Weakened Cholinergic Blockade of Inflammation Associates with Diabetes-Related Depression

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"Melancholia: Fears and despondencies, if they last a long time." —Hippocrates, *Aphorisms*, Section 6.23

Emerging evidence demonstrates association of depression with both immune malfunctioning and worsened course of diverse aging-related diseases, but there is no explanation for the pathway(s) controlling this dual association. Here, we report that in post-reproductive and evolutionarily "blind" years, depression may weaken pathogen–host defense, compatible with the antagonistic pleiotropy hypothesis. In 15,532 healthy volunteers, depression scores associated with both inflammatory parameters and with increased circulation cholinesterase activities, implicating debilitated cholinergic blockade of inflammation as an underlying mechanism; furthermore, depression, inflammation and cholinesterase activities all increased with aging. In the entire cohort, combined increases in inflammation and the diabetic biomarker hemoglobin A1c associated with elevated depression. Moreover, metabolic syndrome patients with higher risk of diabetes showed increased cholinesterase levels and pulse values, and diabetic patients presented simultaneous increases in depression, inflammation and circulation cholinesterase activities, suggesting that cholinergic impairment precedes depression. Our findings indicate that dysfunctioning cholinergic regulation weakens the otherwise protective link between depression and pathogen–host defense, with global implications for aging-related diseases.

INTRODUCTION

Depression is a common illness worldwide, with an estimated 350 million people affected (WHO 2015). The personal cost of depression to afflicted individuals, their families and communities includes significant clinical morbidity, increased mortality, diminished functioning and loss of quality of life (1). An opinion review in *Molecular Psychiatry* (2) presents a positive evolutionary linkage between depression and pathogen–host defense. This hypothesis postulates this linkage as the reason

for depression being sustained through evolution, but the underlying pathway(s) involved remained unexplored. To search for possible mechanistic link(s) between depression and immune reaction in apparently healthy adults, we profiled predictors of residual depression in a cohort of 15,532 apparently healthy working adult volunteers (age = 44.7 ± 11.1). Given the tight involvement of cholinergic signaling in blockade of inflammation (3), we further sought and found significant inter-relationships between cholinergic

parameters and both inflammation and depression. Others found depression and inflammation to worsen the course of diverse medical illnesses (diabetes, stroke, myocardial infarction). However, both may either develop as a consequence of having the disease and/or be a risk factor for its onset (4). To search for aging-related dynamics of these events we chose metabolic syndrome conversion to diabetes mellitus type 2 (DM2) as a case study, and compared the triple association between inflammation, depression and cholinesterase activities which reflect the blockade of inflammation, among other pathways.

MATERIALS AND METHODS

Study Participants

A total of 17,380 persons attending the Tel Aviv Sourasky Medical Center for a routine health examination between September 2002 and September 2010 as

part of periodic health check-ups were asked to participate in the Tel-Aviv Medical Center Inflammation Survey (TAMCIS) (for details regarding this cohort, see also (5)). Participants were recruited individually by an interviewer while they waited for their turn for the clinical examination. The interviewer provided each participant with an explanation of the survey and asked for her/his voluntary participation. In return, participants were promised detailed feedback of the survey's results. Confidentiality was assured, and each participant signed a written informed consent form. All respondents underwent blood sampling (after an overnight fast). For each respondent, the results of these examinations and his/her responses to the study questionnaire were recorded and computerized. The questionnaire covered background, occupational, psychological, and physical morbidity factors.

Depression was assessed by nine items of the Patient Health Questionnaire (PHQ) score (6). Within this test, the PHQ-9 is the depression module, which scores each of the 9 DSM-IV criteria as "0" (not at all) to "3" (nearly every day). PHQ-9 scores of 5, 10, 15 and 20 represented mild or moderate, moderately severe, and severe depression, respectively. Of all participants, only those with full record about their PHQ-9 were included in the final regression model (n = 15,532, 89.3%).

Serum Cholinesterase Analysis

Acetylcholinesterase (AChE) and BChE serum activity levels were assayed in a microtiter plate using an adaptation of the Ellman assay (7). Hydrolysis of 1 mM acetylthiocholine (ATCh, Sigma) was followed by spectrofluorometry (Spectrafluor Plus, Tecan) at 405 nm after 20-min pre-incubation in the dark with (for AChE activity) or without (for BChE activity) 50 µM tetra isopropyl pyrophosphoramide (iso-OMPA, Sigma), a specific BChE inhibitor. Enzyme activities were calculated using 13600 M/cm as the e405 for 5-thio-2-nitrobenzoate. Total cholinergic activity is termed "cholinergic status."

