An anemia of Alzheimer's disease

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Abstract

Lower hemoglobin is associated with cognitive impairment and Alzheimer's disease (AD). Since brain iron homeostasis is perturbed in AD, we investigated whether this is peripherally reflected in the hematological and related blood chemistry values from the Australian Imaging Biomarker and Lifestyle (AIBL) study (a community-based, cross-sectional cohort comprising 768 healthy controls (HC), 133 participants with mild cognitive impairment (MCI) and 211 participants with AD). We found that individuals with AD had significantly lower hemoglobin, mean cell hemoglobin concentrations, packed cell volume and higher erythrocyte sedimentation rates (adjusted for age, gender, APOE-ε4 and site). In AD, plasma iron, transferrin, transferrin saturation, and red cell folate levels exhibited a significant distortion of their customary relationship to hemoglobin levels. There was a strong association between anemia and AD (adjusted OR=2.43 (CI[1.31, 4.54])). Moreover, AD emerged as a strong risk factor for anemia on step-down regression, even when controlling for all other available explanations for anemia (adjusted OR=3.41 95%CI[1.68, 6.92]). These data indicated that AD is complicated by anemia, which may itself contribute to cognitive decline.

Keywords:

Anemia, Alzheimer's disease, Mild Cognitive Impairment, hemoglobin, iron, folate, hematology.

Introduction

115.4 million cases of Alzheimer's disease (AD) world-wide are expected by 2050,[1] an impetus to understand this incurable dementia. AD and unexplained anemia are the most common diagnoses in nursing homes, both with a prevalence of ≈45% in this setting.[2,3] Several reports have associated lower hemoglobin and anemia on a cross-sectional basis with poorer cognition,[4-6] and recently low hemoglobin has featured on a biomarker panel with high diagnostic accuracy for AD.[7] Two prospective studies of elderly populations have reported that anemia and lower levels of hemoglobin were associated with a ≈2-fold increased hazard for developing AD over ≈3 years.[2,8] These studies speculated that low hemoglobin could be a systemic manifestation of AD.

Hemoglobin is the most abundant iron-protein in the body but is also synthesized in cortical neurons, where iron metabolism is severely perturbed in AD. Neocortical neurons in AD have decreased hemoglobin levels[9], and elevated iron levels that promote oxidative damage.[10,11] Genetic evidence supporting a role for abnormal iron-associated metabolism in AD includes a risk polymorphism of hemoglobin[12] and a synergistic risk effect for variants of iron-regulatory genes *transferrin* with *HFE*.[13, 14] APP plays an important role in cellular iron export.[11,15] An increase in IRP1 binding to the APP Iron Responsive Element in erythrocytes in AD compared to age-matched controls indicates that the central abnormality of hemoglobin and iron regulation in AD might be reflected in the blood.[16].

In the present study, we investigated the association of AD with anemia and the causes of anemia in AD. We analyzed iron-related blood biochemistry, hemoglobin and

other blood chemistry values that may impact on anemia in the large Australian Imaging, Biomarker and Lifestyle (AIBL) cohort of healthy controls (HC), mild cognitive impairment (MCI) and AD patients.

Material and Methods

Study design and participants

Recruitment and characteristics of this cohort were previously described[17]. AIBL is a two-site (Melbourne, Perth), prospective study of aging, integrating data from neuroimaging, biomarkers, clinical and neuropsychological measurements and lifestyle information. Volunteers are in cohorts of: a.) AD by NINCDS-ADRDA criteria, b.) Mild Cognitive Impairment (MCI, associated with an increased risk for developing AD), and c.) cognitively healthy individuals (healthy controls, HC) (Supplementary Material, sections Participant Recruitment and Cohort Size for details).

AIBL was approved by the institutional ethics committees of Austin Health, St. Vincent's Health, Hollywood Private Hospital, and Edith Cowan University. Written informed consent was obtained from all study participants.

Procedures

Baseline blood samples were taken from overnight fasting participants, and fractionated or forwarded to clinical pathology laboratories for analysis as described[1] (Supplementary Material, section Biochemistry for details). Anemia diagnosis is detailed in Supplementary Material, section Anemia Diagnosis. All participants were given the Food Frequency Questionnaire developed by the Cancer Council of Victoria (Supplementary Material, Iron and Folate Dietary Intake for details).

Statistical analysis

Analysis was performed with R version 2.15.2 (packages, Supplementary Material, sections Statistical Analysis). Pearson's χ^2 assessed differences in the distributions of the missing data, genders and *APOE ε4* incidence. Analysis of variance (ANOVA) assessed difference in age across the clinical classifications, followed by Tukey honest significant differences to test the pairwise comparisons.

Clinical pathology data were checked for normality by inspection of histograms and quartile-quartile plots. For those that deviated from normality, Box-Cox[18] analysis was performed. The transformed data were then checked for normality.

Analysis of covariance (ANCOVA) was used with age, gender and site as confounding variables (*ApoE- ε 4* genotype was initially included, but was identified as a non-significant main effect and was not included in the final models.) to test the difference between the clinical classifications and continuous data. False discovery rate[19] (FDR) was used to correct for multiple testing. For the analysis of iron, dietary iron was also included as a confounding variable. The analysis of both serum and red cell folate were also adjusted for dietary folate. The results presented are the adjusted means and standard errors (SE). The pairwise analysis was corrected for multiple testing by controlling for FDR. Adjusted p-values are presented (Supplementary Material, sections Statistical Analysis for details).

