Genome-wide analysis of RNA editing levels in human blood identified interactions with mRNA processing genes and suggested correlations with biological and drug-related variables.

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Abstract

Background

A-to-I RNA editing is a post-transcriptional modification catalyzed by ADAR enzymes, that deaminate Adenosines (A) into Inosines (I). Most of known editing events are located within inverted ALU repeats, but they also occur in coding sequences and may alter the function of encoded proteins. RNA editing contributes to generate transcriptomic diversity and it is found altered in cancer, autoimmune and neurological disorders. However, little is known about how editing process could be influenced by genetic variations, biological and environmental variables.

Results

We analyzed the dynamic of RNA editing in human blood using RNA-seq data from 459 healthy individuals and obtained detectable editing levels for 2,079 sites. Analysis of gene expression revealed that *ADAR* is the major contributor to editing, explaining ~13% of observed variability. After removing *ADAR* effect, we found significant associations for 1,122 genes, mainly involved in RNA processing. These genes were significantly enriched in genes encoding proteins interacting with ADARs, including 276 potential ADARs interactors and 9 ADARs direct partners. In addition, association analysis between editing levels principal components and 35 biological and drugs intake variables revealed 24 factors potentially influencing RNA editing in blood, including sex, age, BMI, drugs and medications. Finally, we identified genetic loci associated to global editing levels, including known *ADAR* eQTLs and a small region on chromosome 7, containing *LOC730338* lincRNA gene.

Conclusions

Our data provides a detailed picture of RNA editing and its variability in human blood, giving interesting insights on the mechanisms behind this post-transcriptional modification and its regulation.

Keywords

RNA editing, ADAR1, ADAR2, RNA-seq, Blood

Background

RNA editing is a post-transcriptional process based on the enzymatic deamination of specific adenosines (A) into inosines (I). Since inosine has similar base-pairing properties to guanosine, it is read as guanosine by both splicing and translation machineries, thus generating different RNA molecules from those coded by DNA [1]. RNA editing contributes to the diversification of the information that is encoded in the genome of an organism, thereby providing a greater degree of complexity. Currently, the conversion of A to I is thought to be the most common RNA editing process in higher eukaryotic cells, occurring especially in the brain [2].

RNA editing is catalyzed by adenosine deaminase enzymes (ADARs) [3,4]. In mammals, three members of the ADAR family have been characterized so far. ADAR1 (gene name: *ADAR*) and ADAR2 (gene name: *ADARB1*) are active enzymes expressed in many tissues, while ADAR3 (gene name: *ADARB2*) is expressed specifically in the Central Nervous System (CNS). To date, no functional RNA editing activity has been attributed to this enzyme. The critical role of ADAR enzymes is shown by phenotypes of knockout mice that resulted in embryonic lethality or death shortly after birth [5–7], clearly indicating that A-I RNA editing is essential for normal life and development. In addition, dysregulated RNA editing levels at specific re-coding sites have been linked with a variety of diseases, including neurological or psychiatric disorders and cancer [2,8,9]. Interestingly, ADARs mRNA and protein expression levels do not always reflect RNA editing levels [10]. It has been shown that the subcellular distribution of ADAR enzymes [11] and their interaction with inhibitors [12,13] and activators [14,15] influence ADARs activities.

Originally, A-to-I RNA editing in mammalian cells was described for a low number of mRNAs and it was responsible for deep changes of protein functions. These editing sites were discovered serendipitously comparing DNA and cDNA sequences by direct sequence analysis [16,17]. The number of identified RNA editing sites has largely increased with the diffusion of RNA sequencing

(RNA-Seq) studies based on next generation sequencing technologies (NGS), reaching over two millions of sites. The majority of RNA editing sites is located within intragenic non-coding sequences: 5'UTRs, 3'UTRs and intronic retrotransposon elements, such as ALU inverted repeats [18,19]. With lowering cost of NGS, many RNA-Seq datasets from human tissues, healthy and pathological conditions, have been deposited in sequence databases, available to the scientific community. In parallel, the development of computational pipelines to search for RNA editing sites on RNA-Seq data, allowed a global analysis of the editing reaction, shedding light on its evolutionary conservation [20], tissues specificity [21,22], cellular specificity [23] and its role in diseases such cancer [24] or neurological disorders [9,25].

About 2.5 million editing sites have been identified so far and are listed in RNA editing databases [26,27], but only recently the dynamic and regulation of RNA editing has been systematically investigated in human tissues [22]. However, little is known about how editing process could be influenced by genetic variations, biological and environmental variables [28]. Here, we want to go further in characterizing and understanding the complexity of RNA editing, unveil its variability among individuals and assess possible correlations with gene expression and genetic variations. To this aim, we investigated editing sites from existing RNA seq dataset of whole blood from 459 healthy subjects [29], correlating editing levels with a collection of 35 biological and pharmacological variables, as well as with genes expression and genotyping data.

Results

RNA editing sites detected in human blood samples

To investigate the dynamic of RNA editing in human blood, we used a dataset of 459 subjects, including poly(A)+ RNA sequencing and genotyping data from blood samples and 35 variables describing demographic, pharmacological and phenotypic traits (Additional file 1: Table S1).

After filters, our analysis focused on the editing levels of 2,079 trusted sites (see methods) across 421 genes. These sites are mainly distributed in ALU regions (1,805; 86.5%) and 3'UTR regions (1,234; 59.4%). Overall, we detected 1,268 sites in exons of protein coding genes, including 12 recoding sites (resulting in a missense substitution) and 12 synonymous sites. We also detected 53 sites annotated on ncRNAs (Figure 1a, b). Detailed statistics of the 2,079 trusted sites are reported in Additional file 2, while recoding sites in Table 1.

Considering mean values for each site, detected editing levels range from 0.05 to 1, with most sites showing moderate editing levels between 0.05 and 0.30 (Figure 1c). We also detected 33 sites highly edited (mean value ≥ 0.9), located mainly in intronic regions (Figure 1d). Highly edited sites are reported in Additional file 1: Table S2. To further assess reliability of detected sites, we compared our data with REDIportal database [26], a well-established resources containing multi-tissue estimations of RNA editing levels. The comparison revealed high concordance, with 2,035 and 2,003 overlapping sites when considering the whole REDIportal dataset or blood tissue data, respectively. Measured editing levels in blood showed high concordance (concordance correlation coefficient 0.84, Additional file 1: Figure S1). For the sites included in this study, the editing levels from REDIportal are reported in Additional file 2.

We used Spearman correlation test to analyze correlation in editing level changes across the 2,079 trusted sites and to find sites with co-regulated RNA editing. We found 1,314 significant relationships (FDR < 0.05) involving 629 sites. Correlations were generally low with only 66 sites

with relationships above 0.5 rho value. Correlations become stronger for close sites, especially below 50 bp distance, with 91% of high rho (≥ 0.5) relationships located in this range. No strong negative correlations (rho < -0.5) were observed (Additional file 1: Figure S2).

Genes potentially influencing RNA editing process

We performed regression analysis to identify genes whose expression is associated to the global editing levels, calculated for each subject as the total sum of G bases divided by the total number of bases present at all the 2,079 sites. The analysis revealed 4,719 genes associated to global editing levels (FDR < 0.05). Enrichment analysis on Gene Ontology biological processes (GO-BP) revealed a strong enrichment for genes involved in immune system and interferon signaling (FDR < 1e-6, Figure 2a). Among significant genes, *ADAR* emerged as the top influencing factor, explaining about 13% of the observed variability, while *ADARB1* showed no significant effect (Figure 2b). The influence of *ADAR* was similar on ALU (~10%) and non-ALU (~13%) sites, while *ADARB1* remains not associated also when considering groups separately (Additional file 1: Figure S3). *ADARB2* gene was not detectable in our gene expression data. When the same analysis was repeated removing *ADAR* effect, we obtained 1,122 genes associated to global editing levels (FDR < 0.05), including 376 with a strong association at FDR < 0.01 (Additional file 3). Enrichment analysis on GO-BP and REACTOME pathways revealed that these genes mainly impact ribonucleoprotein complex biogenesis and RNA metabolism / processing (Figure 2c).

