A robust estimation of mosaic loss of chromosome Y from genotype-array-intensity data to improve disease risk associations and transcriptional effects

Juan R González[1,2,3,*], Marcos López-Sánchez[4,5,6], Alejandro Cáceres[1,2], Pere Puig[3], Tonu Esko[7], Luis A Pérez-Jurado[4,5,6,8,9]

[1] Barcelona Institute for Global Health (ISGlobal), Barcelona 08003, Spain.
[2] Centro de Investigación Biomédica en Red en Epidemiología y Salud Pública (CIBERESP), Spain.
[3] Dept. of Mathematics, Universitat Autònoma de Barcelona, Bellaterra (Barcelona), Spain.
[4] Genetics Unit, Universitat Pompeu Fabra, Barcelona, Spain.
[5] Institut Hospital del Mar d'Investigacions Mediques (IMIM), Barcelona, Spain.
[6] Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Spain.
[7] Estonian Genome Centre Science Centre, University of Tartu, Tartu, Estonia.
[8] SA Clinical Genetics, Women's and Children's Hospital, Adelaide, Australia.
[9] South Australian Health and Medical Research Institute & University of Adelaide, Adelaide, Australia.
*To whom correspondence should be addressed - email: juanr.gonzalez@isglobal.org.

Abstract

Accurate protocols and methods to robustly detect the mosaic loss of chromosome Y (mLOY) are needed given its reported role in cancer, several age-related disorders and overall male mortality. Intensity SNP-array data have been used to infer mLOY status but there are discrepancies of reported findings due to the calling uncertainty of the methods and the differences in the tissue-matrix used. We proposed *MADloy*, a method that optimizes mLOY calling by correctly modeling the underlying reference population with no-mLOY status and incorporating B-deviation information. *MADloy* increased the calling accuracy with respect to previous methods in experimentally validated samples and improved the statistical power to detect associations with disease and mortality in simulation studies and real dataset analyses. Using *MADloy i*n 18 individuals, we observed that mLOY cellularity slightly increased in blood after 3 years but was detectable in only 41% samples (7/17) in saliva. We found extreme down-regulation of the entire chromosome Y in kidney and bladder tumors with mLOY, while mLOY in blood was associated with deregulation of DNA replication, repair and recombination pathways. *MADloy* will help to define the mechanisms and roles of mLOY in different tissues as a male-specific marker of disease for personalized medicine.

Introduction

The most common somatic mutation in humans is the mosaic loss of chromosome Y (mLOY) in men. Recent evidence suggests the important role of mLOY in numerous diseases, being a biological factor that contributes to overall male mortality (1, 2) and, therefore, is likely to play an important role in male-specific treatments of disease. In particular, mLOY in blood cells increases with age, and is associated with smoking and with the risk of several age-related disorders, including hematological and non-hematological cancers, macular degeneration (3), Alzheimer's disease (4), major cardiovascular events (5) and suicidal behaviors (6). Despite the accumulating evidence, the mechanisms that trigger mLOY and its clinical consequences are still poorly understood. While susceptibility loci and epigenetic marks for mLOY have been identified (7, 8), larger and more accurate association and functional studies are required; in particular, as some inconsistencies have been observed (9, 10). Therefore, current mLOY calling methods and protocols need to be improved to confirm disease risks and mechanisms. Here, we addressed two main issues that have been proposed to affect mLOY reproducibility, the optimal processing of mLOY calling from SNP array intensity data and the accuracy of detecting mLOY on different tissue-matrices.

A main source of discrepancy can be due to the differences in the methods used to call mLOY status from SNP array intensity data (9, 11). The main signal used for mLOY calling is the log-R-Ratio (LRR) of the SNPs in chromosome Y. As a relative measure of the DNA content of a subject at a genomic locus with respect to a group of individuals, men with mLOY are expected to show low SNP-LRR values across the 56-Mb male-specific region of chromosome Y, which excludes the homologous region between chromosomes X-Y, pseudoautosomal (PAR1 and PAR2) and X-Y transposed (XTR), (12). Computing the mean LLR (mLRR-Y) in the region for each individual, the $mLRR\text{-}Y_{thresh}$ method called mLOY status on those individuals with mLRR-Y lower than the 99% confidence interval of experimentally induced mLRR-Y variation of normal individuals (13). As a threshold dependent method, $mLRR\text{-}Y_{thresh}$ is sensitive on the characterization of subjects with no-mLOY. Assuming that gains of chromosome Y (GOY) are rare, the positive side of the mLRR-Y distribution (centered at the peak) can be used to identify mLOY outliers by reflecting it to the negative mLRR-Y values to define the calling threshold. However, while GOY events are less frequent than LOY events, GOY events can also be relevant particularly in tumor tissues. Furthermore, the presence of few of them is enough to affect the mLOY threshold; in addition, plausible deviations from symmetry in the mLRR-Y

distribution also affect the position of the threshold. These two uncontrolled effects can introduce misclassification in mLOY calling reducing the power to find positive associations (14). Alleviation for mLOY misclassification in the association analyses can be considered if mLRR-Y is taken as a quantitative continuous variable (*mLRR-*Y_{quant}) (11). However, the approach is still suboptimal because the underlying distribution is a clear mixture of individuals with gains, losses or no changes in chromosome Y genetic content, a similar situation found in copy number variation calling (15, 16). Therefore, improvements on the use of mLRR-Y to call mLOY status should include the robust identification of mLOY events as outliers from an mLRR-Y non-symmetric distribution. In addition, current methods have not used the B-allele frequency (BAF) signal in the homologous region between chromosomes X-Y as an independent signal to confirm findings. The deviation of BAF signal in heterozygous probes from its expected value, namely B-deviation, has been robustly used to detect different types of chromosomal events in mosaicism and compute their cellularity levels (Rodriguez-Santiago, 2010). We have therefore developed *MADloy*, a method that integrates robust outlier identification of mLRR-Y values with the use of B-deviation signal to improve mLOY calling. We show the improved performance of the method against current approaches using simulations and real data sets. *MADloy* has been implemented as a Bioconductor package for robust mLOY calling along with visualization functions.

Differences of mLOY detection between blood and buccal smear have also been proposed as a possible source of discrepancy between two large studies that estimated the mortality ratios associated to mLOY (9, 11). To address this issue, we studied 18 individuals with positive mLOY status detected with *MADloy* in blood at base-line and assessed the progression of mLOY cellularity in both blood and saliva in a follow-up visit at 3 years, to determine the extent to which the tissue matrices are comparable for mLOY detection. We finally illustrate the application of *MADloy* in the study of the transcriptional correlates of mLOY in blood and in cancer.

Methods and Materials

mLOY calling

The reference methods for calling mLOY status using genotype-array intensity are those described in (1) and (8). Forsberg et al. (13) proposed to analyze the log R ratio (LRR) values of SNPs probes in the male-specific region of chromosome Y (mLRR-Y) in the 56-Mb region

between PAR1 and PAR2 on chromosome Y (chrY:2,694,521-59,034,049; hg19/GRCh37). An important consideration is the XTR that is shared between X and Y chromosome is also removed from the analysis because XTR can be affected by alterations in chromosome X, then redefining the mLRR-Y region as: chrY 6,611,498-24,510,581; hg19/GRCh37.

Assuming that mLRR values follow a symmetrical distribution across subjects, individuals are LOY-scored based on the threshold that is defined as the lower limit of the 99% confidence interval of the experimentally induced mLRR-Y variation. Forsberg et al. proposed to model the symmetrical distribution by reflecting the positive values of mLRR-Y over its median (method $mLRR\text{-}Y_{thres}$). LRR is computed as the ratio between two intensities and therefore its distribution is likely to be skewed, in addition the presence of large mLRR-Y values representing XYY gains cannot be fully discarded. Wright et al. (8), therefore proposed to use mLRR-Y as a continuous variable to measure the degree of mLOY given by its cellularity content (method $mLRR\text{-}Y_{quant}$). However, it is clear that mLRR-Y is a multimodal distribution and ignoring this feature reduces the power in association studies, as it has been seen when copy number variant status is estimated using continuous intensities.

As an alternative threshold method we proposed to use a robust estimation of the limit that determines a gain in chromosome Y (e.g XYY) that can be seen as an outlier of normal mLRR distribution. This value is estimated by

$$median(mLRR) + 1.2\ IQR(mLRR).$$

The three procedures MADloy based on LRR, mLRR-Y_{thres} and mLRR-Y_{quant} are implemented in the function *MADloy* of *MADloy* package.