Fisher Exact tests were used to analyze the number of subjects above or below a given reference range between two groups. The p-values reported are FDR-adjusted for multiple testing.

Logistic regression was used to assess the odds ratio (OR) of having AD given the anemia state (true/false), and *vice versa*, i.e. OR of being anemic based on the cognitive classification (HC/AD) (Supplementary Material, sections Statistical Analysis for details). To compare the log odds estimates between two logistic regression models, model with all subjects included and one with the subject with anemia of inflammation removed, bootstrapping with 1000 replicates was performed.

Results

Data from the initial baseline cohort[17] were analyzed. The HC group was significantly younger than the MCI and AD groups ($p<0.001$), and the MCI group was significantly younger than the AD group ($p<0.01$). There were significantly more females in the combined cohort, particularly in the HC and AD groups. The AD group had significantly more *APOEε4* allele carriers compared to the HC ($p<0.001$) and the MCI ($p=0.033$) groups, and the MCI group had significantly more *ε4* carriers than the HC group ($p<0.001$) (Supplementary Table 1). There were small numbers of missing biochemical data, but not biased to any clinical group (Supplementary Figure 1).

We initially examined 23 clinicopathology test results deemed relevant to red cell synthesis and iron metabolism. None of the adjusted (gender, site, age) mean values lay outside of the clinical reference ranges, but ANCOVA revealed significant differences across the three clinical groups for hemoglobin, MCH, MCHC, PCV, red cell folate (decreased in AD), ESR and haptoglobin (increased in AD), with a trend to decreased plasma iron in AD (Table 1). While transferrin was not significantly different across the clinical groups ($p=0.905$), transferrin levels declined more steeply with age in AD ($p=0.045$) (Supplementary Figure 2).

Pair-wise comparison of these eight values of interest across the clinical groups (figure 1A-H) revealed that iron, hemoglobin, MCH, MCHC, and PCV were significantly decreased in AD compared to HC, and ESR was significantly elevated in AD compared to HC. MCI values lay between the HC and AD values for iron, hemoglobin, MCH, and MCHC. Only for MCHC were the differences significant between HC and MCI as well as

AD and MCI (HC>MCI>AD). ESR and PCV were significantly different only between MCI and AD (MCI≈HC). In the ANCOVA for plasma iron, dietary iron intake was not a significant confounder and was removed from the final model. While haptoglobin showed a significant difference across the clinical groups, the individual pairwise comparisons showed only a trend towards increase in AD (figure 1H).

Since there was a significant decrease in hemoglobin levels and red cell hemoglobin content in the AD cohort, we analyzed the association between anemia (WHO criteria[20]: males hemoglobin<130 g/L, females hemoglobin<120 g/L) and AD by logistic regression, finding a strong unadjusted association (OR=5.94 95%CI[3.13, 9.94], p<0.001). Adjusted for known major AD risk factors, age, gender and *APOE-ε4* status, the OR for being diagnosed as AD if anemic in this cohort was 2.43 (95%CI[1.30, 4.54], p=0.005) (table 2A), consistent with previous reports.[4,8,21]. Conversely, higher MCHC was protective for AD (OR= 0.62, 95%CI[0.48-0.80], p<0.001, adjusted for age, gender and *APOE-ε4* status).

We then interrogated the reciprocal OR, for being anemic if diagnosed as AD, and controlled for confounders for the risk of anemia. As expected, increasing RDW, urea, ESR and erythropoietin, decreasing WCC, iron and ceruloplasmin all showed an association with anemia within the combined HC/AD cohort (table 2B). Surprisingly, increasing ferritin was associated with anemia, as was increasing red cell folate (but only in AD cases). AD emerged as a strong risk factor for anemia, even when controlling for all other available explanations for anemia (adjusted OR=3.41, 95%CI[1.68, 6.92], p<0.001) (table 2B). Neither iron nor folate dietary intake were confounders. Similarly,

while zinc and copper deficiency are uncommon causes of anemia, plasma zinc and copper were not significant terms in the OR model (not shown). Non-steroidal anti-inflammatory drugs (NSAIDs) may contribute to anemia by promoting GI bleeding, although we found no evidence of blood loss to account for the increased risk of anemia in the AD cohort. Nevertheless, χ^2 tests indicated no bias in the number of subjects on NSAIDs compared to those not on NSAIDs between the anemic and non-anemic groups within in AD and HC cohorts ($p = 0.552$, $p = 0.200$, respectively, Supplementary Table S2). Acetylcholinesterase inhibitors can also potentially cause GI bleeding (by Mallory-Weiss syndrome). χ^2 testing showed no bias in the usage of acetylcholinesterase inhibitors between anemic AD and non-anemic AD subjects ($p = 0.204$, Supplementary Table S3).

Taken together, these data indicate that AD is associated with a proclivity to anemia that could not be explained by known risk factors. To explore possible explanations for the proclivity towards anemia identified in the AD cohort, we analyzed the proportional prevalence of abnormal clinical pathology results (outside the reference range). These were indeed more prevalent in the AD group compared to the HC and MCI groups (13 out of 29 tests, expanded to include white cell sub-population counts, Supplementary Table 4), with no abnormality being more prevalent in the HC cohort. Abnormal results in MCI patients lay in prevalence between HC and AD abnormalities.

