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Leukocyte CCR2 Expression is Associated with Mini-Mental State Examination (MMSE) Score in Older Adults

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Abstract

BACKGROUND: Circulating inflammatory markers may play an important role in cognitive impairment at older ages. Mice deficient for the chemokine (C-C motif) receptor 2 (CCR2) develop an accelerated Alzheimers-like pathology, and CCR2 is also important in neurogenesis. To identify human gene transcripts most closely associated with Mini Mental State Examination (MMSE) scores we undertook a genome-wide and inflammation specific transcriptome screen in circulating leukocytes from a population-based sample.

METHODS: We measured in-vivo transcript levels by microarray analysis in 691 subjects (mean age 72.6 yrs) in the InCHIANTI study. We assessed expression associations with MMSE performance at RNA collection and prior 9 year change in MMSE score in linear regression models.

RESULTS: In genome-wide analysis, raised CCR2 expression was cross-sectionally the most strongly associated transcript with lower MMSE score (beta=-0.16, p=5.1e^{-6}, False discovery rate FDR q=0.077). Amongst inflammatory transcripts, only CCR2 expression was associated with both MMSE score and accelerated decline in score over the preceding 9 years (beta=-0.16, p=5.1e^{-6}, q=0.003; and beta=-0.13, p=5.5e^{-5}, q=0.03; respectively). CCR2 expression was also positively associated with ApoE e4 Alzheimers disease risk haplotype.

CONCLUSIONS: We show for the first time that CCR2 expression is associated with lower MMSE scores in an older human population. Laboratory models of Ccr2-mediated -amyloid removal and regulation of neurogenesis affecting cognitive function may be applicable in humans. CCR2-mediated pathways may provide a possible focus for intervention to potentiate protective reactions to Alzheimers pathology in older people, including for people with adverse ApoE haplotype.

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1 Introduction

Cognitive impairment and dementia at older ages commonly results from accumulating vascular and neurodegenerative pathology in the brain [1] and is experienced by over half of adults aged 85 or over [2]. Major risk factors for cognitive impairments in later life include APOE e4 haplotype [3], but the underlying biological mechanisms are still unclear. Gene expression arrays offer a new approach to identifying the most important molecular mechanisms causing or responding to the pathologies underlying cognitive decline. The most ac-

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cessible tissue for gene expression array analyses in large numbers of older people is circulating blood leucocytes, which are likely to be most sensitive to inflammatory and related mechanisms.

Chronic low-level inflammation has been proposed as a key mechanism underlying cognitive decline and dementia, and has been implicated in the neuropathological cascade leading to late onset Alzheimers disease (LOAD) [4]. Inflammatory factors have been further implicated in cognitive impairment and dementia using mouse models [5]. These include transgenic animals in which chemo-attractant proteins or receptors such as Ccl2 or Ccr2 have been abolished [5]. These genes, associated with the migration of phagocytic and inflammatory macrophages, have been associated with Alzheimers pathology and peripheral atherosclerosis [5-7]. Ccr2-deficient mice show early accumulation of β-amyloid, with premature mortality [7]. Blood-borne systemic factors including CCR2 and CCL11 were also recently implicated in the negative regulation of neurogenesis and cognitive function in rodent studies [8].

The importance of CCR2 and related signaling in human age-related cognitive impairment is unclear. Maes et al (2007) identified leukocyte expression differences between 14 Alzheimers Disease and control individuals in a microarray experiment [9], while Grunblatt et al identified five peripheral blood leucocyte genes whose mRNA correlated significantly with MMSE (Mini Mental State Examination) score in a smaller series of Alzheimers and control individuals [10]. These studies present initial evidence that brain changes in pathologically-specific dementia patients may be detectable using peripheral leukocyte gene expression, but it is currently not known if this also applies to age-associated cognitive decline.

In the current study, we used a genome-wide and inflammation-focused approach to identify the most strongly associated in-vivo transcript levels in circulating leukocytes associated with MMSE score or rate of change in MMSE score in a general population sample of predominantly older people. The MMSE is a widely used measure of cognitive function in prospective epidemiological studies of elderly populations, and is sensitive to moderate or severe cognitive declines, often due to dementia [11,12]. To ensure population relevance, no exclusions for co-morbidity were made in the main analysis.

2 Results

2.1 Cohort Details

Participant characteristics are given in table 1; the study sample had a mean age of 72.6 years (SD: 15.3, range 30 to 104) and 55.2% were female. At RNA collection the mean MMSE score was 25.58 (SD 5.66), 21% of the sample had MMSE scores of 23 or less (8.1% with MMSE of 18 or less), and the mean change in MMSE scores over 9 years was -1.31 (SD = 4.71).