To reduce the number false-positive calls, we propose to use combine the information obtained of mLRR-Y with the B-deviation values in X-Y homologous regions which include pseudoautosomal regions PAR1, PAR2, and XTR regions (12) whenever this data is available. The reason to use B-deviation in addition to LRR is because chromosome Y alterations also alter B-deviation values in the X-Y homologous regions, due to the allelic imbalance between chromosomes X and Y. By setting a B-deviation threshold of 0.05 (we assume an expected BAF of heterozygous probes between 0.45 and 0.55) we can identify whenever the BAF is altered. This information can be used as a quality control criteria by removing from the analysis those samples classified as LOY or GOY using mLRR-Y with normal values of B-deviation who are

indicated as “discordant”. This procedure is implemented in the function *checkBdev* of *MADloy* package. In addition, those samples without alterations in the mLRR-Y values but with alterations in B-deviation classified as “other”, can provide evidences that other types of events can occur in the analyzed sample. We recommend to visually inspecting samples classified as “discordant” or “other”, and only remove those cases having contaminations or other non-LOY events. To this end plots of chromosome X and Y are used as implemented in the functions *plotIndSNPX* and *plotIndLRR* functions.

LRR, mLRR-Y, BAF and B-deviation

mLOY calling was performed on data obtained in PennCNV format for each individual and containing SNP, chromosome, position, LRR, BAF and genotype information. LRR and BAF for Illumina arrays (see EGCUT dataset below) were obtained using GenomeStudio tool (https://www.illumina.com/techniques/microarrays/array-data-analysis-experimental-design/genomestudio.html). For Affymetrix arrays (TCGA dataset), LRR and BAF values were obtained using Affymetrixc Power Tools with the Birdseed v2 algorithm (http://media.affymetrix.com/support/developer/powertools/changelog/index.html) and following the PennCNV-Affy method described in PennCNV webpage (http://penncnv.openbioinformatics.org/en/latest/user-guide/affy/).

For each individual, we computed the 5% trimmed-mean of LRR in autosomes to avoid regions having copy number alterations. The percentage was changed to 25% in the case of cancer samples, where numerous copy number alterations are expected. We then computed the normalized median of LRR-Y (mLRR-Y) with respect to the trimmed-mean of LRR intensity in the autosomes to remove systematic biases across the array. Then, the B-deviation is computed for each sample by selecting the BAF values in the X-Y homologous region (PAR1, PAR2 and XTR) for non-homozygous probes. These probes were selected by setting an upper and lower BAF threshold of 0.8 and 0.2 for normal and variant homozygous genotypes. B-deviation is then calculated as the trimmed mean of the absolute difference between experimental BAF values and 0.5 (i.e expected value) of the heterozygous probes.

Quality control

We perform a quality control of the samples involved in the analyses by removing those samples with large LRR variability to avoid additional variability likely due to technical artifacts.

We followed the Illumina manufacturer's recommendation of filtering samples with high variable LRR in autosomes (standard deviation of LRR > 0.28). This criterion was used for EGCUT dataset. For TCGA and NIA samples, that were genotyped using Affymetrix, we consider as the normal variability of LRR as 2 times the LRR standard deviation of autosomes.

MADloy software

We created *MADloy* (https://github.com/isglobal-brge/*MADloy*), an R package that automates mLOY detection for association studies implementing the methods previously described. The package has been submitted to Bioconductor. The core functions include a pipeline to normalize data, perform quality control and summarize data from PennCNV format containing genome-wide information for LRR values. *MADloy* calls mLOY and gains of chromosome Y (XYY) using NIG distribution. Visualization functions are also implemented to help inspect the LRR and BAF values of each sample. A vignette can be found at https://github.com/isglobal-brge/*MADloy*/tree/master/vignettes.

Experimental validation

Ten random samples belonging to EGCUT cohort were validated using two Multiplex Ligation Probe-dependent Amplification (MLPA) panels, P070 (MRC-Holland Amsterdam, The Netherlands) covering all subtelomeric regions including the two pseudoautosomal regions (PAR1 and PAR2). Probes targeting Y chromosome, and a custom-made panel with probes for SRY and several autosomal loci, were used to assess the copy number status of chromosome Y with respect to the control loci (autosomal and X chromosome). The MLPA reactions were performed as previously described (17) with the some modifications for custom probe selection (18). We used the relative peak height (RPH) method recommended by MRC-Holland. Non-mosaic losses and gains were expected at relative peak height between (0.5, 1.5) for the pseudoautosomal regions (normally disomic), and between (0, 2) for the Y-unique regions (normally monosomic).

Statistical analyses

Association studies of mLOY with age and cancer performed in EGCUT and TCGA data was performed by using generalized linear models (Gaussian and binomial links, respectively). Models with cancer in EGCUT data were adjusted for age to remove the effect of mLOY on aging. Transcriptome data belonging to EGCTU data were obtained using HTA 2.0 microarray.

Transcriptome of TCGA samples were obtained from RNAseq data available at RTCGA package. Both analyses were performed using *limma* package and *voom* method was used to get continuous data from RNAseq experiments of TCGA (19). Models were adjusted for surrogate variables using *sva* package in order to control for possible experimental differences across samples (20). Enrichment analyses were carried out using *GOstats* Bioconductor library (21).

Simulation studies

We performed a number of simulations to determine the power of the association analyses considering mLOY status as continuous (Wright's method), as categorical (Forsberg's and MADloy methods). We considered two main scenarios to assess the power to detect significant associations with continuous (age) or categorical (case/control) outcomes. Data were simulated using functions from the CNVassoc package which is able to simulate gains and losses from continuous data (e.g. LRR) that can be considered as surrogate variable of LOY status in case-control and linear models (22). LRR continuous data was generated by using the mean and standard deviation of LRR in normal and LOY cases and the standard deviation of the outcome using the values observed from EGCUT data. Our aim was to mimic real situations when analyzing correlation between case-control status and mLOY or between age and mLOY. The effect size varied from 1 up to 8 which can be interpreted as age changes for one unit change in the mLOY variable. Sample size varied as (200, 300, 500, 750, 1000, 1500). For categorical outcomes, the magnitude of the effect was measured as an odds ratio (OR). We ran different scenarios varying mean effect as (1.5, 2, 2.5, 3, 3.5, 4) and OR as (1.5, 1.75, 2, 2.5, 3, 3.5, 4).

EGCUT data

SNP data of 682 individuals from three platforms: OmniX Human370CNV and Metabochip arrays were randomly selected from Estonian Gene Expression Cohort (EGCUT, www.biobank.ee). Selected individuals were older than 18 years of age (mean age 51.4+/-18.2 years). EGCUT comprises a large cohort of about 52,000 samples of the Estonian Genome Center Biobank, University of Tartu (23). Data were genotyped using HumanCoreExome array and all the individuals included in the analysis had a genotyping success rate above 95%. Cryptic relatedness was tested with the PLINK v1.07 software. Only one of each detected

relative pairs (up to second cousins) was randomly chosen for the detection of genetic mosaicism. Sample mix-ups were corrected using MixupMapper (24). All studies were performed in accordance with the ethical standards of the responsible committee on human experimentation, and with proper informed consent from all individuals tested. LRR and BAF were generated using GenomeStudio software.

TCGA data

We analyzed 346 paired tumor (n=103) and normal (n=121) male samples of Kidney Renal Clear Cell Carcinoma (KIRC) and Bladder Urothelial Carcinoma (BLCA) belonging to The Cancer Genome Atlas (TCGA) project. Raw data obtained from the Affymetrix Genome-Wide Human SNP Array 6.0 chip were processed with Birdseed v2 algorithm. Clinical data were also downloaded to select male samples and for association analyses. RNAseq data was obtained from RTCGA.rnaseq Bioconductor package that provides counts of 20532 annotated genes in 36 different tumors.