Pulse Measurements

Those followed the procedure detailed under Shenhar-Tsarfaty (5).

Statistical Analysis

Decision tree support tool (8, 9) was employed to construct a tree-like graph model of the predicted effectors of residual depression. The explained variable was residual depression score (summary of 9 items of the PHQ questionnaire) after controlling for age, gender and body mass index (BMI), and the explanatory variables are listed in the supplementary file. The tree provides a hierarchical plot of predictors, such that the higher is more important and each branch divides the rest of the cohort using the calculated cut-point value.

RESULTS

Predictors of Residual Depression

Given the multiple components involved, we selected the decision tree support tool (8, 9) to identify those parameters that are the major contributors to residual depression (the additional depression score compared with that predicted; based on age, gender and body mass index (BMI)). An analysis tree of basal regression was constructed of the residual score of depression (adjusted for age, gender and BMI) for 15,532 cohort members (Supplementary materials). Branches were hierarchically organized, such that the higher is more important and each branch divides the rest of the cohort using the calculated cut-point value noted in the circle (left side = statement in parent circle confirmed). A tree-like hierarchical plot model (Figure 1) highlights inflammatory parameters that emerged as highly significant predictors of residual depression (red circles). The top estimator in the highest tree's 'branch' was self-reported feeling of chronic pain, which can indicate inflammation that is known as a predictor of heightened infection (10). Smoking and
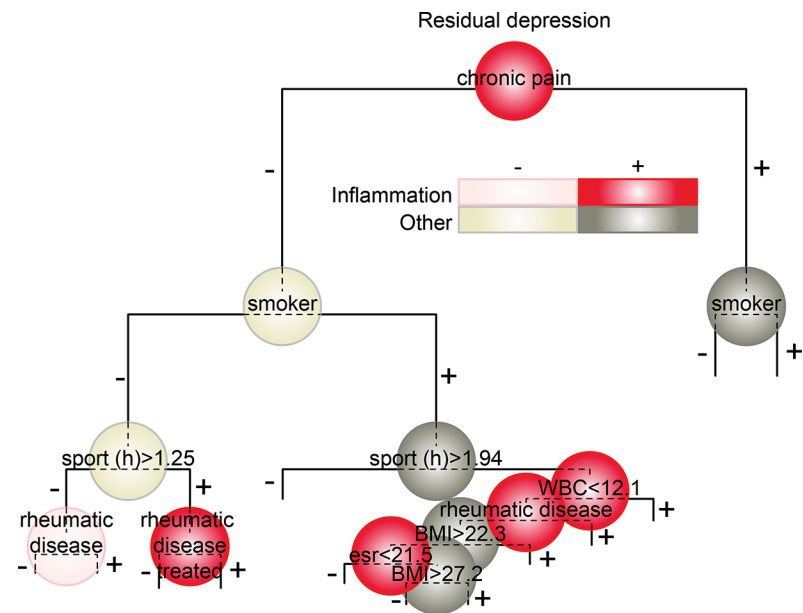

Figure 1. Hierarchical plot of predictors of residual depression. Circles are color-coded by the calculated effect on residual depression (increase or decrease; scale). Of 20 physiological, psychological and medical parameters entered into the model, chronic pain, smoking, sport activity, inflammation parameters, and BMI all emerged as significant predictors. Note that chronic pain increases the residual depression (top row), whereas in pain-free volunteers, smoking increases the residual depression (second row), and over 1.94 exercise hours per week (third row, middle panel) elevated depression scores.

sports activity emerged as the next predictors of elevated residual depression. Since both reflect cholinergic hyper-excitation (11, 12), this suggested relevance of imbalanced cholinergic signaling. The tree model further offers sub-division of the remaining cohort into 'branches' based on the calculated cut-point value of each continuous parameter. Using this division, white blood cell counts (WBCC), waist-to-hip ratio, rheumatic disease, moderate BMI and erythrocyte sedimentation rate (ESR) demonstrated increasing effects on residual depression. Together, these 6 red-highlighted inflammatory predictors in the tree model proposed a direct link of depression with inflammation which likely associates with risk of chronic diseases.