Table 1: This table summarises the population statistics for the 691 participants eligible for our study

Summary Statistics	
Age (years)	**n**
30-49	86 (12.5%)
50-69	98 (14.2%)
70-89	479 (69.3%)
90-104	28 (4%)
Mean age at RNA-collection	72.6 (SD: 15.3)
Gender	**%**
Men	44.8
Women	55.2
MMSE	**Score**
Mean score at RNA-collection	25.58 (SD: 5.66)
Mean change in MMSE (9 years)	-1.31 (SD: 4.71)
Frequency of scores (year 9)	***%***
0-18	8.14
19-23	12.79
24-27	30.52
28-30	48.55
Education	**%**
None	13.31
Elementary	46.02
Secondary	13.46
High school	12.59
University / Professional	14.62
Pack years smoked (lifetime)	**%**
None	55.72
0.1-20	22.87
20-39	14.33
40+	7.09

2.2 Genome wide analysis

In genome wide analysis, only one transcript (CCR2, measured via probe ilmn_1774761) showed a very near significant genome wide association with MMSE score at RNA collection (beta=-0.16, p=5.1e^{-6}; q=0.076; table 2). No probes were significantly associated (q <0.1) with change in MMSE score over the preceding 9 years (table 2), although CCR2 was the most strongly associated transcript (beta=-0.13, p=5.5e^{-5}, q=0.70).

Quantile-Quantile (QQ) plots comparing the observed p-values (-log10) with those that would be ex-

Table 2: The ten probes most closely associated with MMSE score at wave 9 and change in MMSE score (baseline to year 9), ordered by false discovery rate q-value (n observations = 688)

Probe ID	p-value	Coefficient	Beta	95% CIs		q-value	Gene
MMSE score at year 9							
ilmn_1774761	$5.1e^{-6}$	-0.0042	-0.1616	-0.0056	-0.0027	0.076	CCR2
ilmn_2374362	$2.7e^{-5}$	-0.0015	-0.1709	-0.0021	-0.0009	0.204	FAM108B1
ilmn_1791912	$1.1e^{-4}$	0.0052	0.1475	0.0030	0.0074	0.356	SIDT2
ilmn_1758457	$1.2e^{-4}$	0.0008	0.1473	0.0005	0.0011	0.361	TBC1D16
ilmn_1796094	$1.5e^{-4}$	-0.0044	-0.1486	-0.0063	-0.0025	0.381	CD36
ilmn_1693949	$1.7e^{-4}$	0.0011	0.1153	0.0006	0.0015	0.390	UNQ1944
ilmn_1724266	$2.8e^{-4}$	0.0017	0.1382	0.0009	0.0025	0.418	LYPD2
ilmn_1772821	$3.7e^{-4}$	-0.0015	-0.1561	-0.0022	-0.0008	0.430	KIAA1671
ilmn_2313434	$3.9e^{-4}$	0.0013	0.1476	0.0007	0.0019	0.433	TCP1
ilmn_2393573	$4.1e^{-4}$	-0.0019	-0.1073	-0.0027	-0.0010	0.434	RASSF1
Change in MMSE score (9 years)							
ilmn_1774761	$5.5e^{-5}$	-0.0040	-0.1305	-0.0056	-0.0024	0.700	CCR2
ilmn_2374362	$1.5e^{-4}$	-0.0015	-0.1345	-0.0022	-0.0009	0.700	FAM108B1
ilmn_1796094	$2.2e^{-4}$	-0.0047	-0.1146	-0.0067	-0.0026	0.700	CD36
ilmn_1761941	$2.6e^{-4}$	-0.0017	-0.1135	-0.0024	-0.0009	0.700	C4orf18
ilmn_2313434	$2.7e^{-4}$	0.0015	0.1273	0.0008	0.0022	0.700	TCP1
ilmn_1712684	$3.1e^{-4}$	-0.0011	-0.1245	-0.0015	-0.0006	0.700	FAM20C
ilmn_1866887	$3.4e^{-4}$	-0.0011	-0.1290	-0.0016	-0.0006	0.700	BX537605
ilmn_1772821	$5.6e^{-4}$	-0.0016	-0.1277	-0.0023	-0.0008	0.700	KIAA1671
ilmn_1703314	$5.7e^{-4}$	0.0013	0.1167	0.0007	0.0020	0.700	KLHL36
ilmn_1789751	$6.1e^{-4}$	-0.0039	-0.0985	-0.0058	-0.0020	0.700	MFSD1

pected by chance alone (figure 1a) confirmed that large scale disruption to gene expression levels was not a feature of MMSE score at RNA collection or change in score over the previous nine years, although some small deviations from the expected pattern were noted.