The older age of the AD cohort could account for the increased prevalence of abnormal tests, so we created age-matched MCI and HC groups (Supplementary Table 1B) and repeated this analysis. The prevalence of abnormalities for the AD cohort

remained significantly greater than HC for low levels of hemoglobin (18.54 vs 7.47%, AD:HC), PCV (20.49% vs 12.03%), transferrin saturation (9.27% vs 3.78%), serum iron (5.85% vs 1.26%), and serum folate (8.33 vs 1.67%), with elevations of ESR (46.70% vs 33.33%), haptoglobin (42.51% vs 30.25%), ferritin (17.56% vs 7.98%) and TSH (9.80% vs 3.77%) (table 3). Differences in dietary intake could not account for the increased prevalence of serum folate and iron abnormalities observed.

Confining this analysis to age-matched anemia cases only, no clinical pathology abnormality was significantly more prevalent between HC and AD cohorts (Supplementary Table 5). ANCOVA of the hematology and biochemical data, controlling for age and gender, revealed small differences between anemic AD and HC subjects (Supplementary Table 6) for MCHC, lymphocyte count, bilirubin, and transferrin saturation (males only), but did not reveal a clearly different signature between the AD and HC anemic subjects.

As reticulocyte counts were not available, we used plasma erythropoietin levels as an indication of drive for red cell production. As expected, elevated erythropoietin levels were observed in the anemic cohort ($p=0.003$, Supplementary Figure 3), and associated with anemia risk (OR 1.45 CI[1.10, 1.92], table 2B). ANCOVA, controlling for age, gender, site and clinical classification, revealed no significant difference in erythropoietin across the neurological categories ($p=0.103$, table 1).

When anemia cases were categorized on the basis of clinical pathology abnormalities, anemia of chronic inflammation (AI) was the only anemia category significantly more prevalent in the AD group (28.9% of types of anemia) compared to

the age-matched HC group (16.7%, p<0.001; Supplementary Figure 4 and Supplementary Table 7).

AI is characterized by an elevation of interleukin-6 (IL-6).[22] However, IL-6 was not elevated in the AD cohort (Table 1). ANCOVA (adjusted for age, gender and site) revealed an elevation of IL-6 in subjects with anemia (p = 0.005), but no interaction between IL-6, anemia and neurological category (p = 0.826). To determine the extent that the increased risk for anemia in the AD group is accounted for by AI, we compared two logistic models, model 1 (m1) using all subjects and model 2 (m2) where the AI subjects were removed. There was no change in the AD OR for anemia (OR difference [m1 – m2] -0.181 (95%CI[-1.79, 1.67], p = 0.807) when the AI subjects were removed. The risk for anemia in the AD cohort using m2 was still strong (adjusted OR=3.59, 95%CI[1.68, 6.95], p = 0.002). Therefore, AI does not explain the increased prevalence of anemia in AD.

These findings suggest that AD patients may have a defect in hemoglobin synthesis of unknown etiology. To characterize this we examined the relationships between levels of hemoglobin and its substrates: plasma iron, transferrin, transferrin saturation, haptoglobin and red cell folate. Using multiple regression analysis (controlling for age, gender and clinical classification), the relationship between hemoglobin (as a product) and each factor (as a precursor) showed an inverted-U shaped quadratic association (p<0.001) for each clinical cohort. The associations of hemoglobin as a product of either plasma iron, transferrin level, transferrin saturation or red cell folate were all significantly altered in the AD group (p<0.001, figure 2) in that

hemoglobin levels dropped precipitously as the substrate levels approached the margins of the normal range. The haptoglobin-hemoglobin relationship was not affected by clinical classification (p=0.5472). For the relationship between hemoglobin and red cell folate there was a steeper decline in hemoglobin levels in AD patients who had red cell folate levels above 1000 nmol/L (figure 2D), recapitulating the surprising observation that elevated red cell folate increased the risk for anemia in AD patients only (table 2B). These relationships were not significantly perturbed in the MCI cohort.

The puzzling association of higher levels of red cell folate with anemia (table 2B, figure 2D), together with significantly lower red cell folate levels (-13%, table 1, figure 1G) in the AD cohort, could reflect anomalous folate metabolism in AD. Serum folate levels were not significantly changed in AD (table 1). So we analyzed the age-matched cohort for participants who had the mismatched combination of abnormally low serum folate with normal (or elevated) red cell folate. This combination of results was indeed uncommon in the HC group (0.5%) but was significantly more frequent in the MCI (3.8%, p=0.005) and AD (6.6%, p<0.001) groups (Supplementary Table 8). Indeed, in AD it was more common than abnormally low red cell folate (Supplementary Table 8). In addition, the mean ratio of red cell to serum folate, reflecting the exchange of folate between intracellular and extracellular blood compartments, was significantly elevated in anemic AD subjects (figure 1I), with no change in anemic HC and MCI subjects, Since nutritional intake of folate did not explain the increased prevalence of anemia in AD, these data implicate altered exchange of folate between serum and red cells as potentially contributory to the increased risk for anemia in AD.

Discussion

Anemia is an established risk factor for cognitive loss, causally attributed to low oxygenation of obligatorily aerobic cortical tissue.[2,4,5,21]. In the AIBL cohort, people with anemia were 2.40-fold more likely to have AD (adjusting for age and *APOE-ε4*), and anemia emerged as a stronger potential risk factor for AD than aging 5 years (table 2A). These findings are consistent with a prospective study that reported anemia as a risk factor for AD, accelerating cognitive decline to a degree similar to aging 12 years.[8] Therefore, identifying and treating anemia in older age could be imperative. However, our findings indicate that management of anemia in AD may not be straightforward. AI was more common in the AD cohort, but neither AI nor other known causes could explain the increased risk for anemia or lower hemoglobin levels in AD.