Aging-related Changes of Inflammation, Depression and Cholinesterase Activity

A different and independent approach to challenge the aging-related aspect of our theory involved straightforward ANOVA tests. Group division showed positive association between increasing age groups of the screened healthy volunteers and the average severity of their mild but measurable depression symptoms, inflammation readouts and cholinergic enzyme activities (Figure 2 A, C). This was the case in both men and women, albeit with gender-specific differences, such that women in all of these age groups showed higher inflammation and depression values but lower serum cholinesterase activities than men. Supporting the notion of aging relevance, we found that countries with reportedly high pathogen history load (13) also report relatively short longevity compared with those countries with lower pathogen history (Figure 3). Thus, although early life mortality is by far the most prominent determinant of life expectancy (14), the capacity to endure infection in later years is of considerable interest (15).

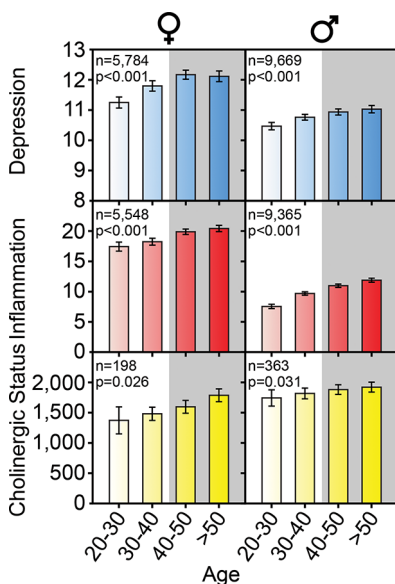Apart from age, we found the inflammatory markers in healthy

Figure 2. Depression, inflammation and cholinergic activities increase with age. Depression scores (sum of 9 items of the "Patient Health Questionnaire" (PHQ)), Inflammation (as reflected by the erythrocyte sedimentation rate, ESR, mm/h) and cholinergic enzymatic activities (Cholinergic status, CS, the total capacity for acetylcholine hydrolysis, nmol substrate hydrolyzed/min per ml (19)) all increase with age in both genders. (Statistics: ANOVA of 4 age groups).

volunteers to increase with depression scores (Figure 4A). To find if inflammation may precede the occurrence of depression we next examined men and women with escalating number of components of metabolic syndrome, who display higher inflammation and a greater risk to develop chronic cardiovascular diseases and diabetes (16). Since cholinesterases determine the levels of acetylcholine (17), which in turn controls inflammation (18), we surmised that elevated cholinesterase activities ("cholinergic status" (19)) would predict lower circulation acetylcholine levels, lower capacity to block inflammation (20) and hence modified susceptibility to infection. Compatible with this prediction, tested individuals showed serum cholinesterase activities that were positively correlated to the numbers of identified
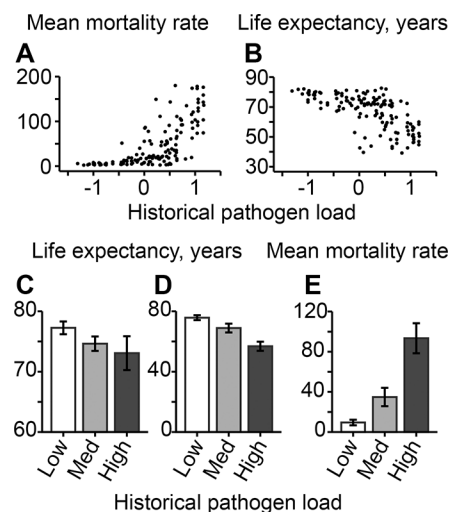
Figure 3. Longer life expectancy in countries with lower historical pathogen load. (A) Mean mortality rate under 5 years (per 1,000 live births) and (B) life expectancy (data from http://data.worldbank.org/) were plotted against historical pathogen load values based on the presence of nine diseases (leishmanias, schistosomes, trypanosomes, leprosy, malaria, typhus, filariae, dengue and tuberculosis) taken from Murray and Schaller score (13). (C) Countries were divided to three equal parts reflecting escalating values of historical pathogen load. This analysis revealed that the higher the pathogen-load the lower total life expectancy ($p < 0.001$). (D) To avoid irrelevant death causes (for example, war or famine) we further focused this analysis of life expectancy on countries with mean life expectancy above 70 ($p < 0.001$). (E) The historical pathogen load and mean mortality under 5 years (per 1,000 live births) scores ($p < 0.001$).

metabolic syndrome components, which is an acceptable measure of their risk of diabetes, (see Figure 4B for mean values, $p < 0.001$ for both genders). Nevertheless, these individuals also showed increasing depression scores ($p < 0.001$ for men and women, data not shown).

