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Implication of the Immune System in Alzheimer's Disease: Evidence from Genome-Wide Pathway Analysis

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Abstract. The results of several genome-wide association studies (GWASs) in the field of Alzheimer's disease (AD) have recently been published. Although these studies reported in detail on single-nucleotide polymorphisms (SNPs) and the neighboring genes with the strongest evidence of association with AD, little attention was paid to the rest of the genome. However, complementary statistical and bio-informatics approaches now enable the extraction of pertinent information from other SNPs and/or genes which are only nominally associated with the disease risk. Two different tools (the ALIGATOR and GenGen/KEGG software packages) were used to analyze a large GWAS dataset containing 2,032 AD cases and 5,328 controls. Convergent outputs from the two gene set enrichment approaches suggested an immune system dysfunction in AD. Furthermore, although these statistical approaches did not adopt *a priori* hypotheses concerning a biological function's putative role in the disease process, genes associated with AD risk were overrepresented in the "Alzheimer's disease" KEGG pathway. In conclusion, a systematic search for biological pathways using GWAS data set seems to comfort the primary causes already suspected but may specifically highlight the importance of the immune system in AD.

Keywords: Alzheimer's disease, APOE, Ca^{2+}, gene, GWAS, immunity, MHC, pathway, polymorphism

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INTRODUCTION

Although mutations in the amyloid-β protein precursor (AβPP), presenilin-1, and presenilin-2 genes have been shown to account for most cases of the early-onset, autosomal dominant forms of AD, the latter account for less than 1% of all AD cases [1]. The genetics of the common form of AD appear to be far more complex and the only unequivocally established genetic risk factor until now was the ε4 allele of the apolipoprotein E (APOE) gene [2].

As is the case in most multifactorial diseases, researchers investigating the genetics of AD have turned to high-throughput or very high-throughput genotyping to analyze case-control studies of hundreds of thousands of polymorphisms. However, the initial genome-wide association studies (GWASs) of case-control collections in AD each examined a relatively small number of cases. To circumvent this limitation, we and others recently performed two large, independent GWASs on over 14,000 individuals. Both studies yielded compelling evidence to suggest that the clusterin (CLU), complement component (3b/4b) receptor 1 (CR1), and phosphatidylinositol-binding clathrin assembly protein (PICALM) genes are all associated with AD risk [3,4]. Nevertheless, if the estimate that 60–80% of AD risk is due to genetic factors is correct, additional genetic susceptibility loci remain to be identified [3–5].

It is important to bear in mind that conventional GWA approaches are primarily based on the application of a highly conservative Bonferroni correction, which ultimately selects only the most highly statistically significant associations. Accordingly, it is legitimate to consider that the "missing" genetic determinants were probably rejected on purely statistical grounds, since they only presented a nominal association with the disease risk. This limitation can be overcome by using meta-analyses of GWASs to gain statistical power (as already been successfully performed in obesity or hypertension, for instance) [6–8]. Other recently developed, complementary statistical and bioinformatics approaches are also capable of extracting pertinent information from SNPs and/or genes nominally associated with disease risk [9–12].

We decided to perform this type of comprehensive analysis on our GWAS dataset. Our main hypothesis was that the genetic determinants of AD are concentrated within one or more specific biological pathways, rather than being randomly distributed. Several methods exist for ranking gene pathways in terms of their involvement in disease susceptibility. A number of computer programs have been developed to test for over-representation of gene ontology (GO) categories (e.g., biological processes) in lists of significant SNPs produced in GWASs. We used the ALIGATOR software to analyze our single-SNP GWAS dataset. This method takes account of multiple sources of potential bias, such as linkage disequilibrium between SNPs, variable gene size, overlapping genes. and non-independent GO categories [13].

However, ALIGATOR (like most techniques based on GO category analysis) is limited by the fact that each functional category is analyzed independently; there is no unifying analysis at the pathway or system level. Furthermore, less than 1% of the GO annotations have been confirmed experimentally [14]. In order to take account of these limitations, we also used the GenGen software package to perform pathway-based analysis of GWA data. This approach is based on use of the KEGG database to detect the over-representation of genes from a specific pathway. It also enables one to define the position of the associated genes in a given pathway [9, 15,16].