2.3 Specific inflammatory analysis

In a targeted analysis on inflammation-related transcripts only, the CCR2 transcript was associated with MMSE score at RNA collection (beta=-0.16, $p=5.1e^{-6}$, q=0.003; table 3) and also with cognitive decline score over the previous nine years, using a false-discovery rate (FDR) of q<0.1 (beta=-0.13, $p=5.5e^{-5}$, q=0.03; table 3). As in the genome wide analysis, other large-scale alterations in the expression of inflammatory genes were not present (after accounting for multiple testing) for either cognitive function or decline, although one large effect (CCR2) was evident on the QQ plot (figure 1b).

2.4 The relationship between CCR2 and MMSE

To understand any non-linearity in the relationship between CCR2 expression and MMSE (and change in MMSE over 9 years, delta-MMSE) we fitted a penalized cubic spline regression model for; A. CCR2 expression and MMSE at RNA collection, and B. CCR2 expression and delta-MMSE over the preceding nine years (see figure 3 for spline regression plots). We found the relationships to be approximately linear throughout the MMSE or delta-MMSE ranges.

2.5 Quantitative real-time PCR validation of CCR2 levels

To validate our microarray results, we also quantified CCR2 expression in a subset of our cohort using quantitative real-time PCR (QRT-PCR). We found that the expression level of CCR2 transcripts as measured by QRT-PCR correlated well with the expression levels measured by microarray analysis (r2 = 0.5, $p=2e^{-4}$).

2.6 Gene set enrichment analysis

No gene sets showed evidence of deregulation in association with MMSE score at RNA collection or change in score using GSEA (Gene Set Enrichment Analysis), at a FDR q-value of <0.1 (table 4).

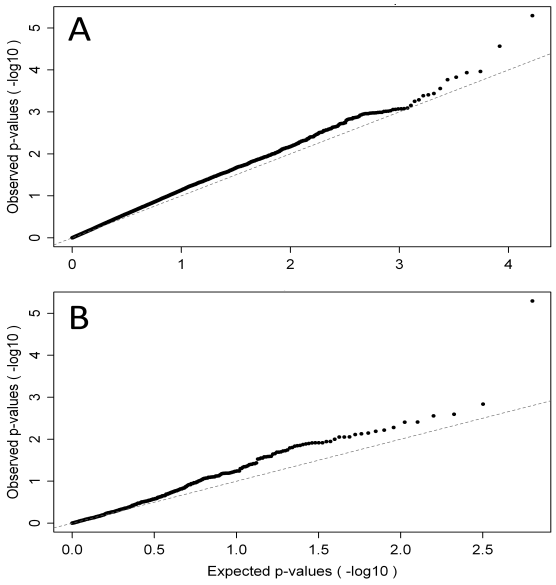

Figure 1: **Quantile-Quantile plots for gene expression analysis of MMSE score at RNA collection** — **A.** The Quantile-Quantile plot for the genome wide analysis (16 571 transcripts) of MMSE at RNA collection is shown. The actual P-values (-log10) obtained are given on the y-axis, plotted against expected P-values (-log10) given on the X-axis. This graph shows potential deviations to the p-value distribution that might be expected by chance. **B.** The Quantile-Quantile plot for the focused analysis of inflammatory genes (635 transcripts) is shown. The actual P-values (-log10) obtained are given on the y-axis, plotted against expected P-values (-log10) given on the X-axis. This graph shows potential deviations to the p-value distribution that might be expected by chance. The positive association with the CCR2 transcript is circled.

2.7 Post-hoc Sensitivity analyses

To determine if the observed association is dependent on the inclusion of some younger people in the predominantly older sample, or dependent on the oldest old, we carried out a sensitivity analysis in subjects aged =70 and <90 years old at RNA-extraction; MMSE score and (logged) CCR2 expression were still strongly associated (beta=-0.15, p = $2e^{-4}$) within this age-range. ApoE genotype was available on n=480 (excluding e2e4 n=4) in our sample: CCR2 expression was positively associated with ApoE risk haplotype (trend test across e2e2, e2e3, e3e3, e3e4, e4e4 groups: coef=0.02, CIs: 0.007 to 0.03, p=0.001). The association between CCR2 expression and MMSE at RNA collection was attenuated but remained significant after additional adjustment for ApoE status (beta = -0.1, p = 0.018). There was no statistical interaction between ApoE status and CCR2 expression.