Given that diabetes is frequently associated with aging, depression and slowly healing infections, we next challenged this concept in 522 diabetic patients. Similar to the published record (21), the depression symptoms in diabetic
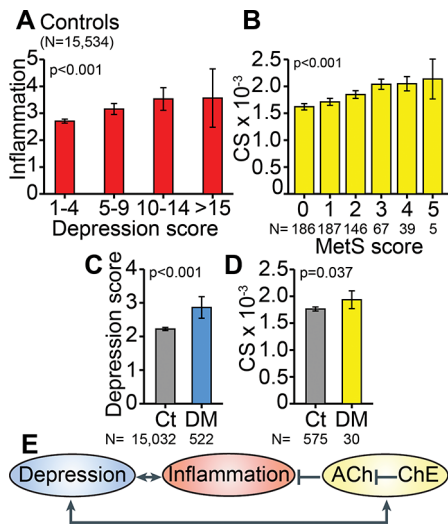
Figure 4. Co-increased depression and inflammation in apparently healthy and diabetic participants. (A) Inflammation associates with depression scores in healthy volunteers. Shown are serum C-reactive protein levels in mg/L as a function of total PHQ score. (B) Cholinergic status increase with the number of metabolic syndrome components. (C) Depression in diabetic patients is higher than in controls. (D) Cholinergic status in diabetic patients is larger than in controls. (E) The vicious cycle of cholinergic blockade of inflammation and depression. With aging, increasing cholinesterase (ChE) activities reduce acetylcholine (ACh) levels, interfering with the blockade of inflammation by ACh and potentiating depression that may reciprocally increase ChE activities.

patients were higher than in apparently healthy controls ($p < 0.001$, Figure 4C). Moreover, diabetic patients showed higher serum cholinesterase activities ($p = 0.037$; Figure 4D) and elevated levels of fibrinogen, a validated measure of inflammation, which likely reflects sterile inflammation and heightened susceptibility for infection (22).

Basal heart rate is another marker reflecting the cholinergic (sympathetic/parasympathetic) balance (5). As expected, basal heart rate increases in diabetic patients (men and women, $p < 0.001$) and in metabolic syndrome ($p < 0.001$, heart rate correlates with

number of components). However, basal heart rate was not correlated to depression score. Moreover, in 227 newly diagnosed diabetes mellitus patients (free of diabetes at the first visit), the heart rate at first visit was already increased to levels comparable to their second visit, and no significant change was observed in their delta heart rate compared with non-diabetic patients, suggesting that their elevated sympathetic tone was present before diabetes diagnosis. Cholinergic impairment thus emerged as an early step in a progressive order of events that may lead to systemic increases in heart rate, inflammation, and then to positively linked depression. Importantly, all of these changes would be blinded in evolutionary terms because they occur in post-reproductive years (see Figure 4E for a scheme of this proposed dynamics).

Triple Associations between Inflammatory, Depression Scores and Metabolic Bio-markers

We next tested our complete cohort for triple associations between inflammatory biomarkers, depression scores and hemoglobin A1c, an established pre-diabetic biomarker (23). Calculated differences between the observed and the expected values for each individual based on their age, gender and BMI were presented as a three-dimensional flow. Participants with exceptionally high WBCC values (reflecting exacerbated inflammatory response) also showed higher hemoglobin A1c levels, reflecting increased risk of diabetes and cardiovascular disease; intriguingly, that fraction of the cohort with the top hemoglobin A1c levels also presented a higher tendency for depression (color-code scale, Figure 5),
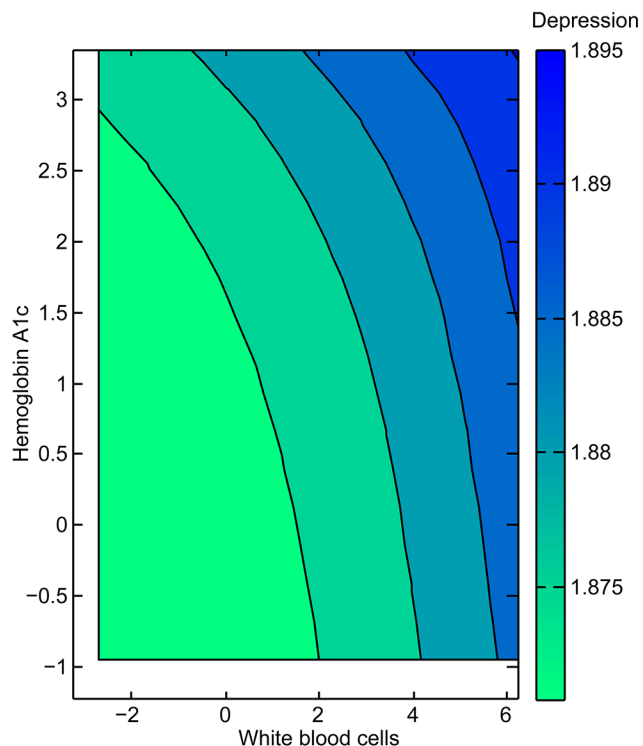