Results

Improved detection of mLOY status

We developed *MADloy*, a bioinformatics tool available as a Bioconductor package, which implements accurate mLOY calling of SNP intensity data using mLRR-Y and B-deviation information across subjects (http://www.github.com/isglobal-brge/*MADloy*). For each sample, MADloy estimates the normalized mLRR-Y given by its ratio with the trimmed-mean of mLRR-Y values in the autosomes. The method uses 5% trimmed-mean to avoid regions having copy number alterations. Calling using mLRR-Y information is given by the identification of unexpected negative values of the mLRR-Y distribution, centered by the expected chromosome Y ploidy for a man (-0.45, one copy), that are lower than 1.2 times the distance of interquantile range. This distance contains ~%95 of the area under a normal distribution. The interquantile range is robust in the present of outliers and therefore is a suitable measure for simultaneous mLOY and GOY calling. The calling is complemented with B-deviation information in the PAR1/PAR2 regions to reduce the number of false positives of mLOY calls and to estimate their cellularity (see Methods). We then examined whether this new calling method, by reducing misclassification and increasing non-biased estimates of effect sizes, can improve the statistical power in association studies.

We first compared *MADloy* and *mLRR-Y*$_{thresh}$ (Forsberg et al) calling methods on 682 males from the Estonian Gene Expression Cohort (EGCUT, www.biobank.ee). Thirty eight individuals (5.6%) were discarded due to large mLRR-Y variability), see Methods. **Figure 1a** shows the ploidy-centered 5% trimmed mLRR-Y values for each subject with their respective calling status using *MADloy*. **Figures 1b and 1c** illustrate the expected pattern of mLRR-Y and BAF of a normal sample, where the mLRR-Y (blue line) is not far from the expected ploidy value that corresponds to one copy (-0.45) (orange line) and the BAF along the PAR1 (vertical blue rectangle) and PAR2 (vertical yellow rectangle) regions is centered at 0.5 (right Y-scale). **Figures 1b and 1c** show the expected pattern of one sample with mLOY, for which mLRR-Y is lower than the ploidy value and a large BAF split is observed. The size of the split is proportional to the cellularity and is measured by B-deviation defined as the absolute difference between BAF values. The expected value of the B-deviation for heterozygous probes is 0.5 when no mLOY is present.

Using *MADloy*, we detected a total of 30 (4.3%) individuals with X-Y allelic imbalance consistent with decreased Y chromosome dosage (**Figure 1a**). Using the *mLRR-Y*$_{thresh}$ method, we called 56 samples with mLOY. A main reason for the difference is that the positive distribution of mLRR-Y is less variable than the negative part breaking the symmetry assumption and increasing the negative threshold at with mLOY is called by *mLRR-Y*$_{thresh.}$ As a result more mLOY individuals are called. **Supplementary Table 1** shows the discordant calls using *MADloy* and *mLRR-Y*$_{thresh}$. We investigated the different sources of discordance at the *mLRR-Y*$_{thresh}$ calling threshold. For instance, we observed that while V39233 has a lower mLRR-Y value than *mLRR-Y*$_{thresh}$, there is no BAF split in PAR1 and PAR2 regions (**Supplementary Figures S1A-B**). In addition, we observed that there are samples with low mLRR-Y due to other causes than mLOY. For instance, **Supplementary Figures S1C-D** shows a case with a BAF split (chromosome X) and LRR values (chromosome Y) consistent with the presence of an additional pair of XX chromosomes, in addition to the XY chromosomes of the individual. These additional X chromosomes are likely due to a contamination of the sample analyzed with another DNA, representing ~26% of the sample analyzed.

To increase the experimental support of *MADloy* calling, we validated by MLPA, the positive mLOY status, as detected by *MADloy*, of 10 randomly selected individuals from EGCUT. The results were fully concordant in all ten individuals. **Figure 2A** shows the LRR and BAF of probes across chromosomes Y and X for one individual. **Figure 2B** illustrates the validation of two

mLOY cases (V32199 and V40611) with different cellularity (30% and 52%, respectively). We observed that for individuals with mLOY the relative peak heights (RPH) of Y probes were clearly reduced compared to the RPH of the X probe (**Figure 2B**).

To address the differences in mLOY detection across different tissues, we asked the extent to which mLOY status in blood could be detected in buccal smear. 18 individuals from EGCUT, who were detected with mLOY using *MADloy* in blood, were randomly selected and re-contacted after 3 years to assess the progression of mLOY in blood and evaluate its detection by *MADloy* in buccal smear (**Table 1**). We observed that mLOY in blood persisted in time (**Table 1)**. Detected from BAF signals, the estimated proportion of cells with mLOY increased in 16 cases (88%) while it decreased in 2 (12%). In saliva, we discarded one individual for low quality of the sample and observed mLOY in only 7 individuals (41%). All cases of mLOY in saliva showed lower cellularity than mLOY in blood at followup. Using the same detection methods and procedures, we therefore confirmed that the detection power of mLOY in blood is substantially decreased if assessed in buccal smear.

mLOY improves the statistical power of association studies

Using a series of simulations we first compared *MADloy* performance with mLRR-Y_{thresh} and mLRR-Y_{quant}. The simulations were performed using an independent simulator for copy number variation, CNVassoc assuming that the distribution of normal and mLOY cases, with the proportions observed for in the EGCUT study, followed those observed for CNVs. We studied the power to detect true associations between mLOY and a quantitative trait. mLOY calling was performed with MADloy and Fosberg's methods and associations were also assessed with mLRR-Y as a quantitative variable (**Supplementary Figure S2**). We found that *MADloy* had the largest statistical power in all scenarios while mLRR-Y_{quant} was the least powerful. In particular, we observed that an association of magnitude 2 per quantitative trait unit was detected with using *MADloy* with a power of 80% in 1,000 individuals while for *mLRR-Y_{thresh}* a magnitude of >2.2 was required for the same power. We also observed similar results for dichotomous traits in case-control studies (**Supplementary Figure S3**). The differences in power were given by a lack of symmetry in the mLRR-Y distribution that resulted in misclassification by *mLRR-Y_{thresh}*. In addition, the simulations confirmed that treating mLRR-Y as quantitative when the underlying distribution is a mixture is a suboptimal approach.

We then compared in real setting the associations with age and cancer risk with mLOY called with MADloy and *mLRR-Y_{thresh}* in EGCUT. In this case we also aimed to evaluate the effect incorporating B-deviation signal in *MADloy* calling. We confirmed the association between mLOY and age. We found that mLOY called by *MADloy* with B-deviation showed the strongest and most significant association with age (beta=21.21, P=2.0x10^{-10}). However, when mLOY status was called using mLRR-Y information only, either by *MADloy* or by *mLRR-Y_{thresh}*, the effect of association was lower (**Figure 3A).** Note that the largest impact in the association was when considering *mLRR-Y_{thresh}* method, suggesting again that the symmetry assumption is less accurate than the interquantile range to define the mLOY calling threshold. We observed analogous results for the association between mLOY and cancer in EGCUT. For MADloy with and without B-deviation we observed similar significant increased risks of any tumor in models adjusted by age (OR=2.89, P= 0.0390, OR=2.86, P= 0.0367). However, for *mLRR-Y_{thresh}*, we did not find statistically significant differences (OR=1.84, P=0.1720), confirming that the loss of power by methods that do not consider the possibility of having GOY nor the B-deviation can affect statistical significance (**Figure 3B**).

We also assessed the different mLOY calling procedures in two cancer studies. We studied the differences in frequency of mLOY status between cancer and constitutional tissues of 346 samples from The Cancer Genome Atlas (TCGA) project. The samples belong to Clear Cell Carcinoma (KIRC) and Bladder Urothelial Carcinoma (BLCA) studies. The genotype data comprised 121 constitutional and 103 tumor samples. We first observed that *MADloy* detected *29* samples with mLOY (8,38%) while 34 were detected by *mLRR-Y_{thresh}*. Thus, *mLRR-Y_{thresh}* shows a more liberal approach to mLOY detection, even under presence of 2 GOY samples (0.6%) that are considered as part of the reference population. However, the GOY samples were called by *MADloy* showing the expected increase in mLRR-Y together with a clear BAF split in the PAR1 and PAR2 regions of chromosome X (**Figure S4**).

We then tested the association with cancer status. We observed again that strongest association was when mLOY was called with the full *MADloy* method (OR=10.6, P=4.65x10^{-6}). Associations when mLOY was called with *mLRR-Y_{thresh}* and were also significant but their P-values were higher in one order of magnitude (P=1.48x10^{-5} and P=1.59x10^{-5}, respectively), in line with previous results and giving further evidence that a more efficient use of the SNP intensity signals in mLOY calling can increment the power to detect associations with phenotypes (**Figure 3B)**.

Application of MADloy on finding transcriptomic signatures of mLOY

Differences in statistical power become crucial when studying the mechanisms underlying mLOY using genomic and transcriptomic data, as few misclassifications can lead to not detecting a relevant genome or transcriptome-wide association. We therefore apply *MADloy* to comparatively analyzed transcriptomic data in cells from individuals with and without mLOY in blood in the EGCUT study and in cancer tissues in the TCGA study.