Idiopathic anemia becomes prevalent in advanced age, and indeed, in the AIBL cohort reversible causes of anemia were rare and idiopathic anemia was the most common cause (Supplementary Figure 4 and Supplementary Table 7). Is it possible that an impairment of hemoglobin production is a systemic manifestation of AD, and that since sub-clinical AD pathology also becomes prevalent above the age of 60, could idiopathic anemia itself be etiologically related to AD? Abnormalities of red cells have been reported in studies of smaller AD cohorts.[23-26] We found that on average, AD red cells are smaller, have less concentrated hemoglobin, sediment faster and have lower folate levels than HC red cells (figure 1). For these results, MCI red cells have values between AD and HC.

As with idiopathic anemia, we could not ascertain a clear etiological explanation for the lowering of hemoglobin in AD, but we detected changes in systemic iron, folate metabolism, thyroid, and inflammation that could adversely impact hemoglobin production. Importantly, the relationships between hemoglobin and its iron-related precursors (serum iron, transferrin and transferrin saturation) were perturbed in AD (figure 2A-C). Unlike HC and MCI, hemoglobin levels in AD declined significantly unless serum iron, transferrin and transferrin saturation were in the middle of the normal range. Therefore, iron-related hemoglobin production may be brittle in AD. The significantly greater prevalence of abnormally high serum ferritin in AD (17.65%) compared to age-matched HC subjects (7.98%, table 3) together with significantly lower plasma iron levels (figure 1A) and increased prevalences of abnormally low serum iron and transferrin saturation (table 3) in AD, argue that iron accumulates in tissue stores and is not adequately mobilized in AD. This is consistent with iron accumulation in the neocortex in AD.[11]

Folate deficiency is a risk factor for both dementia and anemia, and while we observed folate changes in AD, they could not be accounted for by nutritional deficiency or medication. Serum folate levels were not significantly changed In the AD group compared to the age-matched HC group, but subnormal levels were significantly more prevalent (8.33% vs 1.67%, table 3). This might be consistent with a nutritional problem, but red cell folate is a better reflection of long-term deficiency[27] and while red cell folate levels were decreased in the AD group (figure 1G),[28] subnormal levels were uncommon and not more prevalent in AD than in HC (table 3). Alternatively, a systemic defect in

folate metabolism that could contribute to the increased prevalence of anemia in AD was evidenced by the brittle relationship between hemoglobin levels and red cell folate in AD (figure 2D), as well as the puzzling observation that increasing red cell folate strongly increases the risk for anemia in AD but not HC subjects (table 2B). Abnormal folate metabolism has been implicated in AD pathogenesis. A genome wide association study identified a significant association between polymorphisms of methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like protein and the risk for late-onset AD.[29] A proteomic study found that methylenetetrahydrofolate dehydrogenase 1 levels are 32-fold elevated in erythrocytes in AD compared to controls.[26] Folate is susceptible to oxidation, notably by elevated cytosolic iron.[30]

Hypothyroidism might also contribute to the increased incidence of anemia in AD since abnormally elevated TSH was more common (9.80%) than age-matched HC (3.77%, table 3). However, adjusted mean TSH levels were not lower in the AD cohort, indicating that the contribution of hypothyroidism to anemia in AD is limited.

Inflammation may play a role in lowering hemoglobin in AD, since we observed elevated ESR, suppressed serum iron, elevated ferritin, and suppressed transferrin in the AD cohort (tables 1 and 3), and AI was the only diagnostic category for anemia that was significantly more prevalent (+12.2%) in the AD cohort compared to HC (Supplementary Figure 4 and Supplementary Table 7). However, logistic regression analysis excluding AI cases still determined that AD was a risk factor for anemia, and inflammation does not explain the differential tendency to lower hemoglobin levels in AD that we have observed. However, hepcidin and CRP levels in AD may be valuable in

appraising the tendency to anemia.

Therefore, the etiology of the 3.41-fold increase in anemia in AD involves perturbances of multiple systems for hemoglobin synthesis. Hemolysis could be excluded since haptoglobin was elevated in AD (table 1), as others have also reported[31]. While the mechanism of this idiopathic hemoglobin defect in AD remains to be determined, we note that Aβ binds hemoglobin,[32] and hemoglobin accumulates in the hallmark cortical amyloid plaque and congophilic angiopathy of AD.[33] Aβ is enriched in red cell membranes in AD compared to healthy controls,[34,35] and oxidizes red cell hemoglobin,[36] potentially contributing to anemia in AD.

With the caveat that this is a cross-sectional study and the subjects were recruited via advertising, so not a true random sample of the Australian population, our findings identify AD as a novel risk factor for anemia. Since low hemoglobin itself impairs cognition, and is a risk factor for AD, the hemoglobin lesion of AD may not be an epiphenomenon. The interrelationship between AD and hemoglobin warrants closer investigation as a potential target for intervention.

Supplementary information is available at *Molecular Psychiatry*'s website (http://www.nature.com/mp)

Conflicts of interest

AIB and CLM are shareholders in Prana Biotechnology Limited. AIB is a shareholder in Cogstate Ltd and Mesoblast Ltd.