Co-morbidity is very common with cognitive impairment in later life in the general older population, and therefore our approach has been to avoid disease specific exclusions: however, we did examine the effect of removing those diagnosed as having had a stroke (n=76 removed): CCR2 remained associated with MMSE score (beta = -0.14, p=0.001) in the stroke free group.

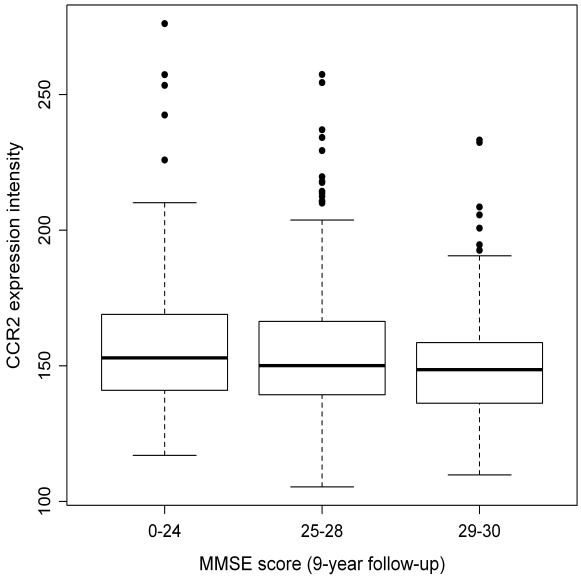

Figure 2: **Boxplot of CCR2 expression by MMSE score at RNA collection** — The boxplot shows CCR2 transcript expression levels (relative units) as plotted on the Y-axis by MMSE score RNA collection on the X-axis.

3 Discussion

In this study, we have investigated associations between blood leukocyte-derived mRNA expression and MMSE scores in an ageing population. CCR2 expression reached inflammation-specific significance (and only narrowly missed genome wide significance after accounting for multiple testing), providing the first human population evidence of likely consistency with the Ccr2 mouse models.

Our finding that ApoE haplotype status, the major inherited genetic risk factor associated with Alzheimers pathology, is associated with CCR2 expression is also consistent with CCR2 signaling having a direct role in cognitive decline in later life in human populations.

The MMSE score is a widely used measure of cognitive impairment in later life, although it has a ceiling effect that limits its ability to detect early subtle cognitive changes, particularly in younger highly educated participants [11,13] and is not considered an appropriate measure to diagnose mild cognitive impairment or

Table 3: The ten inflammation-related probes most closely associated with MMSE score at wave 9 and change in MMSE score (baseline to year 9), ordered by false discovery rate q-value (n observations = 688)

Probe ID	p-value	Coefficient	Beta	95% CIs		q-value	Gene
MMSE score at year 9							
ilmn_1774761	$5.1e^{-06}$	-0.0042	-0.1616	-0.0056	-0.0027	0.003	CCR2
ilmn_2366212	$1.5e^{-03}$	0.0083	0.1267	0.0040	0.0125	0.232	CD79B
ilmn_1677440	$2.6e^{-03}$	-0.0055	-0.1194	-0.0084	-0.0025	0.262	ATP6AP2
ilmn_2276996	$2.8e^{-03}$	-0.0009	-0.1071	-0.0014	-0.0004	0.266	CCR2
ilmn_1785439	$3.9e^{-03}$	0.0042	0.1239	0.0018	0.0066	0.279	CD79B
ilmn_1710017	$3.9e^{-03}$	0.0045	0.1302	0.0019	0.0070	0.279	CD79B
ilmn_1764396	$5.3e^{-03}$	-0.0011	-0.0725	-0.0017	-0.0004	0.288	HDAC4
ilmn_1763875	$6.1e^{-03}$	0.0022	0.0842	0.0009	0.0035	0.291	ABCF1
ilmn_1738767	$6.5e^{-03}$	-0.0012	-0.0780	-0.0019	-0.0005	0.293	PLP2
ilmn_1747227	$7.1e^{-03}$	0.0008	0.1412	0.0003	0.0013	0.295	ADORA1
Change in MMSE score (9 years)							
ilmn_1774761	$5.5e^{-05}$	-0.0040	-0.1305	-0.0056	-0.0024	0.033	CCR2
ilmn_2366212	$2.1e^{-03}$	0.0087	0.1047	0.0041	0.0134	0.305	CD79B
ilmn_1710017	$2.2e^{-03}$	0.0052	0.1013	0.0024	0.0079	0.310	CD79B
ilmn_1677440	$2.9e^{-03}$	-0.0059	-0.0946	-0.0091	-0.0027	0.325	ATP6AP2
ilmn_1747227	$3.2e^{-03}$	0.0009	0.1169	0.0004	0.0015	0.332	ADORA1
ilmn_1737398	$3.3e^{-03}$	-0.0012	-0.0938	-0.0018	-0.0005	0.332	PTPLAD1
ilmn_1785439	$4.1e^{-03}$	0.0046	0.1024	0.0020	0.0072	0.349	CD79B
ilmn_1682312	$6.5e^{-03}$	-0.0025	-0.0726	-0.0040	-0.0010	0.377	CYBB
ilmn_1811049	$6.8e^{-03}$	0.0012	0.0999	0.0005	0.0019	0.379	POU2AF1
ilmn_1771333	$6.9e^{-03}$	-0.0035	-0.0877	-0.0057	-0.0014	0.380	CD47