We searched for transcription correlates with mLOY in blood using the EGCUT study. We analyzed microarray expression data on 682 individuals (compared with age matched controls with no mLOY) and observed 49 deregulated genes p-value $<10^{-3}$, five of which were significant with p-value $< 10^{-5}$. These included *MED16*, *HNRNPKP1*, *TXNDC17*, *TMEM154*, and *CSF2RA,* the only gene in chromosome Y (PAR1). An enrichment analysis using KEGG and GO databases showed several KEGG pathways that were significantly enriched in differentially expressed genes (**Table 2**). Meaningful pathways including DNA replication (p-value=0.004), mismatch repair (p-value=0.02) and homologous recombination (p-value=0.02) were found significant. Interestingly, only two genes located in the Y chromosome (*CSF2RA* and *EIF1AY)* were among the significantly deregulated gene in blood.

Transcriptome-wide association analysis of mLOY in TCGA was also performed with RNA-seq data in two different tumor datasets, the KIRC (Kidney renal clear cell carcinoma) and BLCA (Bladder urothelial carcinoma) studies. In both tumors, we found that 8 of the top-10 down-regulated genes by mLOY where in chromosome Y: *TTTY4C, UTY, TMSB4Y, USP9Y, ZFY, EIF1AY, RSP4Y1* and *TTTY15* (adjusted p-values lower than 1.3×10^{-28}) (**Figure 4**). This strong correlation with expression is fully supportive of the consistency of mLOY calling by *MADloy*, and indicates that mLOY in tumors leads to a detectable drop in transcription or extreme down-regulation of most or the entire chromosome Y across the tissue.

Discussion

Substantial differences in mLOY calling can arise from the different methods used leading to differences in power to detect an effect of mLOY on aging and cancer. We show here that significant improvements in the detection accuracy of mLOY status from SNP array intensity data can be obtained by using a common matrix-tissue and optimization of the calling

methods, by improving the calling threshold in mLRR-Y signal and accounting individuals with chromosome Y gains. Previous methods assumed symmetry in the mLRR-Y distribution and included individuals with gains in the reference population, affecting the threshold to detect mLOY status (13). We observed that individuals with GOY are detectable. Therefore, not accounting for asymmetry in the distribution can increase mLOY false positives, reducing the statistical power in the associations due to misclassification. Interestingly we also observed that treating mLRR-Y as a continuous variable was more detrimental than positive (11). This is likely due to its multimodal nature of its distribution where individuals fall sharply into the normal or mLOY categories.

An additional gain in detection accuracy was observed by an independent analysis of the B-deviation signal (18). While improvements were modest for the associations with age and cancer risk for mLOY in blood, we observed substantial increments in cancer tissues. The inclusion of this B-deviation as an additional signal in mLOY calling allowed us to detect instances where samples are likely contaminated and to confirm individuals with GOY. We have shown that samples classified as mLOY using mLRR-Y with discordant B-deviation values should be visually inspected to define contaminations or other non-LOY events. Removing these cases from the analyses in a quality control phase will reduce misclassification errors and improve statistical power.

It has been suggested that discrepancies in associations between mLOY and disease risk or mortality could also be related to the different tissue source of the DNA, despite significant correlation of mLOY calling in blood and buccal derived DNA from the same individuals (9). We show here that the cellularity of mLOY in blood tends to slightly increase with time, but the concordance with mLOY in buccal smear is only 41%, with lower cellularity in buccal samples in most cases. Therefore, different tissue source could lead to differences in mortality ratios and other associations to mLOY, as previously reported (9, 11). On the other hand, the presence of mLOY across different tissues does suggest a common origin in each individual, consistent with a genetic predisposition that has also been documented by genome-wide association studies (8, 7).

Interestingly, the transcriptomic associations of mLOY are quite different in tumor samples from non-tumor blood samples. In kidney and bladder tumors, extreme down-regulation of the entire Y is the main finding, a logical consequence of the decreased number of

chromosome Y copies. However in blood, only two Y chromosome genes are among the most deregulated ones, and deregulation of autosomal genes mainly affects DNA replication, mismatch repair and homologous recombination pathways. This finding could be related to an increasingly oligoclonal leukocyte cell population in people with mLOY, which is consistent with the possibly compromised immune cell function of circulating leukocytes in people with mLOY as the risk factor for disease (13).

The mLOY is increasingly recognized as a male specific risk factor for multiple diseases and, therefore, its interest in personalized treatment and risk management is likely to increase in the coming years. Specific disease and mechanistic studies require a robust estimation of the mLOY status of individuals. SNP intensity data is already available in large epidemiological studies where the effects of environmental conditions can also be investigated. The ability to call mLOY reliably using these data will increase the reproducibility of the findings We show that *MADloy* is able to replicate the associations of mLOY in blood with cancer and aging while increasing statistical power obtained by standard approaches. The method and the bioinformatic tool presented in this work are easy to use and scalable to large studies. Therefore, with *MADloy* we aim to facilitate comparable studies with large number of individuals to better define the role of mLOY in complex diseases and its underlying mechanisms. *MADloy* allows the re-analysis of thousands of existing GWAS data in public available repositories.

Funding

This research has received funding from the Spanish Ministry of Economy and Competiveness (RTI2018-100789-B-I00). LAPJ lab is funded by the Catalan Department of Economy and Knowledge (SGR2014/1468, SGR2017/1974 and ICREA Acadèmia), and also acknowledges support from the Spanish Ministry of Economy and Competiveness “Programa de Excelencia María de Maeztu” (MDM-2014-0370).

Conflict of Interest

The authors declare no conflict of interest.

References

1. Forsberg,L.A., Rasi,C., Malmqvist,N., Davies,H., Pasupulati,S., Pakalapati,G., Sandgren,J., de Ståhl,T.D., Zaghlool,A., Giedraitis,V., *et al.* (2014) Mosaic loss of chromosome Y in peripheral blood is associated with shorter survival and higher risk of cancer. *Nat. Genet.*, **46**, 624–628.

2. Loftfield,E., Zhou,W., Graubard,B.I., Yeager,M., Chanock,S.J., Freedman,N.D. and Machiela,M.J. (2018) Predictors of mosaic chromosome Y loss and associations with mortality in the UK Biobank. *Sci. Rep.*, **8**, 12316.

3. Grassmann,F., Kiel,C., den Hollander,A.I., Weeks,D.E., Lotery,A., Cipriani,V., Weber,B.H.F. and International Age-related Macular Degeneration Genomics Consortium (IAMDGC) (2019) Y chromosome mosaicism is associated with age-related macular degeneration. *Eur. J. Hum. Genet.*, **27**, 36–41.

4. Dumanski,J.P., Lambert,J.-C., Rasi,C., Giedraitis,V., Davies,H., Grenier-Boley,B., Lindgren,C.M., Campion,D., Dufouil,C., Pasquier,F., *et al.* (2016) Mosaic Loss of Chromosome Y in Blood Is Associated with Alzheimer Disease. *Am. J. Hum. Genet.*, **98**, 1208–1219.

5. Haitjema,S., Kofink,D., van Setten,J., van der Laan,S.W., Schoneveld,A.H., Eales,J., Tomaszewski,M., de Jager,S.C.A., Pasterkamp,G., Asselbergs,F.W., *et al.* (2017) Loss of Y Chromosome in Blood Is Associated With Major Cardiovascular Events During Follow-Up in Men After Carotid Endarterectomy. *Circ. Cardiovasc. Genet.*, **10**, e001544.

6. Kimura,A., Hishimoto,A., Otsuka,I., Okazaki,S., Boku,S., Horai,T., Izumi,T., Takahashi,M., Ueno,Y., Shirakawa,O., *et al.* (2018) Loss of chromosome Y in blood, but not in brain, of suicide completers. *PLoS One*, **13**, e0190667.

7. Zhou,W., Machiela,M.J., Freedman,N.D., Rothman,N., Malats,N., Dagnall,C., Caporaso,N., Teras,L.T., Gaudet,M.M., Gapstur,S.M., *et al.* (2016) Mosaic loss of chromosome Y is associated with common variation near TCL1A. *Nat. Genet.*, **48**, 563–568.