MATERIALS AND METHODS

The GWAS [3]

Genomic DNA samples from 2,344 AD cases were available for analysis, prior to genotype quality control steps. All AD cases were evaluated by neurologists from Bordeaux, Dijon, Lille, Montpellier, Paris, and Rouen and were identified as French Caucasian. A clinical diagnosis of probable AD was established according to the DSM-III-R and NINCDS-ADRDA criteria. Genomic DNA samples of 7,076 controls were available from the 3C study, prior to genotype quality control steps. These controls were known to be dementia-free after four years of follow-up. The 3C Study is a population-based, prospective study of the relationship between vascular factors and dementia. It has been carried out in three French cities: Bordeaux (southwest France), Montpellier (south France), and Dijon (central eastern France). A sample of non-institutionalized, over-65 subjects was randomly selected from the electoral rolls of each city between January 1999 and March 2001 [17]. Written, informed consent was obtained from study participants or, for those with substantial cognitive impairment, from a caregiver, legal guardian, or other proxy. The study protocols were reviewed and approved by the appropriate institutional review boards.

DNA samples were transferred to the French National Genotyping Center (CNG) for genotyping. First-stage samples that passed DNA quality control procedures were genotyped with Illumina Human 610-Quad BeadChips. Samples that had been successfully genotyped for $>$ 98% of the SNP markers were selected for inclusion in the study. SNPs with a call rate $<$ 98%, a minor allele frequency (MAF) $<$ 1% or exhibiting departure from Hardy-Weinberg equilibrium in the control population ($p< 10^{-6}$) were excluded. On the basis of these genetic data, we removed 134 AD cases and 980 control samples because they were found to be first- or second-degree relatives of other study participants or of non-European descent. This process resulted in selection of 537,029 autosomal SNPs genotyped in 2,032 AD cases (mean age: 73.7 ± 8.9; mean age at onset: 68.3 ± 9.0, 34% men), and 5,328 controls (mean age: 73.8 ± 5.4; 34% men).

The SNP-to-gene mapping file

For both ALIGATOR and GenGen analyses (see URLs), gene and SNPs information data for chromosome 1–22 were extracted from NCBI ftp websites (respectively reference assembly, build 36.3 and dbSNP, build 130). Pseudogenes were systematically excluded from analyses. A SNP located between the 5' and 3' ends of the first and last exons of a gene was always assigned to the latter. A SNP located within 20kb of the 5' and 3' ends of the first and last exons of a gene was assigned to the latter, in order to take account of putative regulatory (i.e., expression-modulating) regions. However, if a given SNP was assigned to more than one gene, all the entries were re-analyzed.

ALIGATOR analyses

The ALIGATOR software was developed to test for over-representation of biological pathways (as indexed by GO terms) in lists of significant SNPs from GWASs. The software was implemented and used as described [13]. To define the p values of SNP associations, we used logistic regression to evaluate case vs. control differences. In order to take potential population stratification into account, this process optionally incorporated principal components that were significantly associated with disease status [3]. On the basis of a file containing the full set of our GWAS results (29,200 SNPs nominally associated with the AD risk at $p < 0.05$), three p value categories were then examined (< 0.01, < 0.001, and < 0.0001) (Table 1). As recommended by the tutorial on the ALIGATOR website, we generated up to 50,000 replicate genelists. The replicate genelists, generated from randomly-sampled SNPs, are used to calculate the category-specific p-value. We also tested different numbers of replicate studies (1,000, 2,000, or 5,000). These replicate studies are used to assess significance of the numbers of categories reaching various p-values, as well as study-wide significance levels for individual categories which are corrected for testing multiple non-independent GO categories. This latter parameter slightly modified the results. The data presented in the present report were derived from 50,000 replicate genelists and 1,000 replicate studies.

GWA KEGG pathway analyses

We used the GenGen package as described in the online tutorial (see URLs). To adjust for differences in gene size (i.e., different numbers of SNPs located within or near to each gene) and for linkage disequilibrium between SNPs within the same gene, a two-step correction procedure was performed. Firstly, the raw individual genotype data were analyzed by logistic regression with PLINK software. Again, potential population stratification is taken into account by optionally incorporating principal components that were significantly associated with disease status. As recommended by the software designer (see tutorial), we generated a GWA association result file including SNPs, associated chi2 and P-values for at least 1,000 phenotype permutations. At this stage, the file contained 28,866 SNPs nominally associated with AD at $p < 0.05$. Secondly, we applied the calculate_gsea.pl program, which has been designed to perform pathway-based GWA tests on high-density SNP genotyping data with respect to the KEGG databases (release 17 November 2009) [18]. This program uses (i) the GWA association result file, (ii) a SNP-to-gene mapping file, and (iii) a pathway annotation file to perform pathway-based association tests. The algorithm was adapted from that used in Gene Set Enrichment Analysis software [19]. The method selects the lowest p-value of all the SNPs near a gene but also uses phenotype-based permutation to adjust the statistical significance. Since pathways with only few genes and those with too many can bring some false positives, KEGG pathways containing from 10 to 200 genes were analyzed (as defined by -setmin 10 and -setmin 200 in the calculate_gsea.pl program). Once the most strongly associated pathways have been identified from amongst a set of candidates, the nominal p-values are calculated