related prodromal diagnoses without supplementing it with other cognitive measures, although the population relevance of these clinically-derived constructs remains disputed [14]. Over several years follow-up the MMSE does however provide a useful measure of cognitive impairment and cognitive decline in participants with and without dementia [12], and is the most widely used measure to monitor cognitive function and decline in elderly adults in clinical settings. We therefore consider that it is an appropriate tool to assess age-related differences in cognitive function in our cross sectional population.

Our functional, rather than pathological, classification of participants was important for several reasons. Cognitive decline leads to impairments in activities of daily living, and is a major public health threat. It is also rarely determined purely by one pathophysiological mechanism - mixed dementia is now considered to be the most common form of dementia [15] and functional decline has only a moderate association with the pathological changes of Alzheimer disease [16]. Finally, our use of a large, population-based cohort, rather than from specialist clinics, gave us power to detect moderate biomarker effects that could be of clinical application in population testing and risk reduction: biomarkers of functional cognitive change may hold more clinical application in the population than markers of single pathological processes.

We investigated blood leukocytes in order to identify clinically useful, minimally invasive markers of cognitive function since cortical tissue is inappropriate for the identification of cognitive decline risk or progression in the population. Furthermore, recent studies suggest that circulating systemic factors such as chemokines and their receptors have an important role in neurogenesis and cognitive function in animal models 8. Peripheral inflammation has also been shown to impact negatively on human cognitive function [17] with chronic immune activation in the brain having an association with neurodegenerative disease and cognitive decline [18,19].

There are both similarities and differences between our data and previous studies [9,10,20,21]. It has been suggested that genes involved in cytoskeletal maintenance, cellular trafficking, cellular stress response, redox homeostasis, transcription and DNA repair may be associated with Alzheimers Disease [14] and proteomics studies have suggested that circulating lymphocytes may be promising biomarkers for this disorder [25]. The differences could arise from different patient selection criteria; we used population data rather than data from clinical settings.

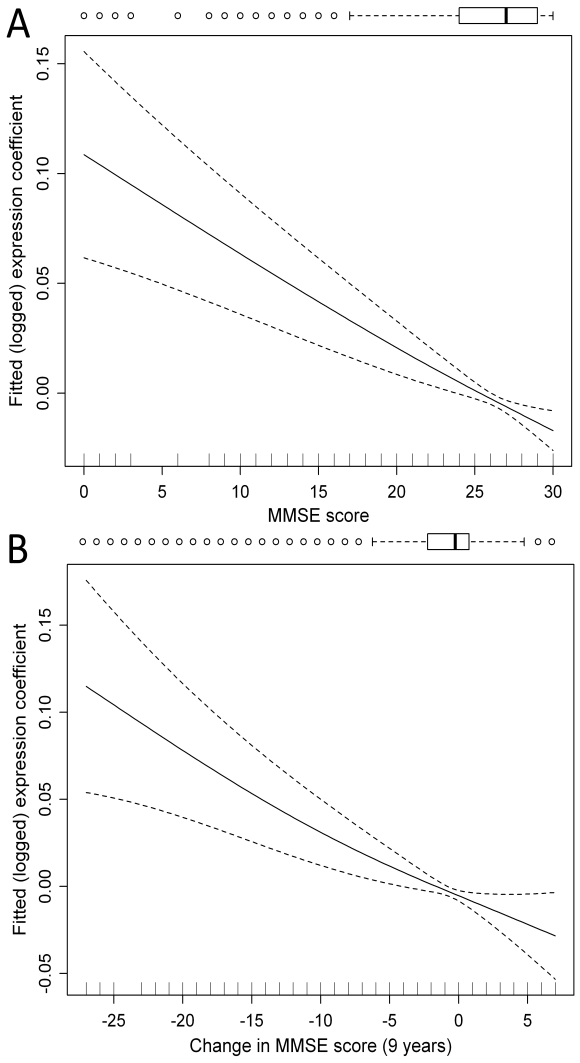

Figure 3: **Cubic Spline plots for linearity of association between CCR2 expression and MMSE** — **A.** CCR2 expression and MMSE at RNA collection. **B.** CCR2 expression and delta-MMSE over the preceding nine years. These penalized cubic regression splines visualize the fitted (adjusted) relationship between CCR2 gene expression and MMSE score, and separately with change in MMSE over 9 years. The relationships are approximately linear after normalization (log-transformed expression data) and adjustment for multiple confounders