To assess possible interactions between ADAR enzymes and genes whose expression is associated with global editing level, we performed network analysis using data on protein-protein interactions from STRING v.10, BioPlex and BIOGRID databases. We created a 415 proteins network including genes significantly associated with global editing levels (FDR < 0.01) that interact with ADAR1, ADAR2 and ADAR3 proteins or one of their first neighbors (Figure 3a). Considering top associated genes with FDR < 0.01 we found 285 out of 376 (76%) interactors. The observed fraction of

ADARs-connected genes represents a significant enrichment compared to random groups (empirical p-value < 1e-06, Figure 3b) and these genes are strongly enriched for RNA binding proteins (Figure 3c). Significant genes also includes 9 genes with direct interactions with ADARs (Table 2). We estimated the role of each node in this network looking at deggre and betweenness values. Degree value account for the number of interaction involving a single node in the network, while betweenness is a measure of centrality based on shortest paths. Node with higher betweenness centrality would have a major role in the network, because more information will pass through that node. Among ADARs proteins, ADAR1 is the main target of network interactions (0.077 betweenness centrality, 72 degree values) compared to ADAR2 and ADAR3 (0.018 and 0.001 betweenness centrality, 29 and 9 degree, respectively). Among associated genes with a direct interaction with ADARs, *ELAVL1*, *RPA1* and *IFI16* act as relevant hub nodes, with betweenness centrality values of 0.137, 0.028, 0.020, respectively (Figure 3d). Detailed network-based statistics are reported in Additional file 4.

Biological factors possibly influencing editing levels

We performed principal component (PC) analysis to extract major dimensions underlying variations in editing levels and to identify correlations with biological / pharmacological variables. Even if the variance explained by single component is generally low (PC1 ~ 0.025), our data revealed 24 factors with a significant correlation (p-value < 0.05) with one of the first 5 PCs (Figure 4 and Additional file 1: Table S3).

Sex, age and BMI, emerged as major contributors to editing variability being associated to PC1 and 2 and influencing also lower components. Substance intake variables, including alcohol, smoke and drugs assumption, were clearly associated to PC3, even if few of the observed associations, namely alcohol intake, thyroid and cholesterol lowering medications, may be influenced by sex biased distribution (Additional file 1: Table S4). As expected from the analysis of genes influencing global

editing, *ADAR* expression level resulted associated to the first 2 PCs, confirming its pivotal role in shaping editing levels variability. Correlation of editing levels for single sites with the first 5 PCs are reported in Additional file 5.

Identification of genetic variants influencing global editing levels

We performed genome wide association analysis between genotyping data of 573,801 SNPs and global editing level to identify SNPs associated to global editing levels in human blood (Figure 5a). Based on GRASP database of known SNP-phenotype associations, the first 100 SNPs with the lowest p-values are involved in 44 human phenotypes with strongest impact on ADAR expression and AraC toxicity (Additional file 1: Table S5). Among these SNPs, 25 SNPs are known eQTLs regulating expression of 19 genes in blood tissue (Additional file 1: Table S6). These genes resulted to be nominally enriched for genes encoding for RNA-binding proteins involved in transcription regulation and response to cytokines (Additional file 1: Figure S4).

After variant clumping, our analysis identified a single significant locus on chromosome 7 (rs856554: p-value 1.86e-7, FDR 0.042), containing the lincRNA gene *LOC730338* (ENSG00000233539) (Figure 5b and Table 3). The SNP rs856554 showed a significant effect on global editing level and seems to influence ADAR expression, despite this effect do not reach statistical significance (Figure 5c). Association results for single SNPs with nominal p-value < 0.05 and for loci after variants clumping are reported in Additional file 6.

Taken together, the 36 known ADAR eQTLs present in genotyping data explain 5.5% of global editing variability (GCTA analysis, p value 3.46e-4) and 5 of them resulted among the top 100 associated SNPs (Additional file 1: Table S7). The effect of the top associated ADAR eQTL (rs6699825) on ADAR expression and global editing level is represented in Figure 5d.

Discussion

The process of A-to-I RNA editing has gained increasing attention in recent years, being implicated in multiple aspects of human physiology and, when dysregulated, in human disorders such as neurological disorders and cancer [2,9,24]. Thanks to advances in next-generation sequencing technology, the prevalence and dynamic of “RNA editome” have been recently characterized across many tissues and developmental stages [18,19,21,22]. However, the published studies are usually limited in the number of samples or lack a detailed characterization of samples that allows assessing the role of biological and environmental factors. Here, we analyzed RNA-seq data from more than 400 blood samples obtained from healthy subjects, provided with detailed annotations of biological and pharmacological variables, in order to investigate the population variability of RNA editing levels and the influence of genetic variations, gene expression and environmental factors on the RNA editing process.

First, we calculated editing levels for more than 2 million known A-to-I sites present in the RADAR database. After filtering, only 2,079 sites resulted with detectable editing levels in at least 100 subjects, supporting the idea that editing process is tissue- or stage-specific [21]. As expected, the majority of these sites are located in inverted repeat ALU sequences [18,19] that facilitate the formation of a RNA double stranded secondary structure with high affinity for ADAR editing enzymes. Interestingly, near 60% of detectable editing sites are located in the 3’UTRs, that are known preferred binding sites for miRNAs. This suggests a potential extensive role of editing process in modulating the miRNA mediated regulation of gene expression in blood. Indeed, RNA editing in the 3’UTRs might introduce nucleotide changes to miRNA target sites or stabilize RNA secondary structure reducing the accessibility for AGO2-miRNAs complex [30].

We identified 24 editing sites located in coding sequences: 12 resulting in synonymous modifications and 12 inducing non-synonymous amino acid changes (re-coding sites). Among the

latter, there were well studied re-coding sites, such as the S/G site of *AZIN1* [8], that mediates the binding to antizyme and cell cycle progression; the G/R site of *BLCAP* [31], that is involved in tge regulation of STAT3 signaling pathway; and the L/R site of *NEIL1* [32], that might modulate the DNA repair capability of the enzyme. Their editing levels range from high (75% of *NEIL1*) to medium-low (14% and 16% for *BLCAP* and *AZIN1*, respectively), indicating that both edited and unedited isoforms are needed for the proper function of the tissues. Interestingly, among the re-coding sites, we also detected sites with an high editing level, such as two sites edited at 70% on the small subunit processome component (*UTP14C*) and two sites edited at nearly 40% on ubiquitin specific peptidase 4 (*USP4*). It is worth to notice that in blood cells several editing sites in 3'UTR and intronic regions reach an editing level of more than 90%. The high efficiency of editing indicates a strong ADARs binding, affecting transcripts stability or structure [33], but the actual functional effect of these fully edited sites remain elusive. Overall, RNA editing process in human blood seems more pervasive than previously reported, prompting for further analyses to understand its biological effects also in healthy subjects.