Table 1
Number of GO pathways selected at different levels of significance using three different levels of associations for SNPs in the GWA data set

P value Criterion For SNPs	No of Top SNPs	No of assigned genes	No of assigned genes with GO	$P < 0.0001$ No of categories	$P < 0.0001$ P value[1]	$P < 0.01$ No of categories	$P < 0.01$ P value[1]	$P < 0.001$ No of categories	$P < 0.001$ P value1
0.0001	113	61	50	33	0.95	9	0.48	1	0.054
0.001	793	362	310	27	1.00	4	0.68	1	0.14
0.01	6423	2151	1766	30	1.00	3	0.70	1	0.20

[1]P values indicated whether signifcantly more GO pathways were identified than expected by chance.

from the permutation procedure and a false discovery rate (FDR) procedure has been used to control the fraction of expected false positive findings below a certain threshold.

RESULTS

Table 1 describes the number of categories reaching significance levels of 0.01, 0.001, and 0.0001 in ALIGATOR for over-representation in our GWAS dataset. The ALIGATOR software allows estimation whether this number of categories was obtained by chance or may results from a real over-representation. In our GWAS data set, no significant level of the excess of over-represented categories was observed (Table 1). However, many of the most significant individual GO categories appeared to be involved in immune processes, whatever the cut-off value used to select significant SNPs from the GWA dataset (< 0.01, < 0.001, or < 0.0001) (Table 2).

As mentioned above, the use of GO annotations presents a number of limitations, particularly the lack of a unified analysis at the pathway or system level. To circumvent this limitation, we used the GenGen software package and the associated KEGG database to detect gene over-representation in a specific pathway. As recommended by GenGen's developers, we first generated a GWA result file (including SNPs and the corresponding chi2 and p values for at least 1,000 permutations) by using a co-dominant model adjusted for principal components. These data were then cross-checked against a KEGG pathway annotation file using the calculate_gsea.pl program. We found that 4,776 SNPs nominally associated with the AD risk were assigned to 1,395 genes involved in 173 KEGG pathways. Following application of the FDR procedure, we identified 5 physiological or disease-related KEGG pathways displaying over-representation of genes associated with the AD risk in our GWA dataset (Table 3).

Remarkably, the top-ranked gene set/pathway was that referenced in KEGG as "Alzheimer's disease": 46 of its 163 genes presented nominal association with the AD risk. This over-representation was significant ($p = 0.001$) after FDR correction (Table 4 and Fig. 1). We also found specific over-representation of genes involved in one or more of KEGG's immune pathways. However, in contrast to the results obtained for the KEGG "Alzheimer's disease" gene set, we observed two potential causes of artificial enrichment in the KEGG immune gene sets. Firstly, given the design of our initial analysis, it is possible that some SNPs can be assigned to different genes (see the paragraph on SNP-to-gene mapping in the Material and Methods section). This assignation can be problematic when the SNP is located within a cluster of genes involved in the same biological pathway. Secondly, some SNPs may be in linkage disequilibrium and thus will ultimately bear the same information – whereas not assigned to the same gene. In order to take into account these two sources of bias in the selected KEGG immune gene pathways, we repeated an analysis in which each SNP was assigned to only one gene (arbitrarily the closest) and by selecting only one SNP in the event of an r^2 value $\geqslant 0.5$ when comparing different SNPs. These processes led to the exclusion of four SNPs. After having controlled for these sources of biases, we were still able to identify two KEGG immune pathways displaying the over-representation of genes associated with the AD risk in our GWA dataset: (i) antigen processing and presentation ($p = 0.04$) and (ii) regulation of autophagy ($p = 0.05$) (Tables 5, 6 and 7).

DISCUSSION

The simple GWA approach used to test hundreds of thousands of markers for association with a specific phenotype has proved to be quite successful in characterizing the major genetic determinants of certain diseases. However, the loci discovered to date do not ac-

Table 2
List of the most signifcantly overrepresented GO categories at different P value criterion for SNPs association with AD risk unsing ALIGATOR