8. Wright,D.J., Day,F.R., Kerrison,N.D., Zink,F., Cardona,A., Sulem,P., Thompson,D.J., Sigurjonsdottir,S., Gudbjartsson,D.F., Helgason,A., *et al.* (2017) Genetic variants associated with mosaic Y chromosome loss highlight cell cycle genes and overlap with cancer susceptibility. *Nat. Genet.*, **49**, 674–679.

9. Forsberg,L.A., Halvardson,J., Rychlicka-Buniowska,E., Danielsson,M., Moghadam,B.T., Mattisson,J., Rasi,C., Davies,H., Lind,L., Giedraitis,V., *et al.* (2019) Mosaic loss of chromosome Y in leukocytes matters. *Nat. Genet.*, **51**, 4–7.

10. Zhou,W., Machiela,M.J., Freedman,N.D., Rothman,N., Malats,N., Dagnall,C., Caporaso,N., Teras,L.T., Gaudet,M.M., Gapstur,S.M., *et al.* (2019) Reply to 'Mosaic loss of chromosome Y in leukocytes matters'. *Nat. Genet.*, **51**, 7–9.

11. Zhou,W. (2019) Stratified analyses of buccal and blood-derived DNA indicate limited tissue-specific difference in the causes or consequences of mosaic chromosome Y loss. *Nat Genet*.

12. Cotter,D.J., Brotman,S.M. and Wilson Sayres,M.A. (2016) Genetic Diversity on the Human X

Chromosome Does Not Support a Strict Pseudoautosomal Boundary. *Genetics*, **203**, 485–92.

13. Forsberg,L.A., Rasi,C., Malmqvist,N., Davies,H., Pasupulati,S., Pakalapati,G., Sandgren,J., de Ståhl,T.D., Zaghlool,A., Giedraitis,V., *et al.* (2014) Mosaic loss of chromosome Y in peripheral blood is associated with shorter survival and higher risk of cancer. *Nat. Genet.*, **46**, 624–628.

14. Wacholder,S., Hartge,P., Lubin,J.H. and Dosemeci,M. (1995) Non-differential misclassification and bias towards the null: a clarification. *Occup. Environ. Med.*, **52**, 557–8.

15. Barnes,C., Plagnol,V., Fitzgerald,T., Redon,R., Marchini,J., Clayton,D. and Hurles,M.E. (2008) A robust statistical method for case-control association testing with copy number variation. *Nat. Genet.*, **40**, 1245–52.

16. González,J.R., Subirana,I., Escaramís,G., Peraza,S., Cáceres,A., Estivill,X. and Armengol,L. (2009) Accounting for uncertainty when assessing association between copy number and disease: A latent class model. *BMC Bioinformatics*, **10**.

17. Os,P.G.C.E.- Van and Schouten,J.P. (2011) Multiplex Ligation-Dependent Probe Amplification (MLPA®) for the Detection of Copy Number Variation in Genomic Sequences. In *Methods in molecular biology (Clifton, N.J.)*.Vol. 688, pp. 97–126.

18. Rodríguez-Santiago,B., Malats,N., Rothman,N., Armengol,L., Garcia-Closas,M., Kogevinas,M., Villa,O., Hutchinson,A., Earl,J., Marenne,G., *et al.* (2010) Mosaic uniparental disomies and aneuploidies as large structural variants of the human genome. *Am. J. Hum. Genet.*, **87**, 129–138.

19. Law,C.W., Chen,Y., Shi,W. and Smyth,G.K. (2014) voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol.*, **15**, R29.

20. Leek,J.T., Johnson,W.E., Parker,H.S., Jaffe,A.E. and Storey,J.D. (2012) The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics*, **28**, 882–3.

21. Falcon,S. and Gentleman,R. (2007) Using GOstats to test gene lists for GO term association. *Bioinformatics*, **23**, 257–258.

22. Subirana,I., Diaz-Uriarte,R., Lucas,G. and Gonzalez,J.R. (2011) CNVassoc: Association analysis of CNV data using R. *BMC Med. Genomics*, **4**.

23. Leitsalu,L., Haller,T., Esko,T., Tammesoo,M.-L., Alavere,H., Snieder,H., Perola,M., Ng,P.C., Mägi,R., Milani,L., *et al.* (2015) Cohort Profile: Estonian Biobank of the Estonian Genome Center, University of Tartu. *Int. J. Epidemiol.*, **44**, 1137–1147.

24. Westra,H.-J., Jansen,R.C., Fehrmann,R.S.N., te Meerman,G.J., van Heel,D., Wijmenga,C. and Franke,L. (2011) MixupMapper: correcting sample mix-ups in genome-wide datasets increases power to detect small genetic effects. *Bioinformatics*, **27**, 2104–2111.

FIGURES

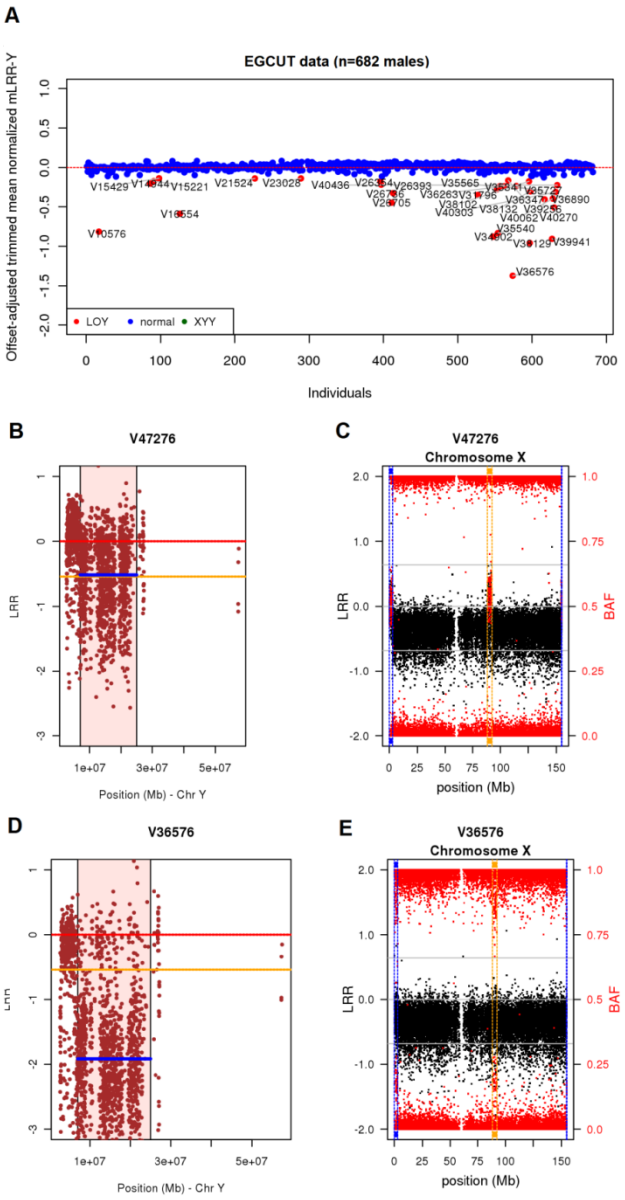

Figure 1. *MADloy* detection in EGCUT data. Figure 1A shows mLOY calling performed using mLRR-Y data by considering our proposed method to robustly detect outliers by considering the possibility of having GOY samples. Figures B and C show the expected behavior of mLRR-Y and BAF data for a normal sample. Figures D and E depicts the expected values of mLRR-Y and BAF data of a mLOY sample. We observe a clear decrease of mLRR-Y (blue line) with respect to the expected value (yellow line) (panel D) and a split BAF in PAR1 (blue box) and XTR (yellow box) regions.