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Measurement (units) [reference range]	HC	MCI	AD	**p-value**
Iron (μmol/L) [M:7-35, F:7-30]	21.93 (0.22)	21.28 (0.52)	20.76 (0.43)	0.079
Transferrin (μmol/L) [M:23-43, F:23-46]	32.96 (0.18)	33.09 (0.45)	33.16 (0.4)	0.890[a]
Transferrin Saturation (%) [M:14 - 53, F:14-48]	27.02 (0.35)	26.37 (0.83)	25.66 (0.69)	0.289
Ceruloplasmin (g/L) [0.17-0.55]	0.312 (0.002)	0.316 (0.005)	0.305 (0.004)	0.122
Ferritin (μg/L) [M:15-200, F:30-370[b]]	120.25 (3.46)	115.08 (8.01)	114.40 (6.75)	0.828
Hemoglobin (g/L) [M:130-180, F:120-160]	140.96 (0.4)	139.93 (0.95)	137.62 (0.82)	0.012
RCC ($x10^{12}$/L) [M:4.5-5.7, F:3.8-5.1]	4.51 (0.02)	4.51 (0.04)	4.44 (0.03)	0.117
MCV (fL) [80-96]	91.35 (0.15)	91.09 (0.36)	90.99 (0.31)	0.642
MCH (pg) [27-33]	31.20 (0.06)	31.00 (0.14)	30.85 (0.12)	0.044
MCHC (g/L) [320-360]	341.54 (0.21)	340.21 (0.51)	338.78 (0.43)	< 0.001
RDW (%) [11-15]	13.36 (0.03)	13.29 (0.07)	13.37 (0.06)	0.650
PCV (L/L) [M:0.4-0.5, F:0.35-0.45]	0.412 (0.001)	0.411 (0.003)	0.402 (0.002)	0.014
ESR (mm/hr) [1-35]	11.07 (0.29)	10.88 (0.7)	13.12 (0.7)	0.029
Serum folate (nmol/L)[c] [7-40]	946.39 (29.03)	847.46 (74.89)	887.74 (65.47)	0.468
Red cell folate (nmol/L) [c] [230-1600]	970.56 (14.67)	901.38 (38.37)	862.96 (31.89)	0.014
B12 (pmol/L) [120-680]	293.78 (4.77)	284.91 (10.96)	291.10 (9.62)	0.817
Bilirubin (μmol/L) [<19]	11.85 (0.15)	11.98 (0.37)	11.37 (0.28)	0.324
TSH (mU/L) [0.1-4.0]	1.60 (0.04)	1.67 (0.1)	1.67 (0.08)	0.817
Haptoglobin (g/L) [0.3 – 2.0]	1.48 (0.03)	1.57 (0.09)	1.63 (0.08)	0.031
Erythropoietin (pg/mL) [d] [32.92 – 222.21	71.25 (2.81)	90.03 (9.33)	73.53 (5.31)	0.103

WCC (x10^9/L) [4-11]	1.73 (0.01)	1.75 (0.02)	1.78 (0.02)	0.122
Urea (mmol/L) [2.5 – 8.3]	1.78 (0.01)	1.77 (0.02)	1.79 (0.02)	0.122[e]
IL 6 (pg/mL) [<5]	1.68 (0.04)	1.85 (0.11)	1.74 (0.08)	0.367

Table 1: Hematological and associated clinical chemistry mean values in the clinical categories. Age, gender and site-adjusted means (standard error) and adjusted p-values (ANCOVA, controlling for false discovery rate [FDR]) are shown for the three clinical classifications.

[a] Interaction between clinical classification and age (p=0.046).

[b] Female ferritin reference range: pre-menopause: 20-220μg/L, post-menopause: 30-370 μg/L.

[c] Adjusted for dietary folate intake.

[d] Erythropoietin analysis performed on the non-imputed data. Reference range as defined by ARUP Laboratories.

[e] Interaction between clinical classification and age (p=0.040).

Factor	Odds ratio (95% CI)	P value
A	for Alzheimer's disease	
Non–anemic, *APOE-ε4* non-carrier, healthy control	Reference	
Age (per 5 yrs)	1.95 (1.71—2.21)	<0.001
APOE-ε4 carrier	5.52 (3.71—8.22)	< 0.001
Female	0.94 (0.63–1.39)	0.741
Anemia	2.43 (1.31—4.54)	0.005
MCHC (per 8 g/L)	0.62 (0.48-0.80)	<0.001
B	for anemia	
Non-anemic, healthy control	Reference	
WCC (per 1.8 x10^9/L)	0.47 (0.30-0.74)	0.001
RDW (per 1%)	1.75 (1.37-2.24)	< 0.001
Ceruloplasmin (per 0.08 g/L)	0.53 (0.32-0.87)	0.012
Ferritin (per 124 μg/L)	1.48 (1.19-1.84)	0.001
Iron (per 7.29 μmol/L)	0.17 (0.09-0.32)	< 0.001
Urea (per 2 mmol/L)	1.75 (1.32-2.32)	< 0.001
ESR (per 12 mm/hr)	1.77 (1.37-2.29)	< 0.001
Erythropoietin (per 74 ng/L)	1.44 (1.10-1.89)	0.008
Red cell folate [HC] (per 518 nmol/L increase)	0.97 (0.60-1.57)	0.914
Red cell folate [AD] (per 518 nmol/L increase)	2.39 (1.36-4.22)	0.002
Alzheimer's disease	3.41 (1.68-6.92)	<0.001

Table 2. Logistic regression analysis of risk factors. The odds ratios for the continuous variables were calculated for the interquartile ranges (except for age) of the

whole cohort. **A.** The odds ratios of having an AD diagnosis in the cohort, with reference to non–anemic, *APOE-ε4* non-carrier, cognitively healthy controls. Anemia and MCHC emerged as significant adjusted risks for AD from the backwards step analysis, together with known risks, age and *APOE-ε4* carrier status. Model fit statistics: Nagelkerke R^2=0.391, c-statistic=84.4%, Somers' Dxy=0.688 and Brier score=0.111. **B.** The odds ratios of being anemic, with reference to non–anemic, cognitively healthy controls. Alzheimer's disease emerged as a risk factor, even when adjusted for all known risk factors for anemia from the backwards step analysis. There was a significant interaction between clinical classification and red cell folate (p=0.002), so odd ratios are presented for both healthy controls and Alzheimer's disease. Model fit statistics: Nagelkerke R^2=0.535, c-statistic=93.2%, Somers' Dxy=0.865 and Brier score=0.039.