Further, we investigated the association of genes expression with global editing level. *ADAR* (encoding ADAR1 enzyme) resulted as the top associated gene and its expression explained about 13% of observed variability in global editing, while *ADARB1* (encoding ADAR2 enzyme) was not associated to global editing level even when ALU and non-ALU sites were considered separately. *ADARB2* (encoding ADAR3 protein) is not expressed in blood cells, excluding that it could have a major negative effect on the editing levels in blood as observed for brain tissues [22]. Thus, ADAR1 emerges as the major contributor to editing process in blood, as already reported for human B cells and other tissues [22,34], while other ADAR enzymes seems to have only a limited effect. Overall, association analysis revealed 4,719 genes that might have a potential effect on the editing process, strongly enriched for genes involved in the immune system and interferon signaling. This supports the association between inflammatory processes and A-to-I editing, that seems mediated by ADAR

expression modulation. Indeed, ADAR1 is present in two main isoforms, a constitutive p110 and an interferon inducible p150 form that is active under an inflammatory response [35]. Moreover, RNA editing, especially ADAR1 activity, are important to modulate innate immunity [36,37]. Modification in the global editing level has been reported after inflammation in mouse and in vitro studies using several inflammatory mediators [38].

When the effect of *ADAR* expression is removed from our analysis, new genes associated to global editing level emerged. These genes are mainly involved in RNA metabolism and ribonucleoprotein complex processing. These results confirmed what recently found after a global analysis of GTeX data [22] and strengthen the role of RNA editing complex in the RNA processing [34].

Associated genes after *ADAR* correction are strongly enriched for potential ADARs interactors, as revealed by network analysis using data from protein-protein interaction databases. Moreover, associated genes interacting with ADARs mainly encode for RNA binding proteins, as revealed by enrichment analysis, suggesting that they could be involved in RNA recognition or assembly of the editing complex. Network analysis showed that ADAR1 is the main target of these interactions, confirming its prevalent role in blood samples, compared to the other editing enzymes. We also identified 9 associated genes with direct interaction with ADARs. Among them, *ELAVL1*, *RPA1* and *IFI16* emerged as relevant hubs in the network, aggregating most of the interactions directed to ADARs proteins. The stabilizing RNA-binding protein human antigen R (HuR), encoded by *ELAVL1*, has been recently proposed as an ADAR1 interactor involved in the regulation of transcripts stability in human B cells [34]. It was unclear if this stabilizing effect is editing depended or not. However, ADAR1-mediated RNA editing of the 3’UTR of cathepsin S enables the recruitment of HuR to the 3' UTR, thereby controlling the cathepsin S mRNA stability and expression in endothelial cells and in human atherosclerotic vascular diseases [28]. The observed association between the global editing level and the *ELAVL1* expression strengthens a general role of RNA editing in RNA stability through the modulation of expression of genes involved in RNA

metabolism. *RPA1* and *IFI16* have never been involved in ADARs activity and represent new interesting partners that could expand the understanding of ADAR function and regulation. *RPA1* gene encodes the largest subunit of the heterotrimeric Replication Protein A (RPA) complex, which binds to single-stranded DNA, forming a nucleoprotein complex that is involved in DNA replication, repair, recombination, telomere maintenance and response to DNA damage [39]. ADAR1 presents Z-DNA binding domains, that are not present in the other editing enzymes [40], helping to direct ADAR1 to active transcription sites and to interact with DNA. Thus, the interaction with RPA1 protein might broaden ADAR1 activity also in the field of DNA repair and maintenance. *IFI16*, interferon gamma inducible protein 16, encodes a member of the HIN-200 (hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats) cytokines family. This protein interacts with p53 and retinoblastoma-1 and localizes to the nucleoplasm and nucleoli [41], where also ADAR enzymes are present. Both IFI16 protein and ADAR1 were associated to response to viral DNA and regulation of immune and interferon signaling responses [41,42]. Future studies will establish the actual functional meaning of these interactions and their role in RNA editing.

Recently, global editing level have been investigated across tissues and in different species [21,22] and also correlated to the genetic background of human population [28]. For the first time, we also investigated the potential impact of demographic, pharmacological and biological factors on RNA editing process genome-wide. Principal component analysis revealed twenty-four variables potentially influencing RNA editing in blood, even if the proportion of editing variability explained is low. In particular sex, age and body mass index (BMI) have the strongest effect on RNA editing levels, being associated to the first principal component. This component is also strongly associated with *ADAR* expression, suggesting that the observed effect of these biological factors could be due to modulation of *ADAR*. This data indicates that, when analyzing editing variations among different groups, such as in case / control studies, gender, age and other biological characteristics should be

taken into account carefully to avoid biased results. Interestingly, the third principal component is associated with variables related to drugs and substances intake, but only weakly with *ADAR* expression, indicating that drugs might modulate editing levels through alternative mechanisms. A role of drugs, in particular antidepressants, have been reported for editing sites on specific neuronal transcripts [43–46]. Our analysis suggests a broader impact of drugs, with several substances able to influence RNA editing process in blood. Even if the contribution of single substances seems small, (PC3 explains ~0.7% of editing variability), substances intake overall may have a larger effect, as suggested by association of global intake variables ("all drugs" and "none treatments") with multiple principal components.

Finally, we analyzed genotyping data to identify SNPs associated to global editing level in human blood tissue. Known *ADAR* eQTLs resulted among the SNPs with the best p-values and, taken together, they explain about 5% of the observed variation in global editing. Our data confirmed that they actually influence expression of *ADAR* in blood and this explain also the observed effect on editing levels. We found a single locus significantly associated with global editing level in blood, localized on chromosome 7 and containing the lincRNA gene *LOC730338.* Long intergenic noncoding RNAs, or lincRNAs, are long RNA transcripts (longer than 200 nucleotides) that have been identified in mammalian genomes mainly by bioinformatics analysis of transcriptomic data. Despite thousands of lincRNAs are now validated, the exact functional role remains unknown for most of them. lincRNAs appear to contribute to the control of gene expression and have a role in cell differentiation and maintenance of cell identity [47]. It has been recently reported in *C. elegans* that lncRNAs are extensively down-regulated in the absence of ADARs as a result of siRNA generation [48]. The authors suggests that ADARs can interfere with the generation of siRNAs by endogenous RNAi and promote lncRNA expression. *LOC730339* expression cannot be measured in our dataset since it lack a poly-A tail, so it is not possible to assess if the associated SNPs observed in the locus could act as eQTLs for this lincRNA. These SNPs seem to have only a marginal

correlation with ADAR expression, so the mechanism that link this locus and *LOC730339* to editing process remain to be investigated.

According to GRASP, HaploReg and GTeX databases, the 100 SNPs with the best p-values also contain several SNPs reported in previous GWAS studies, as well as known eQTLs of genes coding for RNA binding proteins involved in transcription, supporting a co-regulation of RNA editing and transcription and a possible role of editing in several human phenotypes. Overall, this data indicates that genetic variations, especially those associated to ADAR expression, can influence observed editing levels. The analysis of these SNPs should be taken into account when investigating editing levels in different human populations both in physiological and pathological conditions.

Conclusion

Our data provides a detailed picture of RNA editing and its variability in human blood. This study confirms the pivotal role of ADAR1 in the regulation of RNA editing process in blood and suggests new genes, genetic variants, biological and environmental variables that are involved in the RNA editing process. Future studies will be required to confirm and clarify their role and their relationship with the ADAR family enzymes.