Differences with prior proteomic findings in peripheral blood in cognitive decline [22,23] may also be due to small study sizes or the possibility that circulating cytokines previously found associated with cognitive decline may be expressed by activated CNS inflammatory cells and released into the circulation, rather than being expressed by circulating leukocytes [24].

The CCR2 receptor binds CCL2, a protein that is abundantly expressed in macrophage-rich areas of atherosclerotic plaques and in brain microglia [7] resulting in migration of macrophages and brain microglial cells to their site of action. In mouse models, vascular disruption of Ccr2 or Ccl2 leads to a reduction in atherosclerotic plaque formation in ApoE- or LDL-R null mice, even when fed a high fat diet [6,25]. This may be mediated by Ccr2 depletion reducing macrophage infiltration. In mouse brain, Ccr2 knockout has been shown to result in accelerated disease progression, increased mortality and an increase in soluble Aβ assemblies [7,26]. Work on CCR2 gene knockout mice bred on a background of chimeric mouse/human β-amyloid precursor/presenilin over-expression (APP^{Swe}/PS1/$CCR2^{-/-}$) suggested that AD might be associated with a decreased expression of CCR2 26. Although our results at first appear to conflict with this, we suggest that the increased CCR2 levels we note in cognitively impaired individuals probably reflects a reactive increase in the need for chemoattractants in subjects with increased β-amyloid deposition. This is supported by the observation that the ligand for CCR2, CCL2, has previously been reported to be upregulated in the brain of patients suffering from AD [27]. Recent studies have also suggested that CCR2 is with a key modulator of negative regulation of neurogenesis and cognitive function in mice [8]. Our data supports a role for CCR2 in the aetiology of age-related cognitive decline, and indicates that the CCR2 mouse models [7,26] may have particular relevance to the human population, although much more work is needed to confirm this mechanism in human populations.

Key limitations of our study include the possibility that that there are gene expression correlates of cognitive function in specific white cell subtypes which we have not measured separately. These are not however likely to be very marked or common, as we have found only limited overall expression changes. Similarly, as noted above the MMSE score, a widely used clinical measure of cognitive function in older people, suffers from a relative insensitivity to frontal-executive dysfunction and visuo-spatial deficits [28], and a ceiling effect inhibiting sensitive differentiation between medium and high cognitive performers [29]. Our cohort may also be subject to informative loss to follow up, and reflect a higher functioning group, since people with very impaired cognitive function may not have reported for blood sampling in year 9.

We note also that several probes to CCR2 transcripts were present on the chip, but only one showed associations with MMSE score. Differences in the relationship between phenotype and alternative specific probes for the same gene are not atypical in microarray studies. These discrepancies can arise from several factors including differences in binding dynamic of the indi-

Table 4: Biological and molecular functional pathway enrichments for negative correlation with MMSE are presented

Pathway	Size	ES	p-value	q-value
Aromatic compound metabolic process	17	-1.82	0	0.85
Regulation of DNA metabolic process	29	-1.74	0.008	0.95
T cell activation	27	-1.56	0.031	1
Transferase activity transferring glycosyl groups	68	-1.42	0.034	1
Cellular defense response	38	-1.52	0.035	1
Anatomical structure formation	27	-1.55	0.036	1
Positive regulation of lymphocyte activation	15	-1.58	0.039	1
Coenzyme metabolic process	30	-1.44	0.045	1
RNA export from nucleus	17	-1.52	0.045	1
DNA recombination	32	-1.5	0.047	1

vidual probes, differences in the inter-individual intensities between probe signal which can add noise to the data and reduce power and the presence of alternatively-processed isoforms which may bind probes differentially. In the case of CCR2, three alternatively-expressed isoforms exist, with only a single probe, ilmn_1774761, capable of binding to all isoforms. If the effect we note is driven by a specific isoform, only those probes that identify the transcript in question will show a significant association. Since up to 90% of all genes are alternatively spliced, and the full transcriptomic output from any gene is not fully known at present, this can lead to apparent differences in the association of particular probes with disease phenotype in association studies.