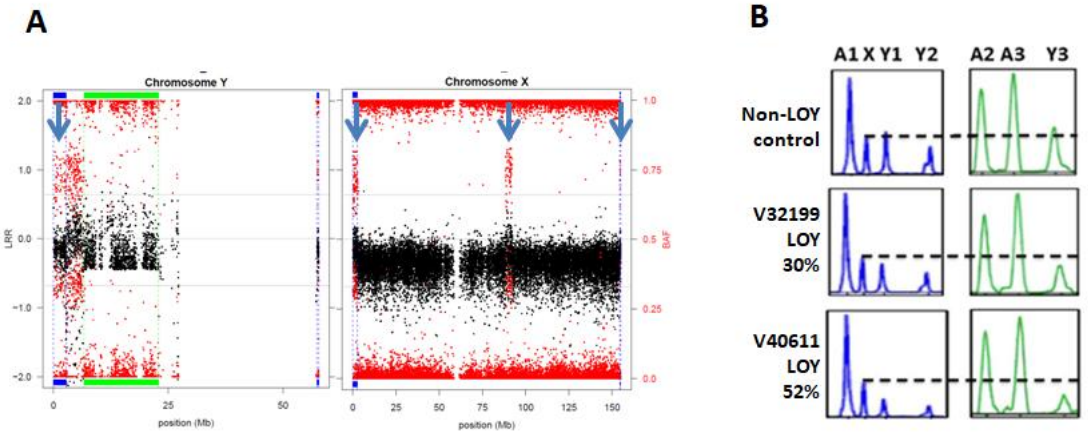

Figure 2. Experimental mLOY validation. A) LLR (black) and BAF (red) values of probes along chromsomes Y and X for an individual with positive mLOY status. Low values of LLR-Y are observed except for the PAR1 and PAR2 regions where a clear BAF split is observed. The BAF split is confirmed in the X chromosome. **B)** MLPA validation of two individuals with mLOY with different cellularity content, compared with a normal individual (top). Two MLPA panels are shown for each individual, showing relative peak height (RPH) for probes in autosomes (A1, A2, A3) and in the X and Y chromosomes (Y1, Y2, Y3). Marking the peak for the chromosome X probe (dotted line), the figure shows a clear reduction in the RPH values of Y probes for the individuals with mLOY.

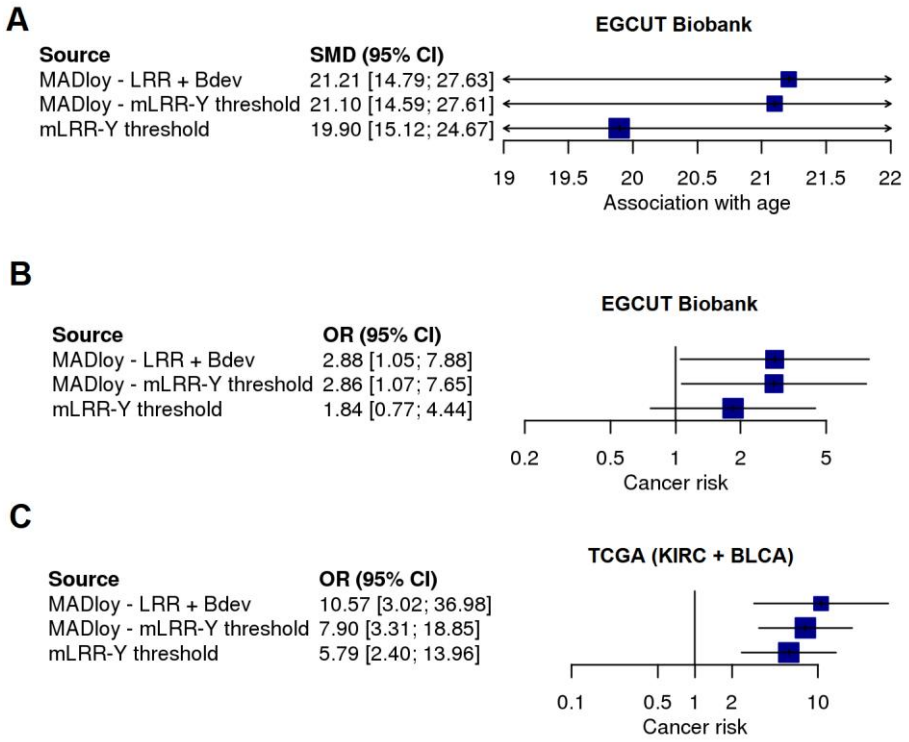

Figure 3. Effect of mLOY misclassification. Effect estimates of three different association studies of continuous (age) and binary phenotypes (cancer) using three different calling methods. Two of them use only LRR information and two different threshold methods and one incorporates the information about B-deviation (Bdev). We observe as the effect is far from the null when using MADloy with both LRR and Bdev.

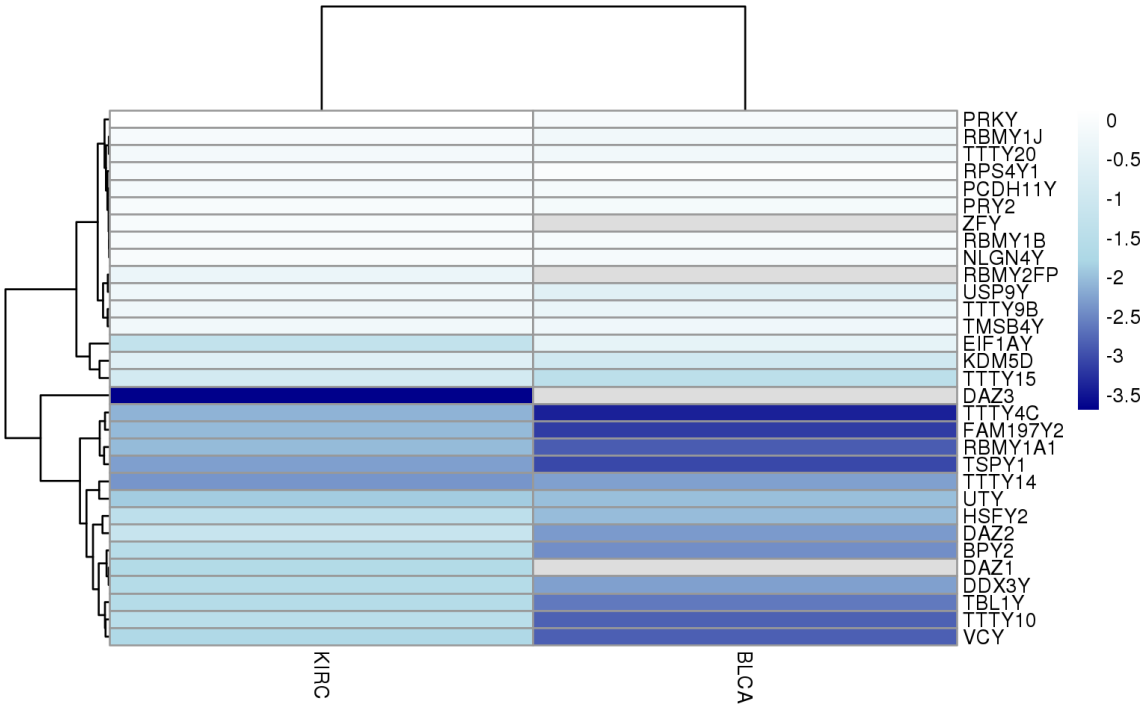

Figure 4. Transcriptomic effects in tumor samples (TCGA). Downregulation of genes in chromosome Y when comparing samples with and without mLOY in KIRC and BLCA datasets of TCGA project.

TABLES

Table 1: Cellularity evolution of LOY detected in blood of 18 individuals after 3 years. At follow-up mosaicism was also tested in saliva (ND: Not Detectable).

	Baseline detection			Follow-up at 3 years	
Sample	**Blood DNA**	**% cells**		**Blood DNA (% cells)**	**Saliva DNA (% cells)**
V15429	Y loss	27		30	ND
V16554	Y loss	52		67	57
V16763	Y loss	41		50	ND
V19232	Y loss	41		53	ND
V22330	Y loss	52		46	ND
V23235	Y loss	37		48	27
V26736	Y loss	42		49	ND
V31014	Y loss	46		48	30
V31220	Y loss	29		39	17
V32199	Y loss	30		40	ND
V32632	Y loss	46		82	ND
V32752	Y loss	32		35	ND
V32850	Y loss	33		43	ND
V34568	Y loss	37		44	ND
V37288	Y loss	31		44	ND
V40611	Y loss	52		29	32
V44342	Y loss	35		37	20
V47558	Y loss	37		39	18

Table 2. Enrichment analysis of blood transcriptomic data in EGCUT samples. Table includes KEGG categories that are over-enriched using differentially expressed genes at false discovery rate (FDR) lower than 0.05. Only terms with size>=5 are shown.