Methods

Description of data

RNA-Seq raw data (aligned reads) was obtained from NIMH repository, NIMH Study 88 / Site 621, dataset 7 (Levinson RNA Sequencing Data). The original data and samples details are described in [29]. This dataset includes poly(A)+ RNA sequencing and genotyping data from blood samples of 922 subjects, 463 MDD patients and 459 control subjects. The present study focuses only on the 459 controls. A detailed phenotypic description including demographic, pharmacological and biological variables is also included for each subject. Among them, we considered only those relevant in at least 30 subjects and not related to MDD clinical evaluation. The 35 variables considered in this study are reported in Additional file 1: Table S1. Moreover, each experiment is annotated with a rich set of technical variables, representing quality metrics of RNA sequencing and characteristics of the blood sample. Normalized gene expression data is given as residuals of ridge regression of log-transformed read counts with 35 technical variables, to remove the effect of experimental biases (see the original paper [29]).

Assessment of editing levels

To reduce the chance of measuring false-positive editing sites, we focused on the set of editing sites reported within RefSeq genes by RADAR database [27] and never seen as Single Nucleotide Variants in the human population according to 1000G phase3 and ExAC v.0.3.1. The original aligned reads were de-duplicated using Picard and the editing levels were then determined genome-wide from BAM files using REDITools [27] with the following parameters:

```
REDItoolDenovo.py -t25 -m20 -c10 -q25 -O5 -l -V0.05 -n0.05
```

The raw data contains editing levels for 2,027,557 sites. To ensure that we were looking at true editing events, we filtered out sites occurring in regions were incorrect alignments could have generated artifacts in editing detection: known pseudogenes from GENCODE v25; segmental duplication with ≥ 99% identity; single exon genes, that are often retrotransposed genes with high similarity to the corresponding parent gene. The filtered dataset resulted in 709,184 sites.

Finally, we decided to retain only sites with detectable editing levels in at least 100 subjects for subsequent quantitative analysis, resulting in a final dataset of 2,079 sites (trusted sites dataset).

Comparison with REDIPortal dataset

We compared editing levels detected in our dataset from blood samples with similar data obtained from REDIPortal [26]. Editing levels were retrieved directly from REDIPortal database, containing RNA editing values calculated from 55 body sites of 150 healthy individuals from GTEx project. Mean editing levels of our 2,079 trusted sites were compared with corresponding data reported for blood tissue in REDI portal. To assess concordance between the two datasets, we calculated concordance correlation coefficient between mean editing values detected in our data and reported in REDPortal blood tissue for overlapping sites.

Correlation between editing levels across sites

Using Spearman correlation test, we analyzed correlation of editing levels across the 2,079 trusted sites. Each site was analyzed against all other sites for a total of 4,322,241 tests. FDR correction was used to account for multiple tests. Corrplot R packages were used to analyze correlation matrices and generate correlation plots.

Association between global editing level and gene expression

To investigate which genes could influence the editing process, we used robust linear regression to assess the association between gene expression levels and the overall editing level in each subject. For each subject, we calculated the sum of editing levels across all the 2,079 trusted sites and used this as measure of editing process. The sum of editing levels was computed as the total number of reads with G at all trusted sites as compared to the overall coverage at all trusted sites. The sum of editing levels was determined also for Alu sites and non-Alu sites, separately. Only genes with at least 10 reads in at least 100 subjects in our dataset were tested for association, resulting in 14,961 addressable genes. To choose the set of phenotypic, biological and pharmacological variables to include as covariates in regression analysis, a stepwise model selection by AIC was performed. The 7 included variables are indicated in Additional file 1: Table S1. Moreover, since there was a correlation between the variance observed at each editing site and its sequencing coverage for sites with coverage below ~ 40 X (Additional file 1: Figure S4), the log2 of reads count was also included as covariate in the analysis. The strength of the association was determined by ANOVA test comparing the null ('background') model that includes only the set of covariates with the full model (covariates plus normalized expression levels). FDR was used to correct for multiple tests. Subsequently, association analyses were repeated including ADAR expression as additional covariate, to remove the effect of ADAR expression.

Gene set enrichment analysis and gene network analysis

The impact of associated genes on biological functions and cellular pathways was investigated using hypergeometric test. We tested the over-representation of pathways among the subset of significant genes at 5% FDR level compared to all expressed genes. Enrichment analysis was performed separately for the following sets from MSigDB v.6.0: cellular pathways from REACTOME and the three main Gene Ontology categories (Cellular Components, GO:CC; Biological Process, GO:BP; Molecular Function, GO:MF). To verify if the proteins encoded by

these genes could interact with ADAR proteins, the major enzymes involved in RNA-editing, we explored human protein-protein interaction (PPI) data. First, we created a comprehensive human PPI network combining data from 3 different sources: BioPlex 2.0 [49], BioGRID 3.4.15 [50] and STRING 10.0 [51]. For the BioGRID dataset, only interactions marked as physical were taken in to account, whereas for the STRING dataset only interactions with a combined score above 400 and physical/biochemical evidences were considered. Proteins of the ubiquitin gene family were removed from the network, resulting in a final PPI dataset with 22,913 proteins (nodes) and 833,686 interactions, containing 108 direct interactors of ADARs (ADAR1, ADAR2 and ADAR3 proteins). We assessed the number of genes strongly associated with global editing level (FDR < 0.01) interacting with ADARs or one of their first neighbors. To test the significance of these overlap, we performed a random test on the overall set of 14,961 genes addressable in our RNA-Seq data (background genes). We randomly sampled among background genes 1 million groups of N genes (N = 376) and for each simulated group we counted how many elements interacts directly with ADARs or one of their neighbors. Empirical p-value was then calculated as the number of test resulting in an equal or higher number of interactors. Cytoscape v.3.4.0 [52] was used to visualize the PPI network and calculate network related statistics.

<u>Principal Component (PC) analysis of editing levels and identification of biological factors correlated with PCs</u>

The principal components of editing levels were determined on the complete data using the prcomp package R. To identify the number of PCs to account for, we evaluated the percentage of explained variance by the top 30 PCs, and identified the 5th component as the point at which the explained variance plateaus. To compute principal components, the missing values of the sites were imputed using a nonparametric imputation method based on random forest (missForest R package [53]).

To investigate which biological and pharmacological variables could influence editing levels in blood, we studied the correlation between the 35 variables listed in Additional file 1: Table S1 and the first five PCs. Kruskal-Wallis test, Mann-Whitney-Wilcoxon test and Pearson's product-moment correlation test were used to assess association for categorical, binary and continuous variables, respectively. To identify which sites were most correlated with each PC, we analyzed the loadings, that could be interpreted as correlation coefficient between the original variables and components. Moreover, given a high number of sites and low loading values, to deepen the role of each site in the computation of the PCs, we performed the Pearson correlation test. We considered a "moderate" correlation when its absolute value was between 0.3 and 0.5 and the test passed the Bonferroni threshold, while a "weak" correlation was considered when the correlation absolute values ranged between 0 and 0.3 and the respective p-values were significant for Bonferroni correction.

Association study for SNPs and global editing levels

To identify SNPs associated to global editing level, we analyzed genotyping data and global editing levels in the 459 human blood samples. Starting from genotypes provided in the original dataset [29], we filtered raw data removing SNPs strongly deviating from Hardy-Weinberg equilibrium (fisher test p-value < 1e-4) and with a minor allele frequency below 0.10, to ensure that the least represented genotype accounts for at least 5 individuals. The final dataset contained 573,801 SNPs. We used plink linear association analysis with additive model, including the same 7 covariates used for analysis of gene expression (see above, Additional file 1: Table S1). FDR was used to correct for multiple tests. After association analysis, we used GCTA [54] to evaluate the impact of ADAR known eQTLs on observed global editing levels, using the same set of covariates included for the plink association analysis. The top 100 associated SNPs were overlapped with GRASP 2.0 [55] database, to assess their role in human phenotypes and diseases. We then evaluated genes potentially regulated by the top 100 SNPs based on known blood eQTLs from HaploReg 4.1

[56] and GTeX [57]. Enrichment analysis was conducted for potentially regulated genes on GO:BP, GO:MF, GO:CC and REACTOME pathways using hypergeometric test. Background gene group was obtained as all genes with a known eQTL among all the 573,801 tested SNPs. To identify significant loci associated to global editing level, we performed variant clumping based on the association results, using plink with 1Mb window and 0.5 R2 thresholds. In this way all SNPs in 1Mb window and with R2 ≥ 0.5 are grouped together around the index SNP, that is the SNP with the lower association p-value.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

RNA-Seq raw data and description of subjects are available from NIMH repository, Study 88, but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available.