Future work should first seek to replicate our CCR2 finding, and explore associations with the full range of isoform specific probes for the main target genes. Work should also seek to gather more evidence on which of the near significant probes may in fact be associated with cognitive function. Future prospective studies will also provide valuable information as to the role of chemo-attractant proteins in age-related cognitive decline. Functional work in humans is also needed to clarify the mechanisms involved in the raised expression of CCR2 we observed in those with lower or declining MMSE scores. Studies of the role of CCR2 in specific forms of dementia (Alzheimers, vascular, Lewy Body etc, or mixed) would also help characterise the role of CCR2. If raised expression of CCR2 is a helpful but insufficient response to the accumulation of -amyloid, then attempts to increase this response further may potentially be effective. Studies of the role of CCR2 in vascular disease and pro-inflammatory response would also be informative. Our findings raise the possibility that CCR2 expression levels may also be associated with early, pre-symptomatic cognitive changes, although a more sensitive approach than change in MMSE score would be required to detect this.

To conclude, we have carried out the largest assessment of in-vivo leukocyte human gene expression alterations in conjunction with MMSE measured cognitive function in a predominantly older population, to date. We identified associations between MMSE score and CCR2 transcript levels, which may reflect the key role CCR2 plays in the removal of -amyloid and in regulation of neurogenesis. Work is now needed to confirm our findings, establish whether these reflect the proposed mechanisms and relate the circulating transcriptome changes to specific forms of brain pathology.

4 Material and Methods

4.1 Study cohort

We used InCHIANTI, a population-based study of aging [30], which has followed older persons over a 9 year period to assess normal aging using both interviews (conducted at the participants home by experienced interviewers) and blood samples (in the study clinic, all patients fasted for 8 hours prior to collection). Peripheral blood samples for RNA extraction were collected from participants at the 9 year follow-up (2008/9). Cohort demographics are given in table 1. Ethical approval was granted by the Instituto Nazionale Riposo e Cura Anziani institutional review board in Italy. Participants gave written informed consent to participate and for sample collection after having received an extensive description of the procedures, purposes and potential risks of the study. RNA was extracted from each sample using the PAXgene Blood mRNA kit (Qiagen, Crawley, UK) according to the manufacturers instructions. To ensure population relevance, no exclusions for co-morbidity were made in the main analysis.

4.2 Whole transcriptome scan

Whole genome expression profiling of the samples was conducted using the Illumina Human HT-12 microarray (Illumina, San Diego, USA), data processing was carried out using the Illumina and Beadstudio software (Illumina, San Diego, USA) as previously described [31]. Baseline intensities were calculated as mean and SD computed over all beads for a particular probe. QC steps included correction for local background effects, removal of outlier beads, computation of average bead signal and SD for each probe and gene, calculation of detection p-values using negative controls present on the array, quantile normalization across arrays, check of outlier samples using a clustering algorithm, and checks of positive controls. Subject level QC steps included removing individuals where the expression intensity was +/- 3 standard deviations from the mean. All microarray experiments and analyses complied with MIAME guidelines [32]. Following microarray data QC steps, 16,571 transcripts gave reliable signals above background ($p<0.01$) in >5% of the sample population and were therefore eligible for analysis.

4.3 Cognitive assessment

Participant interviews and clinical assessment, including the Mini-Mental State Examination of global cognitive function (MMSE), were undertaken at baseline, and at years 3, 6 and 9 of follow-up. The MMSE is an assessment of global cognitive function widely used in both hospital and community settings as a dementia screening tool [33,34].

4.4 Sample and statistical analysis

From 733 blood samples collected, RNA quality and microarray QC steps resulted in loss of 35 participants from the analysis. From the remaining 698 participants, 7 were excluded due to absence of MMSE data, yielding 691 individuals. Three further individuals were dropped from the regression models due to incomplete leukocyte data. (See table 1 for summary statistics of the cohort included in the analyses). All the remaining 688 individuals were included in the analyses, irrespective of other pathologies.

Associations between gene expression and MMSE score at RNA collection were analysed using multiple regression models adjusted for the following potential confounding factors: age in years (as a continuous variable), gender, highest level of education received (in five categories: none, elementary, secondary, high school, and university/professional), lifetime pack-years smoked (in four categories: none, 0.1 to 20 years, 20 to 39 years, and 40 plus years), blood leukocyte type (neutrophil, lymphocyte, monocyte, eosinophil percentages) each as continuous variables, hybridisation and amplification batch (in 10 and 14 categories respectively), and study site; participants lived in either a rural village (Greve) or an urban site (Bagno a Ripoli).

