "4029-87202-v1"
    a. Response: The file has been named 're-edited 4029-87202-v1'. There has been no change in the content of the manuscript except for a brushing up of English, and the addition of 'Summary' based on the re-review comment that was made
(7) Figure file names should identify the figure and panel.
e.g. "Figure 1 Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ..."
    a: Response: The corrections have been made

Trust these corrections are in order