The datasets supporting the conclusions of this article are included within the article (and its additional files). Editing levels calculated from RNA-Seq are available in the GitHub repository, https://github.com/gedoardo83/RNA_editing_blood.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

EG, AB conceived and designed the analysis. EG, CS performed bioinformatic and statistical analysis. MG acquired the data. MG and CM provided intellectual input and conceptual advice and revised the paper. EG, AB, CS wrote the paper. All authors read and approved the final manuscript.

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Tables

Table 1. Editing levels detected for the 12 recoding sites identified in human blood

Site id	Gene	Strand	Aa change	Alu	Editing Minimum	Editing Mean	Editing Maximum
chr3_49354819	*USP4*	-	Gln->Arg	no	0.13	0.45	0.82
chr3_49354856	*USP4*	-	Lys->Glu	no	0.15	0.36	0.65
chr3_49398423	*RHOA*	-	Lys->Arg	yes	0.19	0.33	0.64
chr4_2835556	*SH3BP2*	+	Arg->Gly	no	0.05	0.08	0.16
chr4_2940026	*NOP14*	-	Asn->Ser	yes	0.1	0.23	0.56
chr4_77979680	*CCNI*	-	Arg->Gly	no	0.05	0.08	0.19
chr8_103841636	*AZIN1*	-	Ser->Gly	no	0.07	0.16	0.45
chr13_52604264	*UTP14C*	+	Ser->Gly	no	0.24	0.64	1
chr13_52604880	*UTP14C*	+	Gln->Arg	no	0.45	0.85	1
chr15_75646086	*NEIL1*	+	Lys->Arg	no	0.27	0.73	1
chr16_3292200	*MEFV*	-	Stp->Trp	yes	0.05	0.16	0.36
chr20_36147563	*BLCAP*	-	Gln->Arg	no	0.05	0.14	0.33

Table 2. Network based statistics for the 9 ADARs direct partners

Gene	Betweenness centrality	Degree	Association adjusted p	Associated genes rank
ELAVL1	0.137	168	0.0312	840
HDLBP	0.001	15	0.0400	986
HNRNPUL1	0.007	39	0.0004	10
IFI16	0.020	66	0.0055	246
RPA1	0.028	96	0.0140	496
SDAD1	0.002	19	0.0253	713
SUZ12	0.007	52	0.0022	110
THOC1	0.002	17	0.0184	587

USP39	0.002	20	0.0190	596

The table reports betweenness centrality and degree values for the 9 genes directly interacting with ADARs in PPI databases and significantly associated to global editing levels (adjusted $p < 0.05$). Adjusted p-values are calculated as FDR corrected p values from robust regression of global editing level and gene expression. The rank position among top associated genes is also reported.

Table 3. Top 4 SNPs associated to global editing level define a locus on chromosome 7

SNP	**Chr**	**Position**	**A1**	**Beta**	**p value (FDR clumped association)**	**R2**	**Gene (distance)**
rs856554*	7	46760129	G	0.00352	1.87×10^{-07} (0.042)	-	*LOC730338*] (23.4kb)
rs856589	7	46734307	A	0.00326	2.44×10^{-07}	0.73	[*LOC730338*]
rs6463347	7	46780614	C	0.00328	5.14×10^{-07}	0.76	*LOC730338*] (43.9kb)
rs856565	7	46721854	A	0.00327	8.16×10^{-07}	0.88	[*LOC730338*]

For each SNP the table reports distance from *LOC730338* gene. Gene name within square brackets indicate SNPs located within the gene, while single bracket indicate 3' distance. Index SNP is marked with * and the FDR of association for LD-clumped association analysis is reported. R2 with the index SNP is reported for other SNPs in the locus.

Figure legends

Figure 1. Distribution of 2,079 trusted editing sites analyzed in the study

(a) Distribution of the 2,079 trusted editing sites within ALU regions and (b) based on functional classification. (c) Density plot representing overall distribution of editing levels. (d) Density plots of editing levels for different editing site categories and ALU/non-ALU sites.

Figure 2. Association between gene expression and global editing levels

We analyzed association between global editing levels and gene expression for 14,961 human genes. (a) Gene set enrichment analysis by hypergeometric test on GO-BP categories and REACTOME pathways revealed that associated genes are mainly involved in immune system response mediated by interferon I and alpha / beta. (b) When we analyze distribution of global editing levels and *ADAR* gene expression, *ADAR* expression levels explains ~ 13% of observed variability. No significant effect is observed for *ADARB1* expression. *ADAR* and *ADARB1* expression levels are reported as residuals of ridge regression with technical covariates (see description of data in methods section). The graphs report adjusted p-value and R2 value from robust regression analysis. (c) The 1,122 genes associated to global editing level after removing *ADAR* expression effect were enriched for genes mainly involved in ribonucleoprotein and RNA processing.

Figure 3. Genes associated with global editing levels are enriched for ADAR interactors

(a) Reconstructed PPI network including ADARs and best genes significantly associated with global editing levels (FDR < 0.01). Among these genes, we observed 285 potential ADARs interactors, including 9 direct partners of ADARs proteins. (b) Boxplot of number of ADARs interacting genes observed in 1M random simulations. The observed number of interactions (285)