	Below Reference Range							Above Reference Range						
	Total	HC	MCI	AD	p-value	Adj p-value	Pairwise HC v AD p-value	Total	HC	MCI	AD	p-value	Adj p-value	Pairwise HC v AD p-value
Fe [238, 108, 205]	3.09%	1.26%	1.85%	5.85%	0.016	0.093	0.009	2.00%	2.10%	0.93%	2.44%	0.735	0.948	
Transferrin [238, 108, 205]	2.36%	1.26%	3.70%	2.93%	0.299	0.630		1.27%	1.68%	1.85%	0.49%	0.425	0.750	
Transf'n Sat'n [238, 108, 205]	5.26%	3.78%	0.93%	9.27%	0.003	0.029	0.020	7.80%	5.88%	11.11%	8.29%	0.225	0.607	
Ceruloplasmin [238, 108, 205]	0.91%	1.67%	0.00%	0.49%	0.284	0.630		0.73%	1.26%	0.93%	0.00%	0.257	0.623	
Ferritin [238, 108, 205]	2.72%	2.94%	1.85%	2.93%	0.888	1.000		11.98%	7.98%	10.19%	17.56%	0.008	0.058	0.004
Hemoglobin [241, 109, 205]	11.35%	7.47%	6.42%	18.54%	0.000	0.012	0.001	3.42%	2.07%	9.17%	1.95%	0.004	0.029	1.000
RCC [241, 109, 205]	21.80%	21.16%	16.51%	25.37%	0.193	0.589		4.50%	3.32%	5.50%	5.37%	0.460	0.750	
MCV [241, 109, 205]	0.90%	0.41%	0.00%	1.95%	0.220	0.607		7.21%	6.64%	5.50%	8.78%	0.563	0.821	
MCH [241, 109, 205]	1.26%	0.41%	0.00%	2.93%	0.036	0.187	0.052	18.20%	21.16%	15.60%	16.10%	0.304	0.630	
MCHC [241, 109, 205]	0.36%	0.00%	0.00%	0.98%	0.174	0.562		0.00%	0.00%	0.00%	0.00%	1.000	1.000	
RDW [241, 109, 205]	0.00%	0.00%	0.00%	0.00%	1.000	1.000		6.85%	5.81%	5.50%	8.78%	0.414	0.750	
PCV [241, 109, 205]	14.23%	12.03%	7.34%	20.49%	0.003	0.029	0.019	2.70%	2.49%	5.50%	1.46%	0.130	0.503	
ESR [240, 107, 197]	1.10%	1.67%	0.00%	1.02%	0.503	0.774		33.82%	33.33%	11.21%	46.70%	0.000	0.000	0.006
WCC [241, 109, 205]	5.59%	5.39%	9.17%	3.90%	0.158	0.539		1.44%	1.66%	1.83%	0.98%	0.725	0.948	
Neutrophils [241, 109, 205]	3.60%	4.15%	4.59%	2.44%	0.507	0.774		1.44%	1.66%	2.75%	0.49%	0.230	0.607	
Lymphocytes [241, 109, 205]	25.41%	23.24%	29.36%	25.85%	0.466	0.750		0.54%	0.00%	1.83%	0.49%	0.050	0.242	0.460
Monocytes [241, 109, 205]	0.18%	0.41%	0.00%	0.00%	1.000	1.000		2.16%	2.90%	1.83%	1.46%	0.601	0.832	
Eosinophils [241, 109, 205]	0.00%	0.00%	0.00%	0.00%	1.000	1.000		2.70%	1.24%	5.50%	2.93%	0.075	0.335	
Basophils [241, 109, 205]	0.00%	0.00%	0.00%	0.00%	1.000	1.000		0.90%	1.24%	0.00%	0.98%	0.728	0.948	