P value Criterion For SNPs	GO ID	Type	Total genes in category	Nb of genes on list	Category-specific p-value	Study wide p-value[1]	Function
$P < 0.01$	48261	Biological process	5	3	0.00012	0.2	Negative regulation of receptor-mediated endocytosis
	32395	Molecular function	8	6	0.00052	0.532	MHC class II receptor activity
	3918	Molecular function	4	3	0.00086	0.703	DNA topoisomerase (ATP-hydrolyzing) activity
	42613	Cellular component	12	7	0.00118	0.806	MHC class II protein complex
	43190	Cellular component	9	4	0.0014	0.846	ATP-binding cassette (ABC) transporter complex
	31307	Cellular component	6	3	0.00154	0.871	Integral to mitochondrial outer membrane
	2504	Biological process	15	7	0.0017	0.893	Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II
	5132	Molecular function	8	3	0.00184	0.904	Interferon-alpha/beta receptor binding
	6268	Biological process	13	4	0.0019	0.914	DNA unwinding during replication
	6955	Biological process	542	73	0.00212	0.947	Immune response
$P < 0.001$	33344	Biological process	13	4	0.00004	0.14	Cholesterol efflux
	42613	Cellular component	12	4	0.00018	0.4	MHC class II protein complex
	2504	Biological process	15	4	0.00022	0.49	Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II
	2455	Biological process	30	4	0.00096	2.05	Humoral immune response mediated by circulating immunoglobulin
	34379	Biological process	5	2	0.0012	2.53	Very-low-density lipoprotein particle assembly
	34377	Biological process	7	2	0.00158	3.33	Plasma lipoprotein particle assembly
	65005	Biological process	8	2	0.00162	3.41	Protein-lipid complex assembly
	32395	Molecular function	8	3	0.00162	3.41	MHC class II receptor activity
	5319	Molecular function	55	7	0.00174	3.69	Lipid transporter activity
	31490	Molecular function	6	2	0.0023	4.8	Chromatin DNA binding
$P < 0.0001$	2455	Biological process	30	3	0.00002	0.054	Humoral immune response mediated by circulating immunoglobulin
	19724	Biological process	48	3	0.00012	0.136	B cell mediated immunity
	16064	Biological process	46	3	0.00012	0.136	Immunoglobulin mediated immune response
	51235	Biological process	47	3	0.00024	0.209	Maintenance of location
	2449	Biological process	58	3	0.00034	0.267	Lymphocyte mediated immunity
	2460	Biological process	64	3	0.00034	0.267	Adaptive immune response based on somatic recombination of immune receptors built from immuno
	2250	Biological process	64	3	0.00034	0.267	Adaptive immune response
	2443	Biological process	64	3	0.00048	0.344	Leukocyte mediated immunity
	6959	Biological process	64	3	0.00092	0.478	Humoral immune response
	17038	Biological process	93	3	0.00108	0.519	Protein import
	50778	Biological process	83	3	0.00116	0.539	Positive regulation of immune response

[1]P values indicated whether the signifcantly GO pathways were potentially identified by chance.

count for the complete heritability for most of the studied phenotypes. It has been suggested that since (i) GWASs are probably insufficiently powered to detect small main effects (false negatives) and (ii) gene-gene interactions are likely to play a role, the full potential of highly complex GWAS datasets may not yet have realized [6]. Gene set enrichment analyses can address this complexity by considering multiple loci simultaneously and relating them to known functional annotations. Hence, pathway analyses can lead to new discoveries overlooked in simple, single-SNP tests and thus successfully identify associations with pathways involved in pathogenesis.

This context prompted us to perform gene set enrichment analyses of a GWAS dataset featuring 2,032 AD cases and 5,328 controls. Using the GenGen software

Table 3
KEGG pathways signicantly overrepresented in our GWA data set

KEEG Ref.	Total No of genes[1]	No of genes[2]	No of selected genes[3]	Nominal P-value	FDR P-value	Ranked list of over-represented gene sets/pathways
hsa05010	163	148	46	0.001	0.001	Alzheimer's disease
hsa04140	34	31	16	0.003	0.007	Regulation of autophagy
hsa04650	131	127	64	0.002	0.02	Natural killer cell mediated cytotoxicity
hsa04622	81	78	30	0.003	0.02	Antigen processing and presentation
hsa04612	71	64	29	0.001	0.03	RIG-I-like receptor signaling

[1]number of genes assigned to the pathway in the KEGG database.
[2]number of genes for which a SNP in the GWA database has been assigned.
[3]number of genes presenting a SNP nominally associated with AD risk and finally retained for estimation of overrepresentation.