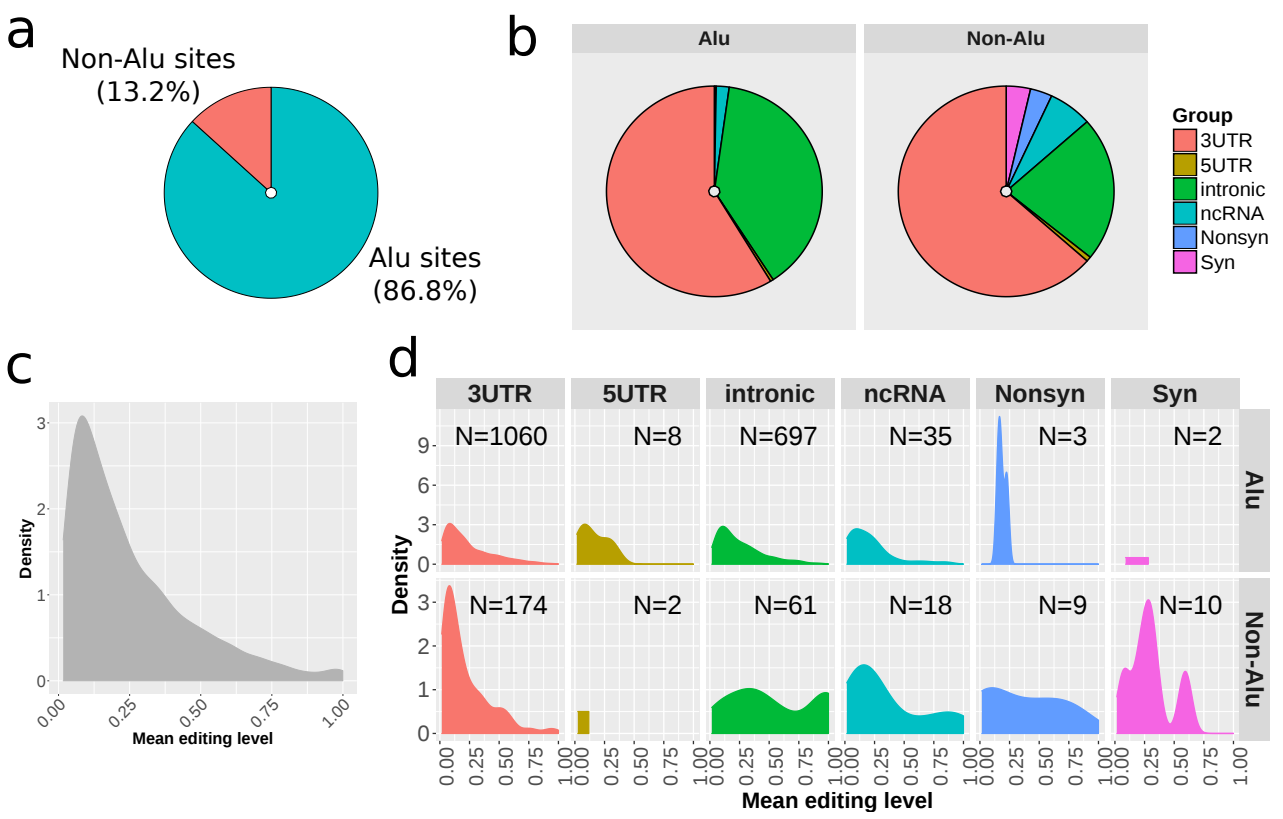
resulted in empirical p-value < 1e-6. (c) ADARs interactors are strongly enriched for RNA binding proteins in GO-MF categories. (d) Distribution of degree and betweenness centrality values among network nodes are represented by violin plots. ADAR1 protein has a major role (higher values) among ADAR proteins. Among ADARs direct partners, ELAVL1, RPA1 and IFI16 showed high values of degree and betweenness centrality, suggesting a central role in the network.

Figure 4. Impact of biological / pharmacological factors on editing levels

We analyzed correlation between 35 biological / pharmacological variables and principal components (PCs) calculated from editing levels of 2,079 trusted sites. The heathmap represents strength of association, with significant p values < 0.05 colored in yellow-red scale. Sex, age and BMI are the strongest factors, correlated to PC1, while substance / drug intake variables were mostly associated with PC3. Variance explained by each PC is represented by the bar plot in the upper side, while association of *ADAR* expression with each PC is represented in the lower panel.

Figure 5. Association study for SNPs and global editing levels

(a) Manhattan plot representing the association between 573,801 SNPs and global editing levels, where black line represents threshold for the top 100 SNPs (p value ~ 10e-4). (b) Detailed view of genotyped SNPs located in the region at chromosome 7 that showed genome-wide significant association with global RNA editing level. Known GWAS associations for human phenotypes from GRASP database are reported in the lower panel. (c) The top associated SNP (rs856554) showed a significant effect on global editing level and seems to influence also *ADAR* expression, despite this effect is not significant. (d) The top associated *ADAR* eQTL (rs6699825) showed significant effect on both. P values reported in box-plots are based on Tukey post-hoc pairwise test in ANOVA. *ADAR* gene expression level is reported as residual of ridge regression with technical covariates (see description of data in methods section).