Urea [240, 108, 205]	0.18%	0.00%	0.00%	0.49%	0.566	0.821		16.46%	14.58%	12.04%	20.98%	0.082	0.341	
Creatinine [240, 108, 205]	0.00%	0.00%	0.00%	0.00%	1.000	1.000		15.01%	15.00%	13.89%	15.61%	0.927	1.000	
Serum folate [240, 108, 204]	4.89%	1.67%	5.56%	8.33%	0.003	0.029	0.001	2.54%	2.50%	3.70%	1.96%	0.603	0.832	
Red cell folate [241, 109, 205]	3.42%	2.90%	3.67%	3.90%	0.826	1.000		5.77%	6.22%	5.50%	5.37%	0.971	1.000	
Vitamin B12 [240, 108, 205]	3.44%	2.08%	3.70%	4.88%	0.258	0.623		6.51%	5.00%	7.41%	7.80%	0.432	0.750	
Bilirubin [239, 108, 205]	0.00%	0.00%	0.00%	0.00%	1.000	1.000		11.41%	10.88%	16.67%	9.27%	0.147	0.534	
TSH [239, 108, 204]	2.36%	2.09%	1.85%	2.94%	0.817	1.000		5.81%	3.77%	2.78%	9.80%	0.011	0.072	0.012
Haptoglobin [238, 106, 207]	2.36%	2.52%	3.77%	1.45%	0.346	0.683		33.39%	30.25%	22.64%	42.51%	0.001	0.017	0.008
IL-6 [170, 82, 144]	0.00%	0.00%	0.00%	0.00%	1.000	1.000		10.35%	7.65%	12.20%	12.50%	0.280	0.630	
Erythropoietin [110, 48, 101]	11.20%	10.91%	6.25%	13.86%	0.139	0.750		10.42%	7.27%	12.50%	12.87%	0.353	0.683	

Table 3: Frequency of abnormal clinicopathology results in age-matched clinical cohorts. Frequency of results above or below the normal reference ranges, within each clinical diagnosis (healthy control, HC, mild cognitive impairment, MCI, age-matched to Alzheimer's disease, AD). P-values are from Fisher's exact test for independence, testing across HC vs AD, and after FDR (Adj p-value). The pairwise comparison was only performed for where there was a significant difference across all three diagnoses. Bold reflects values that were significantly different on pairwise comparison; italic reflects near-significant pairwise comparison.

Figure legends

Figure 1. Changes in hematological and associated clinicopathology adjusted-values in Alzheimer's disease. Shown are pair-wise comparisons of clinicopathology values that are significantly different (on ANCOVA, Table 1) or showing a trend (iron) across the cohorts. A: iron, B: hemoglobin, C: mean cell hematocrit, D: mean cell hemoglobin concentration, E: erythrocyte sediment rate, F: packed cell volume, G: red cell folate, previously reported[28], H: haptoglobin, I: the ratio of red cell to serum folate (rfol/sfol). Shown are the adjusted means (±SE) and p-values from ANCOVA adjusted for site, age and gender. HC, healthy controls; MCI, mild cognitive impairment, AD, Alzheimer's disease.

Figure 2. The relationship of blood hemoglobin to precursor levels is perturbed in AD. Lines of best fit from linear regression models for hemoglobin as a product of A: plasma iron, B: plasma transferrin (quadratic relationship), C: transferrin saturation, D: red cell folate. Each shows a quadratic relationship. For all plots, age is fixed at the population mean (72.2 years). The grey shaded regions are the 95% confidence intervals. Solid lines- healthy controls, dotted lines- mild cognitive impairment, dashed lines- Alzheimer's disease. The red vertical lines indicate the min and max of the reference ranges.

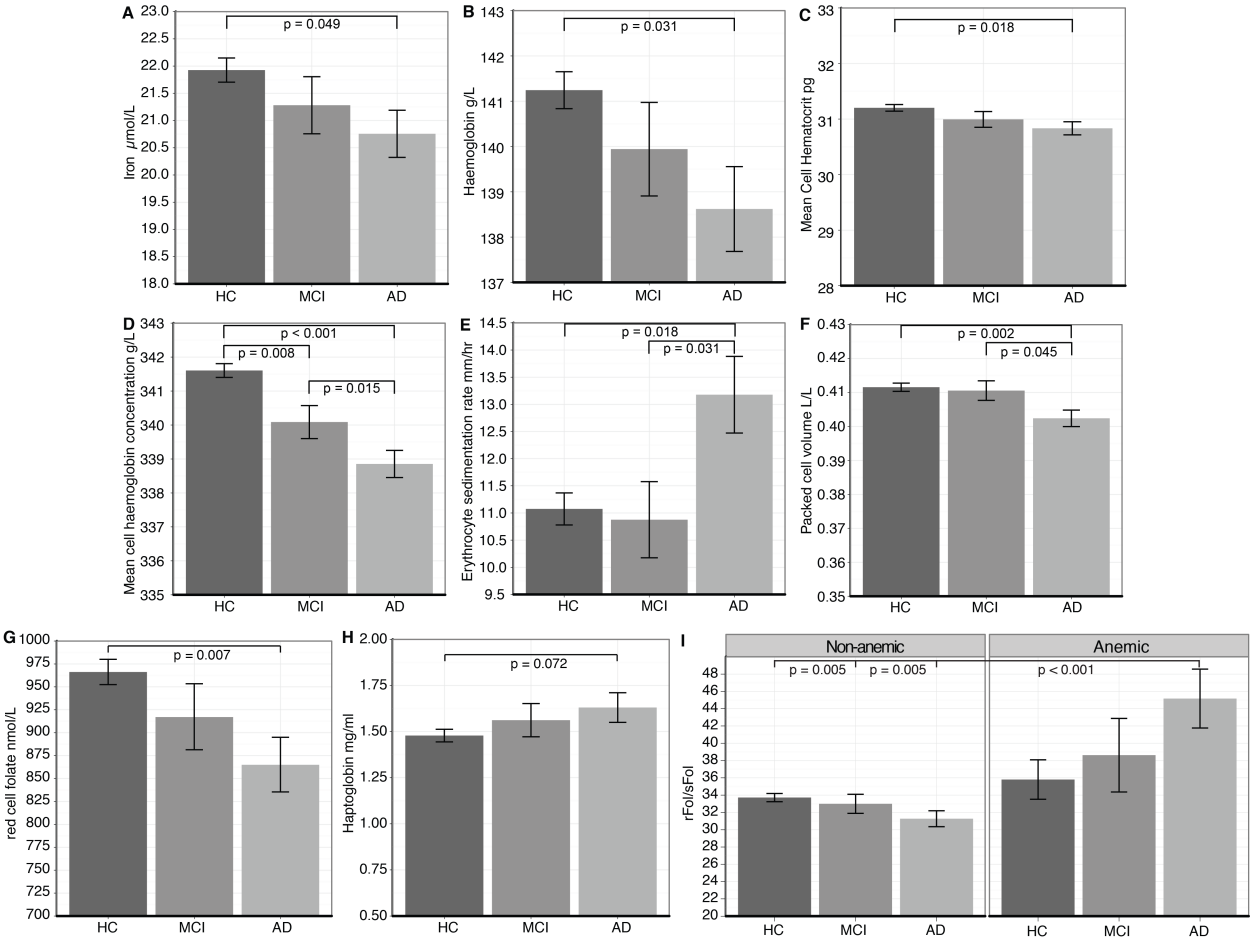
A
Iron µmol/L
p = 0.049
HC
MCI
AD
B
Haemoglobin g/L
p = 0.031
C
Mean Cell Hematocrit pg
p = 0.018
D
Mean cell haemoglobin concentration g/L
p < 0.001
p = 0.008
p = 0.015
E
Erythrocyte sedimentation rate mm/hr
p = 0.018
p = 0.031
F
Packed cell volume L/L
p = 0.002
p = 0.045
G
red cell folate nmol/L
p = 0.007
H
Haptoglobin mg/ml
p = 0.072
I
Non-anemic
Anemic
rFol/sFol
p = 0.005
p = 0.005
p < 0.001