Count	Size	FDR	OR enrich	Term	Genes
3	32	0.0039	10.76	DNA replication	MCM4, RPA1, RPA3
2	22	0.0204	10.18	Mismatch repair	RPA1, RPA3
3	64	0.0264	5.08	Antigen processing and presentation	CD4, HSP90AA1, NFYC
2	26	0.0280	8.47	Homologous recombination	RPA1, RPA3
3	71	0.0345	4.55	Hematopoietic cell lineage	CD4, CSF2RA, ITGA2B
3	81	0.0480	3.95	Ribosome	RPS15, RPS24, RPSA
1	5	0.0497	24.94	Vitamin B6 metabolism	PSAT1

Robust estimation of mosaic loss of chromosome Y with genotype-array-intensity data: cellularity evolution, disease risk association and transcriptional consequences

Supplementary Material

Juan R González[1,2,3,*], Marcos López-Sánchez[4], Alejandro Cáceres[1,2],
Pere Puig[3], Tonu Esko[5], Luis A Pérez-Jurado[4,5,6]

id	mLRR	MADloy (LRR)	mLRR-Y_{thres}	MADloy (LRR + Bdev)
V17493	-0.1	normal	LOY	discordant
V25393	-0.09	normal	LOY	other
V26411	-0.11	normal	LOY	other
V26498	-0.08	normal	LOY	discordant
V38138	-0.1	normal	LOY	discordant
V38996	-0.08	normal	LOY	discordant
V39073	-0.11	normal	LOY	discordant
V39233	-0.13	normal	LOY	discordant
V40705	-0.1	normal	LOY	discordant
V45177	-0.09	normal	LOY	discordant

Table S1: Discordant calls of EGCUT data sets. Data of some one case with discordant and other classification by MADloy are visually inspected in Figure S3.

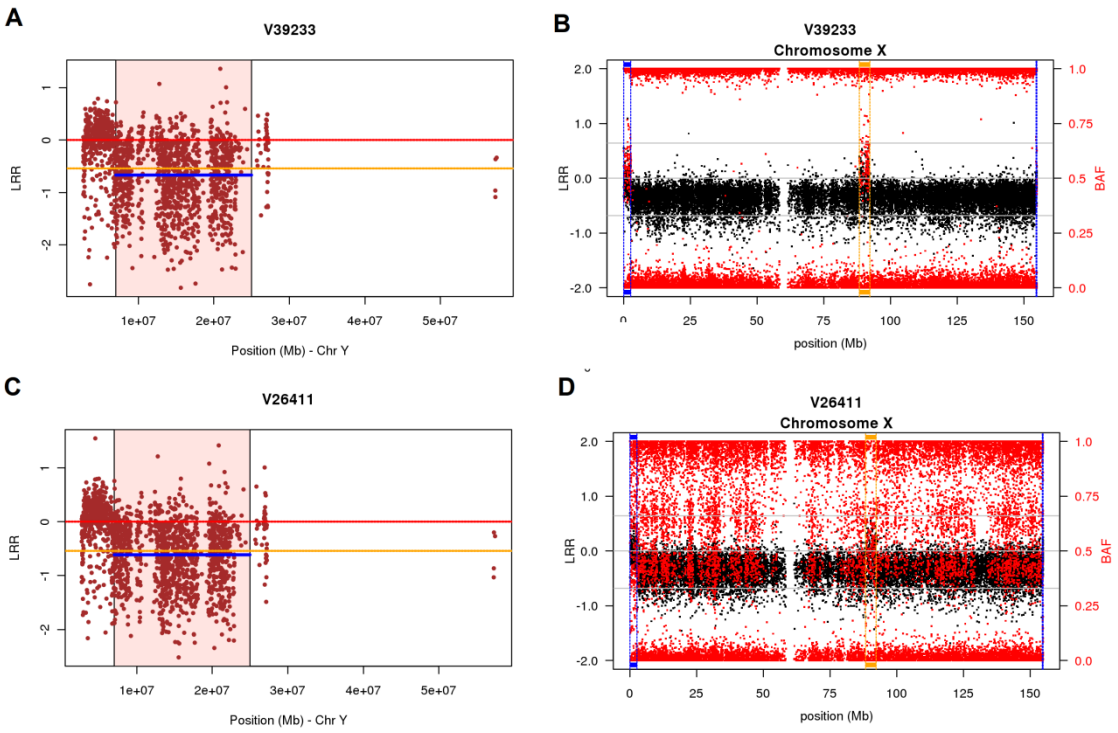

Figure S1: Panels A and C show LRR and mLRR-Y of chromosome Y. Panels B and D show LRR and BAF of chromosome X of two samples from ECGUT dataset that were called as “discordant” (V39233) and “other” (V26411) MADloy. Sample V39233 (panels A and B) has been classified as “discordant” due to a decreased LRR (panel A, blue line) than expected (panel A, orange line) but no clear BAF split is visible in PAR1, PAR2 (panel C, Blue boxes) and XTR (panle C, orange box) in chromosome X. Sample V26411 (panels C and D) has been classified as “other” due to a decreased LRR(C, blue) than expected (panel C, orange line). However, by looking at chromosome X plot (panel D), it can be seen a 2-band BAF split caused by the presence of an XX sample contamination that will also decrease the expected chromosome Y dosage in the sample.

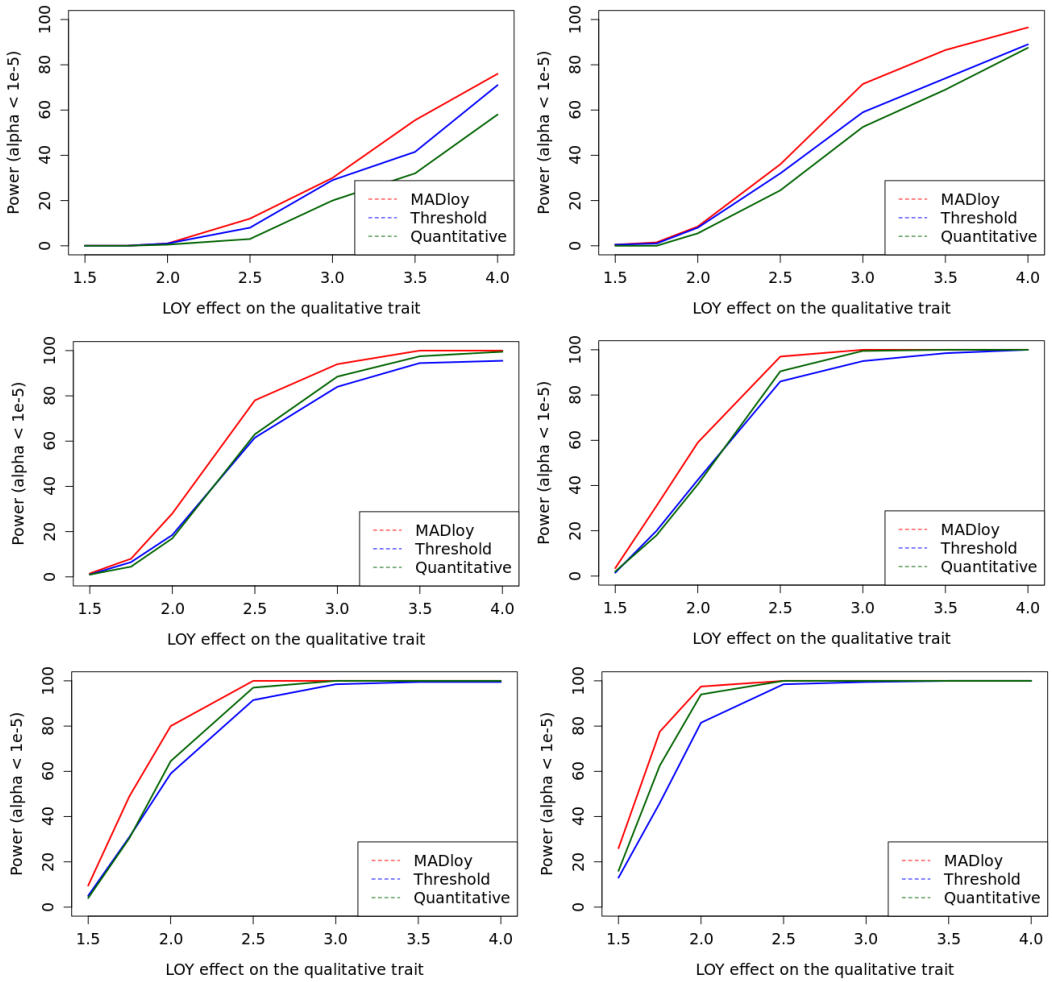

Figure S2: Simulation results for quantitative traits (e.g. age) for different methods. MADloy: uses mLRR-Y information, Threshold: mLRR-Y_{thres} (Fosberg et al., 2014), and Quantitative: mLRR-Y_{quant} (Wright et al., 2017). Each panel shows the results for different sample sizes (from left to right and top to bottom: 200, 300, 500, 750, 1000, 1500). The study assess the power (p-value<10^{-5}) to detect differences in the mean of the quantitative trait in the range 1.5 up to 4. The scenarios and how data are simulated are described in the Methods Section of the main manuscript.