a
Non-Alu sites
(13.2%)
Alu sites
(86.8%)
b
Alu
Non-Alu
Group
3UTR
5UTR
intronic
ncRNA
Nonsyn
Syn
c
Density
Mean editing level
d
3UTR
5UTR
intronic
ncRNA
Nonsyn
Syn
N=1060
N=8
N=697
N=35
N=3
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N=174
N=2
N=61
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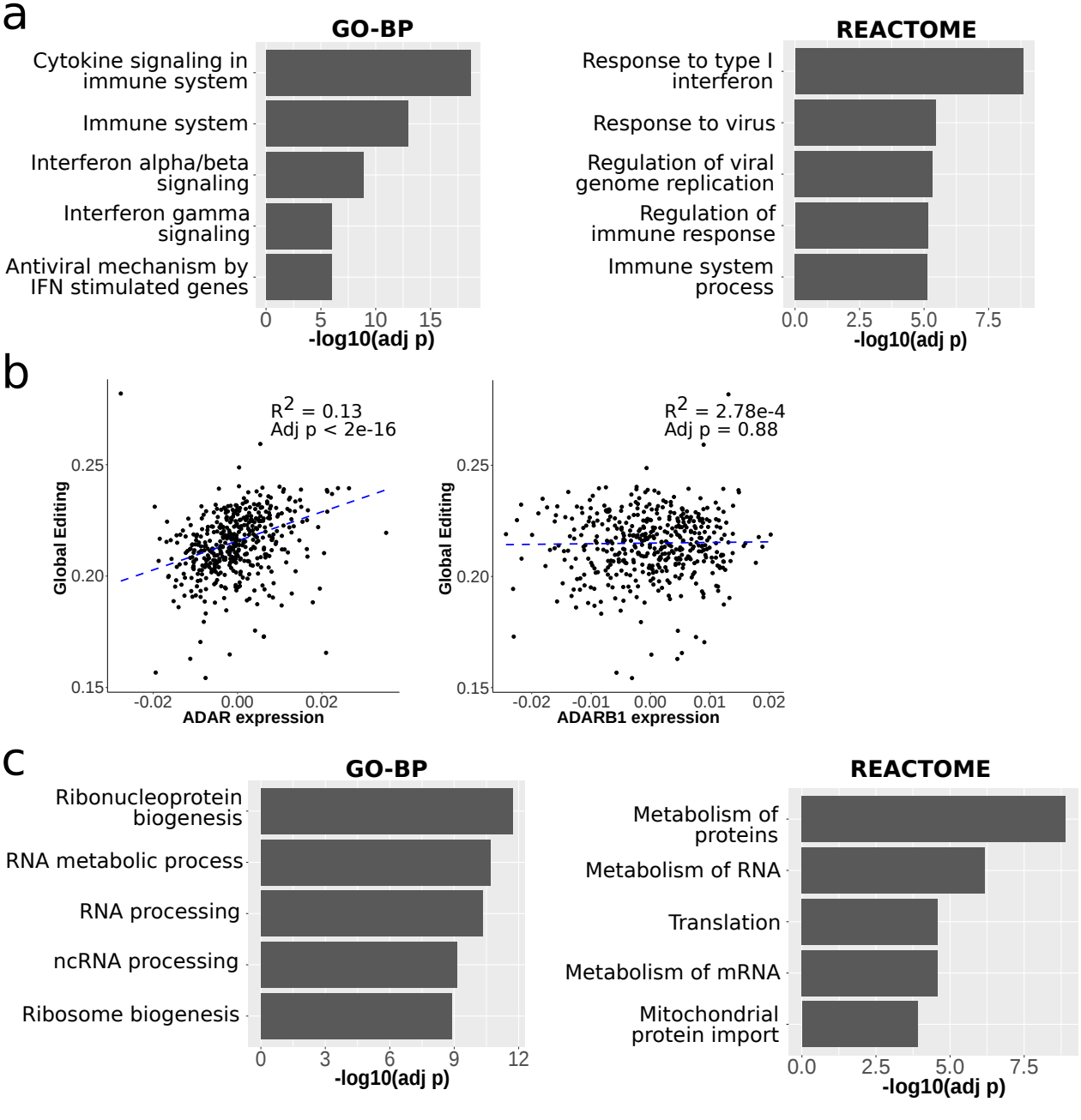
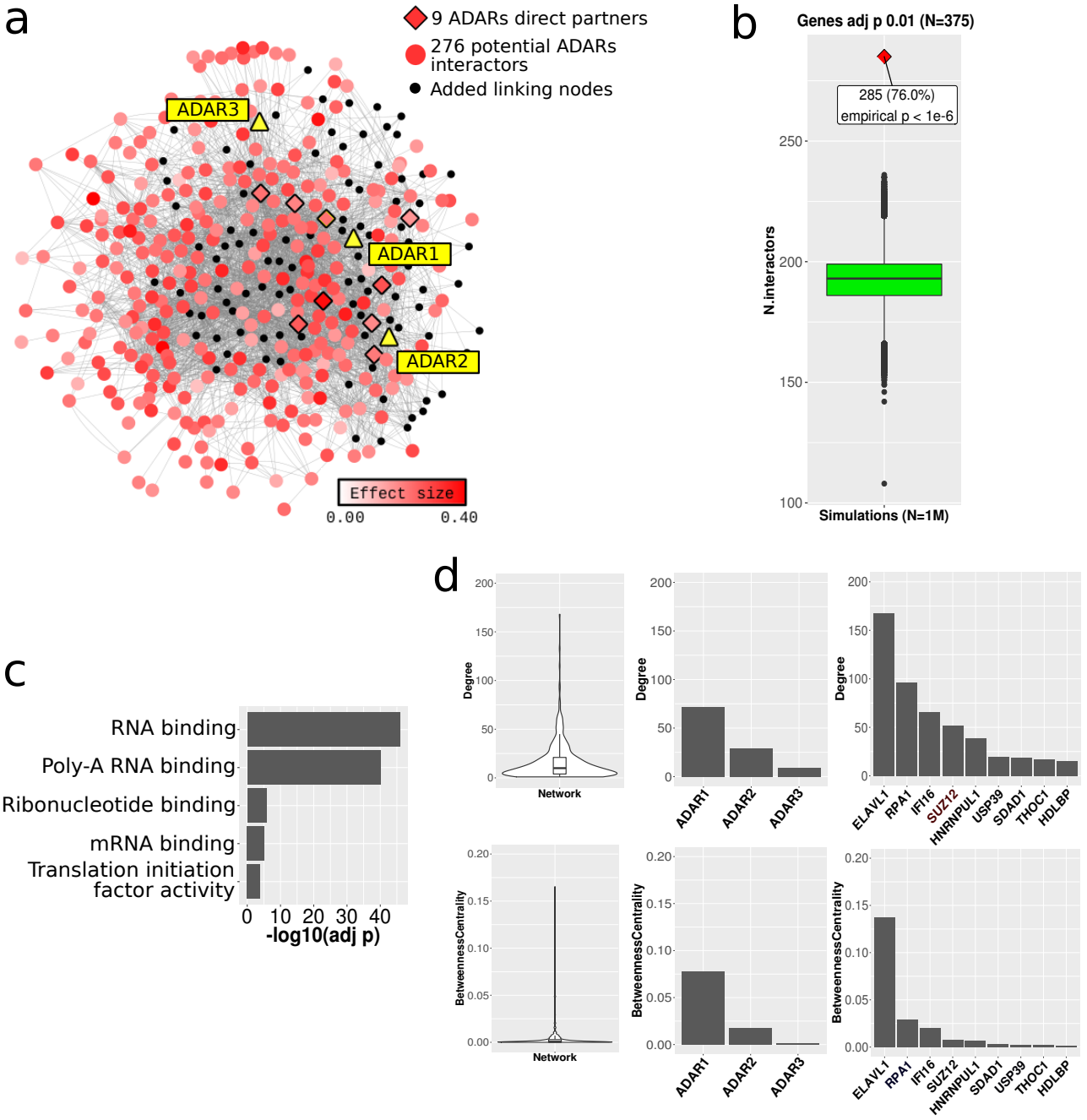
N=9
N=10
Alu
Non-Alu
Density
Mean editing level


a
GO-BP
Cytokine signaling in immune system
Immune system
Interferon alpha/beta signaling
Interferon gamma signaling
Antiviral mechanism by IFN stimulated genes
0
5
10
15
-log10(adj p)
REACTOME
Response to type I interferon
Response to virus
Regulation of viral genome replication
Regulation of immune response
Immune system process
0.0
2.5
5.0
7.5
-log10(adj p)
b
R2 = 0.13
Adj p < 2e-16
Global Editing
0.25
0.20
0.15
-0.02
0.00
0.02
ADAR expression
R2 = 2.78e-4
Adj p = 0.88
Global Editing
0.25
0.20
0.15
-0.02
-0.01
0.00
0.01
0.02
ADARB1 expression
c
GO-BP
Ribonucleoprotein biogenesis
RNA metabolic process
RNA processing
ncRNA processing
Ribosome biogenesis
0
3
6
9
12
-log10(adj p)
REACTOME
Metabolism of proteins
Metabolism of RNA
Translation
Metabolism of mRNA
Mitochondrial protein import
0.0
2.5
5.0
7.5
-log10(adj p)