Figure 5. Triple association of hemoglobin A1c, inflammation and depression scores. Association of Hemoglobin A1c, white blood cells counts (WBCC) and depression score (PHQ). Each score presents the differences between the observed and expected value for individuals with the same age, gender and BMI. Depression scores (color code) were Square-root transformed. Note the increasing tendency for depression (blue) in those participants with higher values of both hemoglobin A1c and WBCC.

suggesting that depression may be functionally linked to inflammation and hence to elevated risk of chronic disease.

DISCUSSION

Our study is compatible with the prediction that impaired cholinergic regulation may precede and exacerbate the development of inflammation and depression. Our findings further support the identification by Raison and Miller of single nucleotide polymorphisms in cholinergic-related genes associated with major depression (24–28) and with the beneficial use of vaccination to reduce inflammation in successful independent aging (29). Recently we found that anxiety (which shares many symptoms with depression) ignites exacerbation of pre-existing neuro-immune risks (5), compatible with our previous reports of co-increasing severity of symptoms, shrinkage of the hippocampus and inflammation biomarkers in patients with post-traumatic stress disorder, who tend to be depressed (30). Importantly, PTSD patients also show impaired pathogen–host defense (31, 32), extending the prediction of a link between depression and pathogen–host defense to the wider context of stress-related diseases.

In his initial antagonistic pleiotropic hypothesis, Williams predicted that if a gene potentiated both reproduction in early life and aging in later life, then senescence would be adaptive in evolution (33); our current findings extend this hypothesis by proposing that physiological links, such as that between cholinergic and immune functions, may exert antagonistic pleiotropy effects that would be sustained through evolution regardless of the associated damage to carriers at post-reproductive years. We found the theory presented by Raison and Miller to be compelling and convincing; nevertheless, 6 out of the 8 inflammatory parameters we tested, but none of the unrelated 17 parameters introduced into our model emerged as relevant to the residual depression. This points at the link between cholinergic and immune functions as an antagonistic pleiotropy feature, especially given the added risk of cardiovascular disease and diabetes that is attributed to inflammatory load (16, 34). Supporting a causal role in this link for the cholinergic blockade of inflammation, we also found similar and gender-characteristic age-related escalation in depression, inflammation and cholinergic status scores that is unlikely to be simply coincidental. Furthermore, we observed a seemingly causal sequence of events for cholinergic malfunctioning, inflammation and depression, compatible with recent reports linking cholinergic regulation with post-stroke recovery (20, 35), with risk of major adverse cardiac events (36) and with fear-induced risk of cardiac health (5). Additionally, carriers of cholinergic mutations and/or polymorphisms that interfere with microRNA suppression of AChE may be specifically prone to such risks (37, 38).

CONCLUSION

Taken together, our findings suggest that the age-related increases in serum cholinesterase activities of healthy adults (39) reflect an antagonistic pleiotropic weakening of the cholinergic blockade of inflammation that may co-elevate the risk of both pathogenic infections and depression. Current public medicine in Western societies avoids much of infant mortality due to acute inflammatory events (15); correspondingly, future challenges for extending life expectancy (12) require new solutions for limiting the damage caused by inflammation events and which we find to be exacerbated by depression at the post-reproductive years. Human health at the post-reproductive years should therefore be interrogated with the same intensity as at early development.

DISCLOSURE

The authors declare they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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