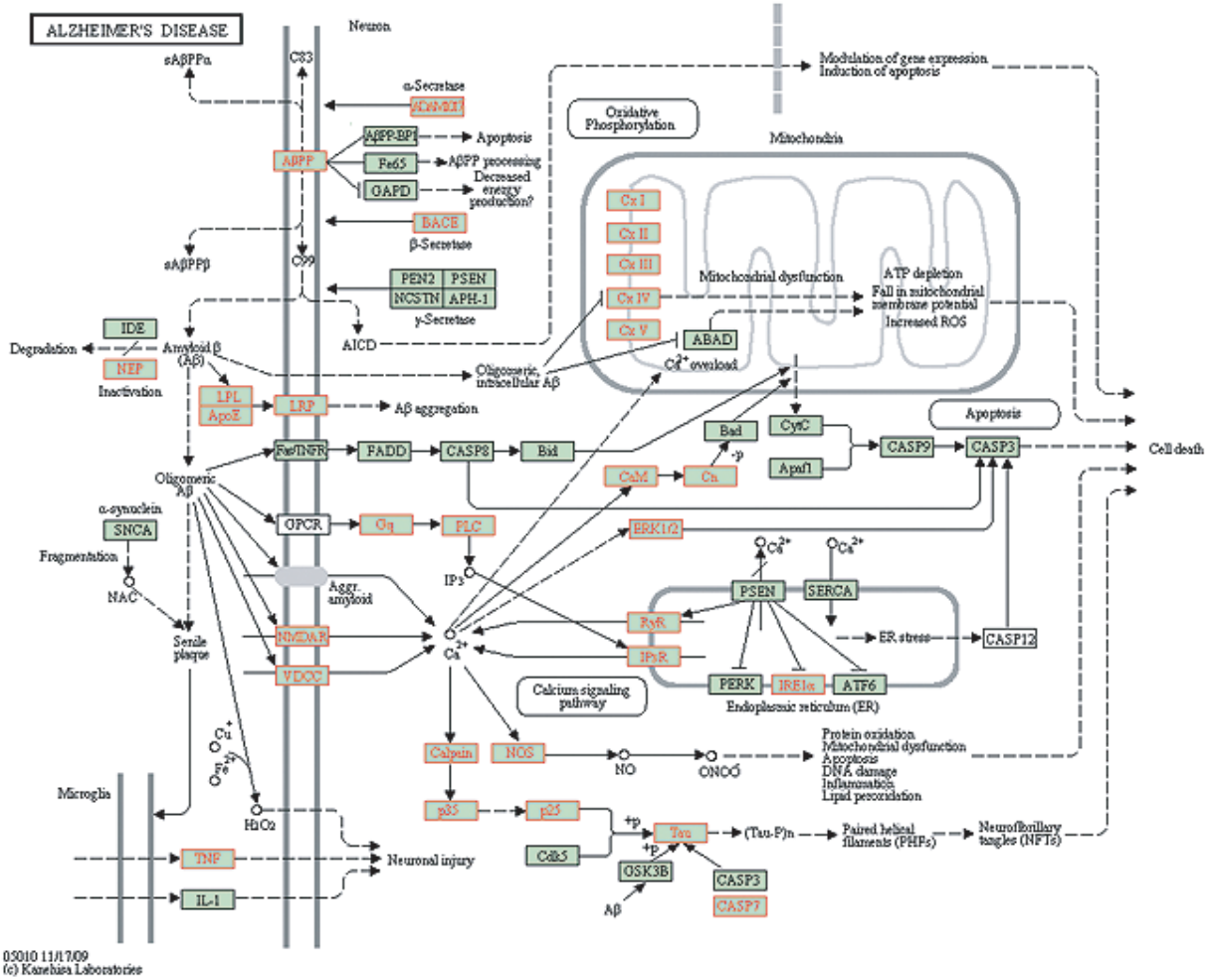

Fig. 1. A depiction of the "Alzheimer's disease" pathway in the KEGG database. Proteins or complexes encoded by genes nominally associated with the AD risk and selected for over-representation by the GenGen package are indicated in red.

package, we observed an over-representation of genes associated with the AD risk in the KEGG "Alzheimer's disease" pathway. Since the gene set enrichment analysis does not make any *a priori* hypotheses about the biological functions involved in the disease process, our findings appear to be relevant and should advance our current understanding of AD. For instance, genes coding for α-secretase (ADAM10 [20]) or β-secretases

Table 4
list of genes nominally associated with AD risk and defined using the GenGen software as significantly involved in the hsa05010 KEEG pathway (Alzheimer's disease)