a
9 ADARs direct partners
276 potential ADARs interactors
Added linking nodes
ADAR3
ADAR1
ADAR2
Effect size
0.00
0.40
b
Genes adj p 0.01 (N=375)
285 (76.0%)
empirical p < 1e-6
N.interactors
250
200
150
100
Simulations (N=1M)
c
RNA binding
Poly-A RNA binding
Ribonucleotide binding
mRNA binding
Translation initiation factor activity
0 10 20 30 40
-log10(adj p)
d
Degree
Network
ADAR1
ADAR2
ADAR3
ELAVL1
RPA1
IFI16
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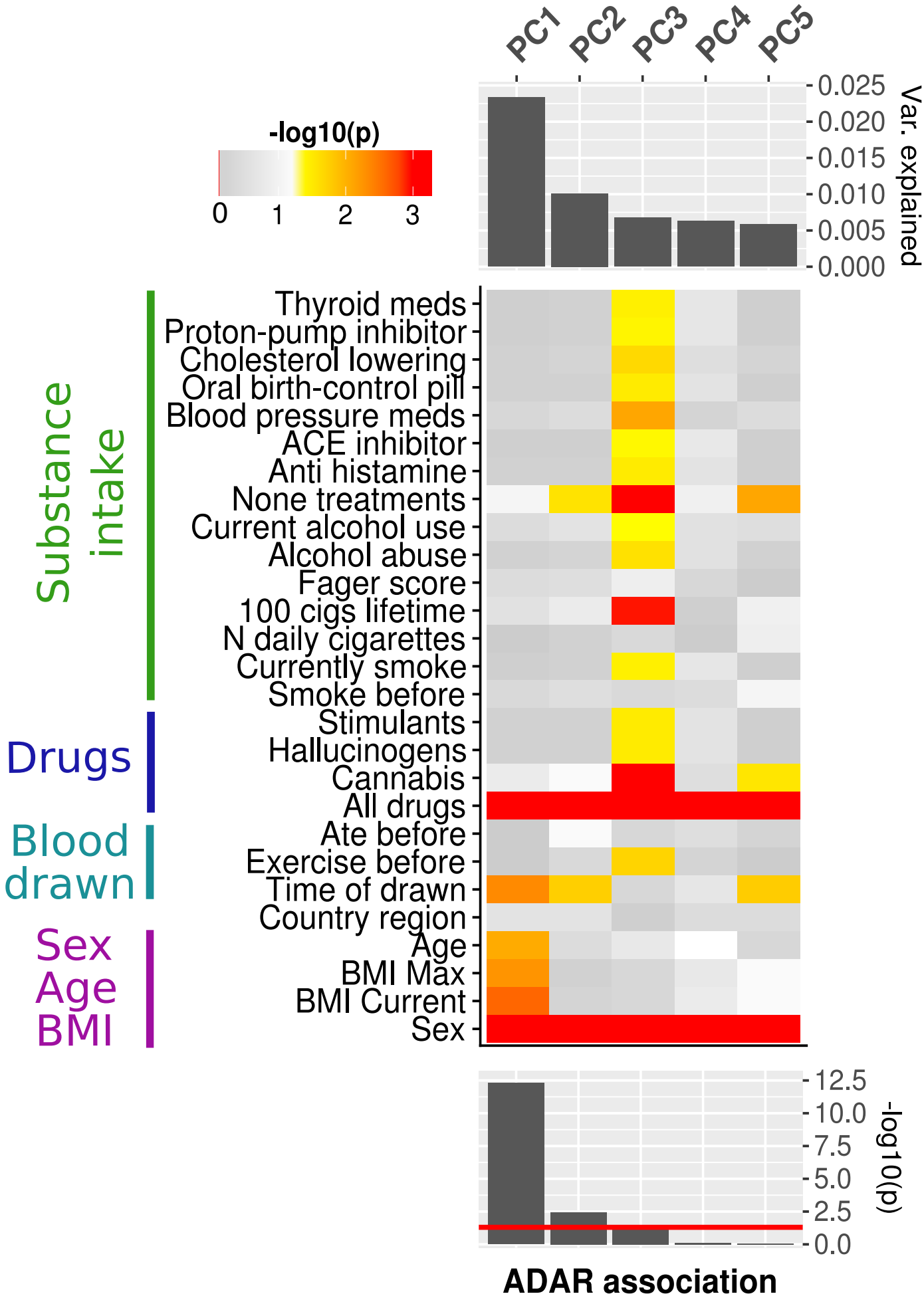
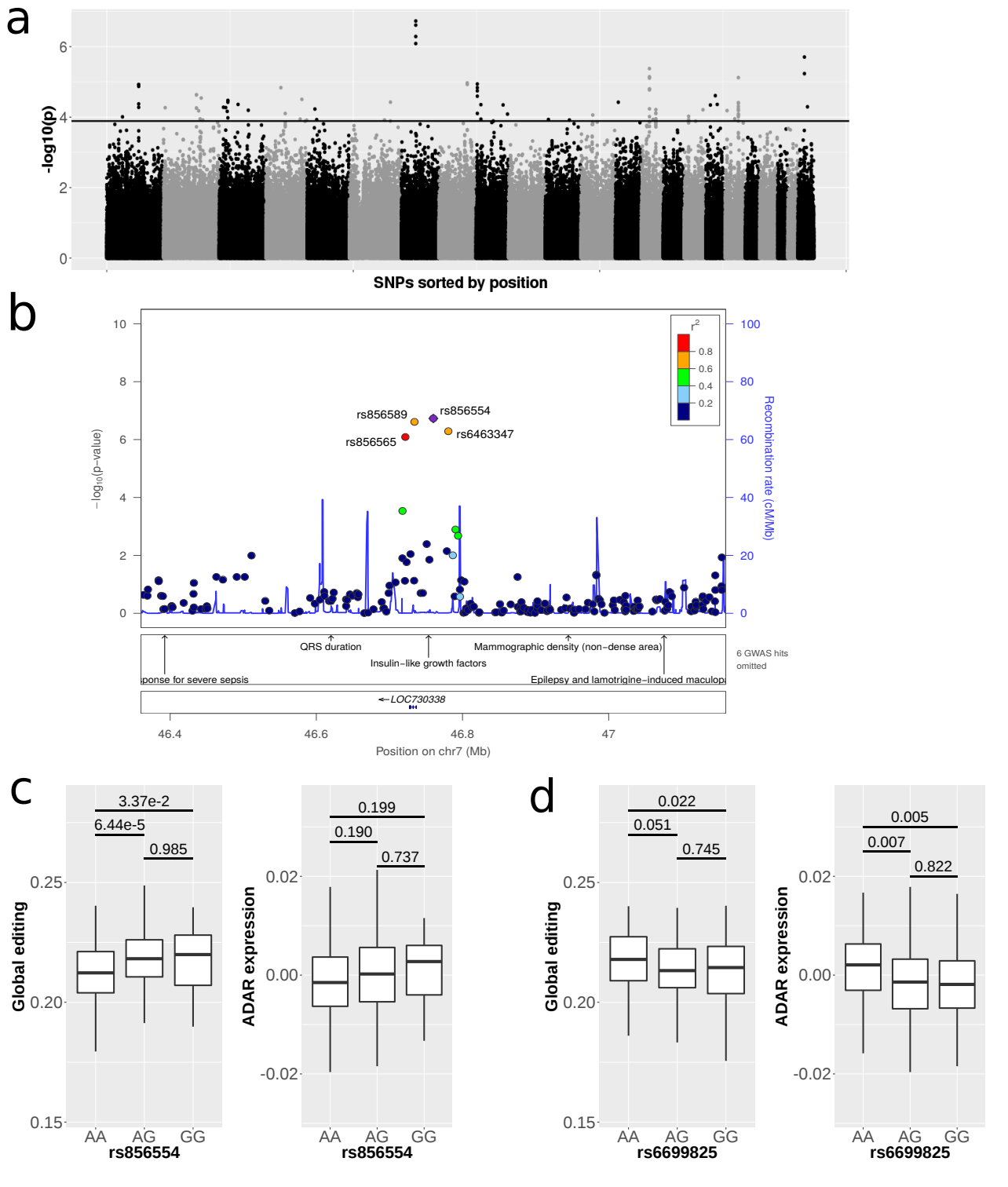
HNRNPUL1
USP39
SDAD1
THOC1
HDLBP
BetweennessCentrality


PC1
PC2
PC3
PC4
PC5
0.025
0.020
0.015
0.010
0.005
0.000
Var. explained
-log10(p)
0
1
2
3
Substance intake
Drugs
Blood drawn
Sex
Age
BMI
Thyroid meds
Proton-pump inhibitor
Cholesterol lowering
Oral birth-control pill
Blood pressure meds
ACE inhibitor
Anti histamine
None treatments
Current alcohol use
Alcohol abuse
Fager score
100 cigs lifetime
N daily cigarettes
Currently smoke
Smoke before
Stimulants
Hallucinogens
Cannabis
All drugs
Ate before
Exercise before
Time of drawn
Country region
Age
BMI Max
BMI Current
Sex
12.5
10.0
7.5
5.0
2.5
0.0
-log10(p)
ADAR association


a
-log10(p)
SNPs sorted by position
b
rs856589
rs856554
rs856565
rs6463347
r2
0.8
0.6
0.4
0.2
−log10(p-value)
Recombination rate (cM/Mb)
QRS duration
Mammographic density (non−dense area)
Insulin−like growth factors
6 GWAS hits omitted
Epilepsy and lamotrigine−induced maculop
LOC730338
Position on chr7 (Mb)
c
3.37e-2
6.44e-5
0.985
Global editing
AA
AG
GG
rs856554
0.199
0.190
0.737
ADAR expression
rs856554
d
0.022
0.051
0.745
Global editing
rs6699825
0.005
0.007
0.822
ADAR expression
rs6699825