Figure 1

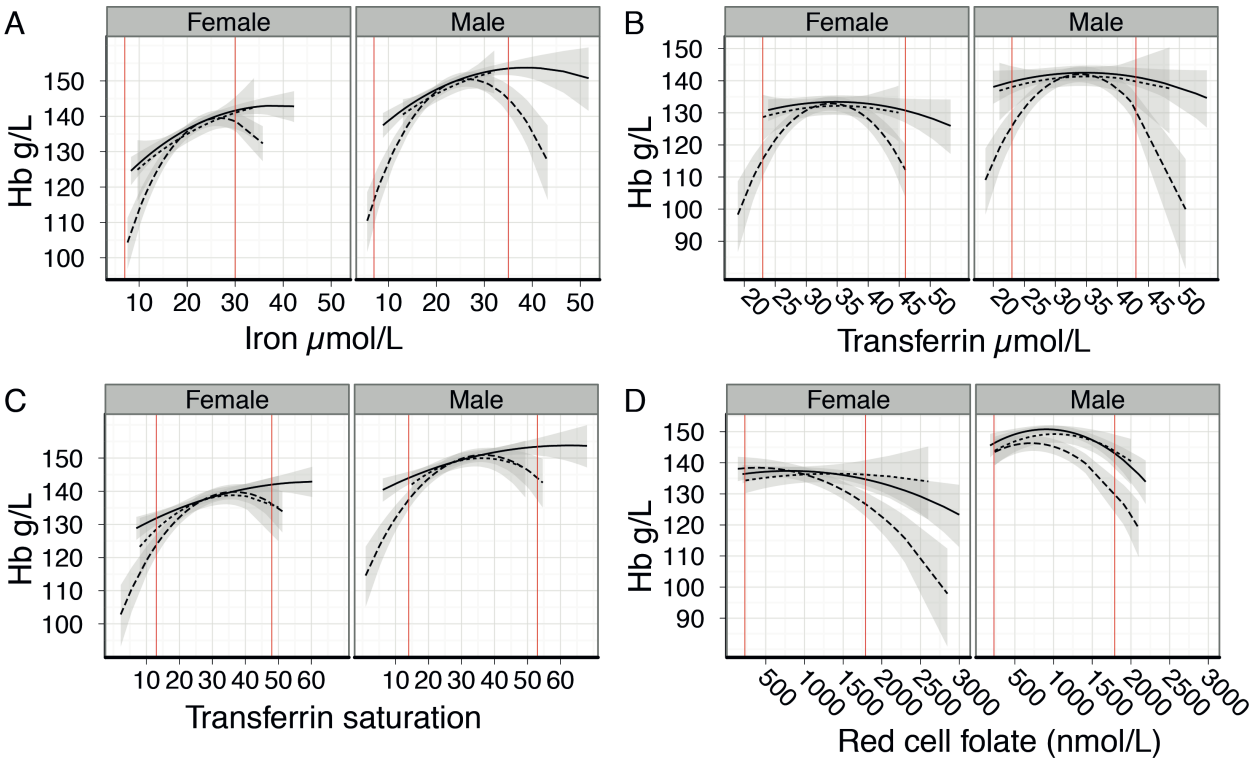
A
Female
Male
Hb g/L
150
140
130
120
110
100
10 20 30 40 50
Iron μmol/L
B
Female
Male
Hb g/L
150
140
130
120
110
100
90
20 25 30 35 40 45 50
Transferrin μmol/L
C
Female
Male
Hb g/L
150
140
130
120
110
100
10 20 30 40 50 60
Transferrin saturation
D
Female
Male
Hb g/L
150
140
130
120
110
100
90
500 1000 1500 2000 2500 3000
Red cell folate (nmol/L)

Figure 2

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