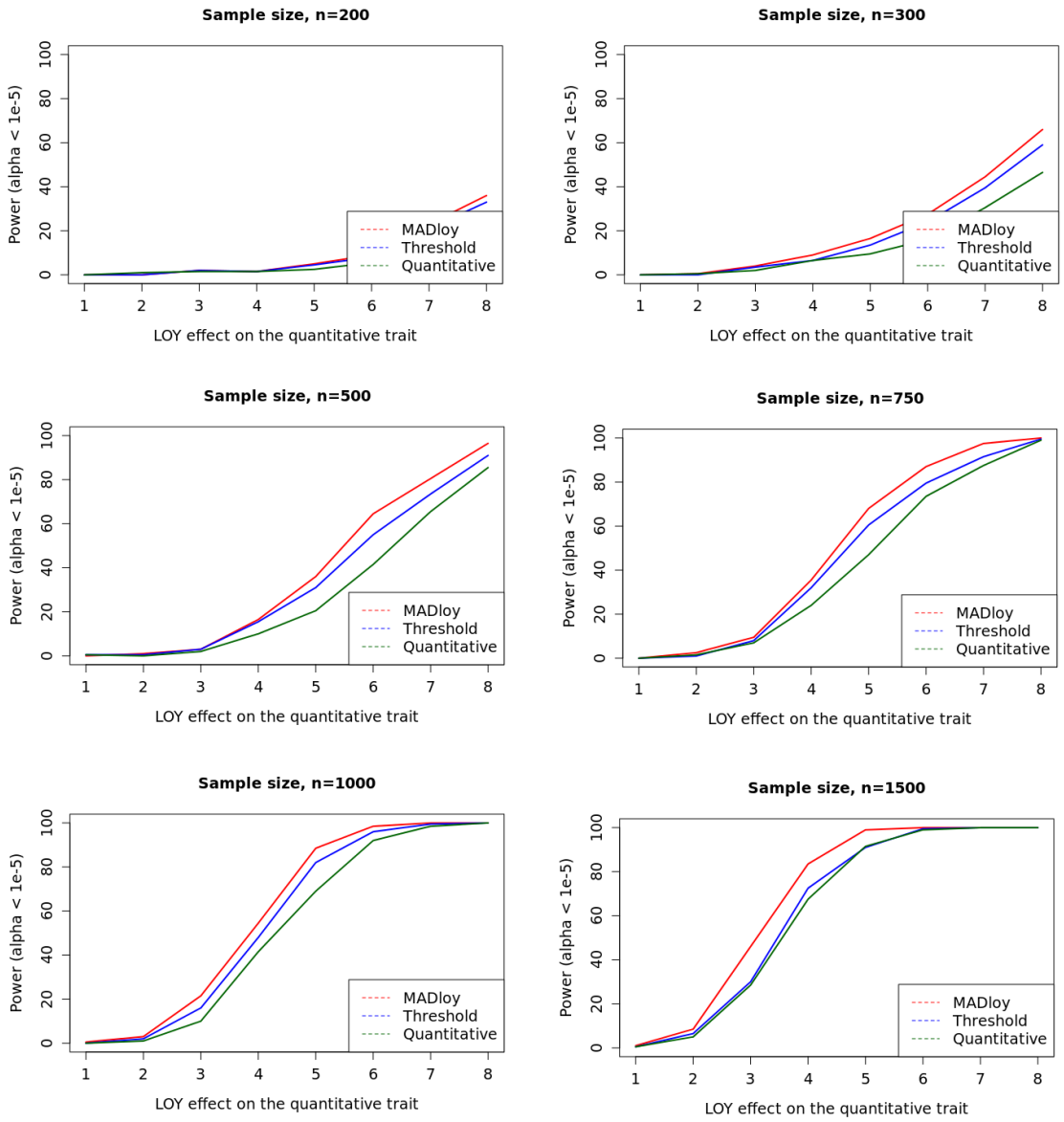

Figure S3: Simulation results for binary traits (e.g. case/control) for different methods. MADloy: uses mLRR-Y information, Threshold: mLRR-Y_{thres} (Fosberg et al., 2014), and Quantitative: mLRR-Y_{quant} (Wright et al., 2017). Each panel shows the results for different sample sizes (from left to right and top to bottom: 200, 300, 500, 750, 1000, 1500). The study assess the power (p-value<10^{-5}) to detect differences between cases an controls at different levels (odds ratios ranging from 1 up to 8). The scenarios and how data are simulated are described in the Methods Section of the main manuscript.

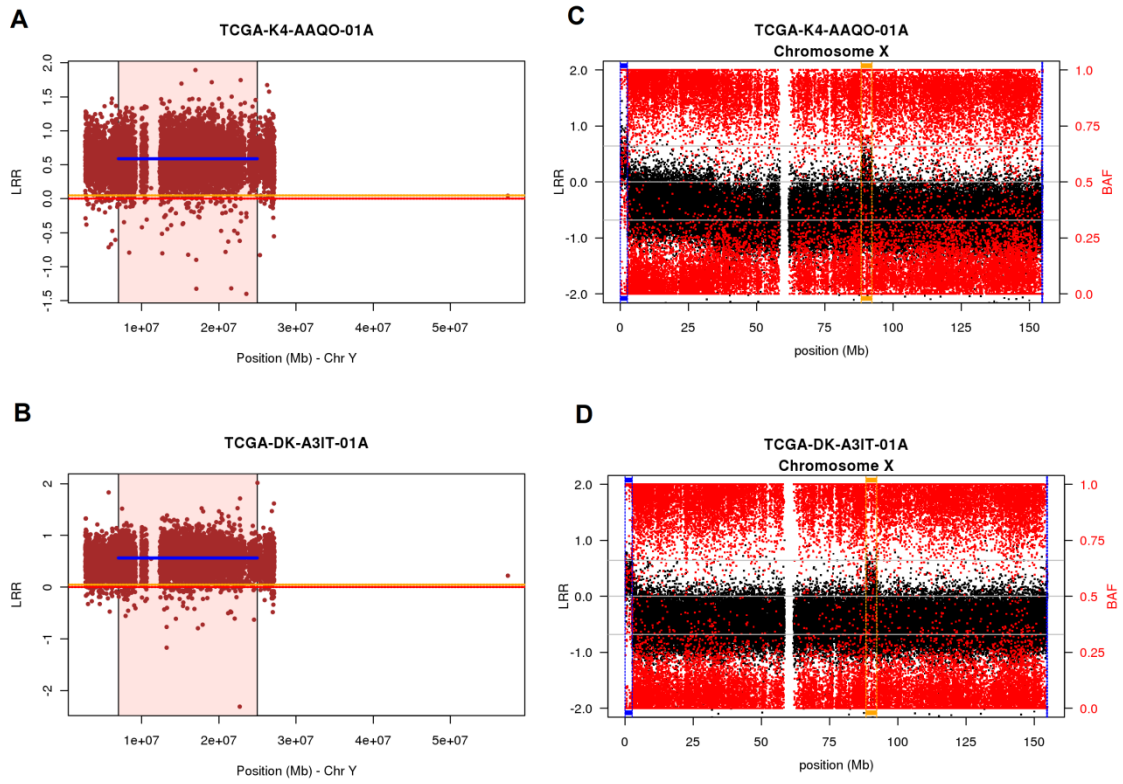

Figure S4: Panels A and C show LRR and mLRR-Y of chromosome Y. Panels B and D show LRR and BAF of chromosome X of two samples from TCGA dataset who were called as GOY. Sample TCGA-K4-AAQO-01A has an increased mLRR-Y (blue line panel A) which is far from the expected value (orange line panel A). Additionally, the LRR and BAF plot of chromosome X (panel C) shows a BAF split in the PAR1, PAR2 (blue boxes) and XTR (orange boxes), compatible with the allelic imbalance between the two Y and one X chromosome in the sample analyzed. The same is observed for sample TCGA-DK-A3IT-01A, where the mLRR-Y (panel B) and the LRR and BAF plot of chromosome X (planel D) shows the same pattern.