GENE	best associated SNP	P-value
APOE	rs2075650	3,6E-110
PLCB4	rs6086834	5,8E-04
PLCB1	rs6086570	9,1E-04
CDK5R1	rs756785	1,7E-03
MAPT	rs1467967	1,9E-03
RYR3	rs16957135	2,6E-03
ADAM10	rs653765	2,7E-03
ITPR2	rs17477122	2,9E-03
CACNA1C	rs4765898	4,0E-03
NDUFS6	rs750495	4,8E-03
MME	rs2016848	5,5E-03
GRIN2B	rs12818068	6,7E-03
ITPR1	rs2291597	7,0E-03
BACE2	rs6517656	7,1E-03
ITPR3	rs4713646	7,6E-03
BACE1	rs560564	8,3E-03
COX4I2	rs6060454	8,5E-03
COX7B2	rs9291291	8,5E-03
NOS2A	rs3794764	9,3E-03
APP	rs462281	1,0E-02
CALM1	rs1058903	1,1E-02
PPP3CA	rs17030795	1,1E-02
CACNA1D	rs3796349	1,2E-02
NDUFS2	rs10797094	1,2E-02
NOS1	rs12099598	1,2E-02
COX7A2L	rs1981664	1,2E-02
CASP7	rs11196449	1,2E-02
LPL	rs4466415	1,3E-02
PLCB3	rs915987	1,4E-02
SDHC	rs4272646	1,6E-02
TNF	rs3132452	1,7E-02
NDUFA9	rs4147683	1,8E-02
CACNA1S	rs3767498	1,9E-02
GNAQ	rs7033572	2,0E-02
CAPN1	rs17743381	2,2E-02
UQCRC2	rs11648723	2,3E-02
NDUFV3	rs4148972	2,3E-02
GRIN2C	rs7219247	2,3E-02
ERN1	rs17688326	2,4E-02
NOS3	rs3918227	2,5E-02
COX6B2	rs11084396	2,5E-02
LRP1	rs1800159	2,6E-02
ATP5C1	rs1244447	2,7E-02
MAPK1	rs2298432	2,8E-02
UCRC	rs16988025	2,9E-02
CAPN2	rs751128	3,0E-02

(BACE1 and BACE2 [21]) have SNPs nominally associated with the AD risk in their vicinity (i.e., in the gene itself or within 20kb of the 5' and 3' ends of the gene's first and last exons). This observation may indicate that changes in the expression or function of proteins directly involved in AβPP metabolism may slightly modify the risk of developing AD in sporadic, late-onset forms of AD. This overrepresentation of genes associated with AD risk in the "Alzheimer's disease" KEGG pathway seems also to point out Ca^{2+} signaling involvement (Fig. 1) [22]. This observation is of particular interest, since much biological evidence has suggested that impaired Ca^{2+} signaling is involved in the physiopathology AD (by modulating AβPP metabolism [23] or in modulating toxicity linked to amyloid-β (Aβ) peptide exposure [24], for example).

In addition to these well-documented processes, our study suggests that the regulation of autophagy and antigen processing and presentation are also involved in AD. Autophagy has already been suspected of playing a role in AD [25]. Firstly, this biological process seems to be induced but impaired in neurons in the AD brain, since autophagic vacuoles accumulate dramatically in dystrophic neuritis [26]. Secondly, it has been suggested that autophagy can protect neurons from Aβ-induced apoptosis [27]. In fact, it is not clear whether autophagy has causative or a protective role or whether induction is a consequence of the disease process [28].

Interestingly, endogenous presentation of an epitope derived from proteins on MHC class II can be mediated by autophagy [29]. We found that the KEGG "antigen processing and presentation" pathway also displays over-representation of the genes associated with the AD risk in our GWA dataset. One can postulate that Aβ peptides are endocytosed by antigen-presenting cells, processed into fragments that are bound to MHC molecules and presented to T lymphocytes. Antigen presentation can lead to B-cell stimulation and then production of Aβ-specific auto-antibodies. In conclusion, specific immune responses could appear to be capable of inducing Aβ degradation and may constitute a natural line of defense against harmful accumulation of the Aβ peptides. Of course, such mechanisms have been reported in Aβ peptide-immunized AD patients: individuals have shown a dramatic reduction of amyloid deposition when compared with non-immunized individuals [30]. However, natural antibodies against Aβ peptides are present in the sera of AD patients and in non demented individuals [31]. These auto-antibodies inhibit Aβ peptide aggregation *in vitro* [32], and it has been observed that in non-amyloid-immunized AD patients, auto-antibodies against Aβ peptides may help reduce the plaque burden and increase the numbers of phagocytic microglia [32]. Altogether, these data can be interpreted as an Aβ peptide antibody-dependent activation of immune response. Furthermore, even if the CLU and CR1 genes are not annotated in the KEGG immune response pathways we picked-up, this hypothesis would be in accordance to our initial results in-

Table 5
immune KEGG pathways signicantly overrepresented in our GWA data set after correction for artificial cluster enrichment and LD control

KEEG Ref.	Total No of genes[1]	No of genes[2]	No of selected genes[3]	Nominal P-value	FDR P-value	Ranked list of over-represented gene sets/pathways
hsa04612	81	70	22	0.002	0.04	Antigen processing and presentation
hsa04140	34	28	11	0.005	0.05	Regulation of autophagy

[1]number of genes assigned to the pathway in the KEGG database.
[2]number of genes for which a SNP in the GWA database has been assigned.
[3]number of genes presenting a SNP nominally associated with AD risk and finally retained for estimation of overrepresentation.