Separate linear regression models were fitted for each of the full set of 16,571 probes which passed QC in the discovery dataset. We have expressed the effect sizes of the associations in standardized betas to aid in the interpretation (expression intensities vary from probe to probe, and the normalization procedures further affect the interpretability of coefficients). We controlled for the effect of multiple testing by measuring the statistical significance of each association using both the p-value and the q-value. The q-value quantifies significance in terms of the false discovery rate rather than the false positive rate [35], and forms a measure of how likely a particular p-value is to represent a genuine association. Expression levels were taken to have a significant association with MMSE score at RNA collection, or cognitive decline from baseline 9 years previously, if the association achieved a nominal p-value $=0.05$ and a q-value <0.1. Our study was powered to detect expression differences of <0.25 SD for the 16,571 transcripts studied, allowing us to detect moderate expression differences between groups.

4.5 Inflammatory gene scan

The large number of transcripts tested in the whole genome scan yields a very stringent requirement for statistical significance, resulting in a substantial risk of type II (false negative) error. Since leukocytes are an inflammation-related tissue, we carried out a sub-analysis focusing on a smaller subset of genes involved in inflammatory response only. The genes of interest were identified using the Molecular Signatures Database (MSigDb) [36], with search terms for inflammatory/immune-related gene pathways as defined by the Gene Ontology (GO) project (www.geneontology.org). 44 inflammation-related genesets were returned, comprising 635 unique probe IDs, which equate to 425 unique genes with available data in our cohort (Supplementary table 1). Linear regression models were carried out as described above. This study is powered to detect expression differences of <0.22 SD for the inflammation transcript set.

4.6 PCR validation

The expression of the CCR2 gene in a subcohort of 100 individuals selected at the extremes of the MMSE spectrum was validated by the use of a custom real-time PCR assay (probe and primer sequences available on

request). Reaction mixes included 5 µl 2x TaqMan universal master mix (no AMPerase) (Applied Biosystems, Foster City, USA), 30 µl dH2O and 2 µl cDNA template. PCR amplifications were performed on the ABI 7900HT platform (Applied Biosystems, Foster City, USA). Cycling conditions were 50°C for 2 minutes, 94.5°C for 10 minutes followed by 40 cycles of 97°C for 30 seconds and 57.9°C for 1 minute. The expression of each gene was measured in triplicate for each sample. Gene expression relative changes were quantified using the $2^{-\delta\delta Ct}$ method [37] relative to the geometric mean of the GUSB, B2M and PPIA endogenous controls. The correlation between quantifications achieved using microarray and by real-time PCR was then assessed using linear regression.

4.7 Pathway Analysis

We also performed GSEA to identify gene sets/pathways associated with cognitive function, according to the method of Subramanian et al [36]. Molecular or biological function pathways were identified using Gene Ontology gene sets from the molecular signature database (MSigDB) (http://www.broadinstitute.org/gsea/msigdb/index.jsp). Gene set size filtering excluded gene sets containing less than 15 or more than 500 genes. Genes were ranked according to the magnitude of their association with cognitive function, and representation of each gene set within this ranked list was analysed. Gene sets significantly overrepresented at the top or bottom of the ranked list were taken to be significantly associated with cognitive function. A signal-to-noise metric was used to rank genes, and gene set enrichment scores were calculated using a weighted enrichment statistic. One thousand random permutations of the phenotype label were used to calculate the empirical p-values of each pathway compared to the p-values that would be ascertained by chance. Gene sets with a nominal p-value$<$0.01 and q-value$<$0.1 were considered associated with cognitive impairment or cognitive decline.

4.8 Spline regression

Using R package mgcv [38] we fitted an adjusted generalized additive model (GAM) using a smoothed penalized cubic regression spline; the smoothing parameter was chosen automatically by cross-validation. The same data and covariates that made up the original generalized linear regression (GLM) screen were used. GAM is applied here because it allows non-parametric fits, and thus if an aberrant relationship between expression and age had existed it could be highlighted.

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7 Contributions

LWH oversaw the validation experiments, interpreted the data and co-wrote the manuscript. RBS aided in study design interpreted the data and contributed to the manuscript. DL added clinical input, aided in study design and contributed to the manuscript LP carried out the multivariable regression analysis and contributed to the manuscript AF carried out the real-time PCR validation of CCR2 levels WH oversaw the statistical analysis, and contributed to the manuscript.. DH carried out the microarray experiments. JMG aided in design of the InCHIANTI study and contributed to the manuscript. SB oversees the InCHIANTI study and contributed to the manuscript. AS oversaw the microarray experiments. LF organized the sample cohort and contributed to the manuscript. DM managed the project, interpreted the data, co-wrote the manuscript and contributed funding.

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