Table 6
list of genes nominally associated with AD risk and defined (A) without and (B) with controlling for artificial gene cluster enrichment and LD between SNPs as signifciantly involved in the hsa04140 KEEG pathway (Regulation of autophagy) using the GenGen software

A. GENE	best associated SNP	P-value
IFNA14	rs1330320	4,7E-04
IFNA10	rs4977686	2,0E-03
IFNA16	rs4977686	2,0E-03
IFNA4	rs4977686	2,0E-03
IFNA7	rs4977686	2,0E-03
ATG7	rs2454505	2,5E-03
IFNA8	rs1330322	4,0E-03
IFNA21	rs7037868	4,8E-03
IFNA17	rs12337907	5,8E-03
IFNA1	rs7864960	7,5E-03
IFNA6	rs10119678	1,1E-02
PRKAA2	rs2179761	1,3E-02
PIK3R4	rs11713445	1,3E-02
IFNA13	rs1224392	1,4E-02
IFNA2	rs1224392	1,4E-02
IFNA5	rs28383775	1,4E-02
B. GENE	best associated SNP	P-value
IFNA14	rs1330320	4,7E-04
IFNA7	rs4977686	2,0E-03
ATG7	rs2454505	2,5E-03
IFNA8	rs1330322	4,0E-03
IFNA21	rs7037868	4,8E-03
IFNA16	rs12337907	5,8E-03
IFNA1	rs7864960	7,5E-03
PRKAA2	rs2179761	1,3E-02
PIK3R4	rs11713445	1,3E-02
IFNA13	rs1224392	1,4E-02
IFNA5	rs28383775	1,4E-02

volving CLU and CR1 as major genetic determinants of AD in influencing susceptibility to late onset forms of the disease through a role in Aβ clearance [3]. Furthermore it is worth noting that CR1 might act as either activator or inhibitor of B cell and T cell functions [33]. Interestingly CR1 is mainly expressed in the choroid plexus in the brain [34] and the choroid plexus could represent a site for lymphocyte entry in the CSF and brain, and for presentation of antigens [35].

Despite these interesting results, our study suffered from the usual limitations of gene set enrichment analyses [11]. The latter tend to highlight genes that contain many SNPs or indeed any pathway that contains several large genes. Conversely, these analyses tend to overlook pathways that only contain small genes. To compensate for this problem, we used two approaches based on permutation testing, needed to account for this size bias. Furthermore, the quality of the biological databases used in gene set enrichment approaches strongly influences the relevance of the resulting outputs. We addressed this specific point by using two different databases: GO and KEGG. The GO database mainly relies on computer prediction but also includes human annotation. It provides a broad spectrum of gene sets for testing enrichment. However, as previously mentioned, GO annotation analyses are limited (i) by the fact that each functional category is analyzed independently (in the absence of a unifying analysis at the pathway or system level) and (ii) because less than 1% of the GO annotations have been confirmed experimentally [14]. This is why we also used the KEGG pathway database; in contrast to GO, it is manually compiled on the basis of biological evidence [17]. Interestingly, both approaches highlighted the innate immune system (even though the over-representation of the immune GO categories in the ALIGATOR analyses was not significant). Furthermore, the fact that we observed significant enrichment of genes featured in the "Alzheimer's disease" gene set supports the pertinence of our results.

Lastly, gene set enrichment approaches have (in part) been developed to detect potential genetic determinants that are rejected on purely statistical grounds as a result of only nominal associations with the disease risk. However, it is likely that false positive associations are also included in these analyses and may lead to biased gene set enrichment by chance. Even though this specific point is difficult to assess, we crosschecked our data with the results obtained in another recent large AD GWAS [4]. However, the available online data were limited, since these only included SNPs associated at

Table 7
list of genes nominally associated with AD risk and defined (A) without and (B) with controlling for artificial gene cluster enrichment and LD between SNPs as signifciantly involved in the hsa04612 KEEG pathway (Antigen processing and presentation) using the GenGen software

A. GENE	best associated SNP	P-value
IFNA14	rs1330320	4,65E-04
HLA-DRB1	rs9270856	4,77E-04
HLA-DRA	rs3135344	5,44E-04
HLA-DPA1	rs2105929	8,08E-04
HLA-DOA	rs9277015	1,26E-03
IFNA10	rs4977686	1,97E-03
IFNA16	rs4977686	1,97E-03
IFNA4	rs4977686	1,97E-03
IFNA7	rs4977686	1,97E-03
HLA-E	rs1264456	3,50E-03
HLA-DOB	rs7767167	3,78E-03
IFNA8	rs1330322	3,97E-03
LTA	rs35969216	4,42E-03
IFNA21	rs7037868	4,77E-03
IFNA17	rs12337907	5,78E-03
TAP1	rs4148870	5,85E-03
TAP2	rs4148870	5,85E-03
NFYB	rs10778309	6,80E-03
IFNA1	rs7864960	7,52E-03
HLA-DQB1	rs7755224	8,53E-03
HLA-F	rs1610603	8,88E-03
CANX	rs7734102	8,94E-03
HLA-DQA1	rs9272105	8,98E-03
IFNA6	rs10119678	1,08E-02
HLA-B	rs2523619	1,19E-02
CIITA	rs4072865	1,21E-02
IFNA13	rs1224392	1,37E-02
IFNA2	rs1224392	1,37E-02
IFNA5	rs28383775	1,38E-02
CREB1	rs2551645	1,76E-02
HLA-DMA	rs714289	1,82E-02
HLA-DMB	rs714289	1,82E-02
CTSB	rs1736090	1,90E-02
HSPA6	rs1801274	2,10E-02
KIR3DL3	rs12151161	2,18E-02
B. GENE	best associated SNP	P-value
IFNA14	rs1330320	4,7E-04
HLA-DRB1	rs9270856	4,8E-04
HLA-DRA	rs3135344	5,4E-04
HLA-DPA1	rs2105929	8,1E-04
HLA-DOA	rs6933546	1,7E-03
IFNA7	rs4977686	2,0E-03
HLA-E	rs1264456	3,5E-03
HLA-DOB	rs7767167	3,8E-03
IFNA21	rs7037868	4,8E-03
IFNA8	rs7025006	5,6E-03
IFNA16	rs12337907	5,8E-03
NFYB	rs10778309	6,8E-03
IFNA1	rs7864960	7,5E-03
HLA-DQB1	rs7755224	8,5E-03
CANX	rs7734102	8,9E-03
HLA-DQA1	rs9272105	9,0E-03
HLA-B	rs2523619	1,2E-02
TAP2	rs2071544	1,3E-02
IFNA13	rs1224392	1,4E-02
CREB1	rs2551645	1,8E-02
HLA-DMB	rs714289	1,8E-02
CTSB	rs1736090	1,9E-02

the $p < 0.001$ level. Nevertheless, we found that 5 genes in the KEGG "Alzheimer's disease" gene set (APOE, GNAQ, BACE2, RYR3, and ITPR2) and 5 genes in the KEGG "antigen processing and presentation" pathway (HLA-DRB1, HLA-DRA, HLA-DOB, HLA-DQA1, and TAP2) were associated with the AD risk in both GWASs. Interestingly, the 6p21.3 region gene region (containing the MHC) has been already described as being associated with the AD risk in both by genome-wide linkage analyses and conventional candidate gene approaches [36,37]. Furthermore, it has been reported that the HLA-DRA gene is under-expressed in the AD brain, compared with controls [38]. Taken as a whole, these data support the association of the MHC region with the AD risk and the involvement of an inflammatory process in AD. Importantly, we are aware that clusters of genes with similar functions can create spurious evidence that multiple genes in a pathway are associated with the disease. However, we attempted to limit this potential bias in two ways: (i) we repeated the analyses by assigning each SNP to only one gene and (ii) we controlled for linkage disequilibrium in this region. These precautions suggest that the HLA-DRB1, HLA-DRA, HLA-DOB, HLA-DQA1, and TAP2 signals are more or less independent.

In conclusion, the validity and the strength of the present results are strongly dependent on the inherent limitations of gene set enrichment approaches. Furthermore, *in vitro* and *in vivo* experiments will be necessary to clearly understand how these genes could take place in the AD physiopathological process. Finally, it is important to bear in mind that these results will remain hypothetical until they are validated in other large GWASs. At this stage, a meta-analyses of GWASs would be particularly powerful. However, gene set enrichment analyses of our large GWAS appear to confirm the prime suspects in AD: AβPP metabolism and Ca^{2+} signaling. In addition, our data specifically highlight the importance of the innate immune system in the pathophysiology of this disease.

URLs

Aligator: http://x004.psycm.uwcm.ac.uk/∼peter/.

dbSNP: http://www.ncbi.nlm.nih.gov/projects/SNP/.

Gengen: http://www.openbioinformatics.org/gengen/.

KEGG: http://www.genome.jp/kegg/.

NCBI ftp: ftp://ftp.ncbi.nih.gov/.

Plink: http://pngu.mgh.harvard.edu/∼purcell/plink/.

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