Instructions
ENIGMA-CNV working group, v2.2

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Purpose:
This document describes the different steps for joining ENIGMA-CNV including data analysis and submission.

Please address any questions to: enigmacnvhelpdesk@gmail.com

Joining ENIGMA-CNV with data – short overview

Welcome to the ENIGMA-CNV working group! We are pleased to have you onboard.

Steps involved in joining:

A. Administrative – sign up for ENIGMA-CNV to the working group chairs.

Analysis and data submission:
B. Structural imaging data processing
-minimal protocol
-extended protocol
C. CNVs
- call and visualize
D. Covariates
E. Send data or request a secure transfer-link from enigmacnvhelpdesk@gmail.com (preferred) and/or i.e.sonderby@medisin.uio.no

Note: All files including this instruction and helper-files are available at:
https://github.com/ENIGMA-git/ENIGMA-CNV

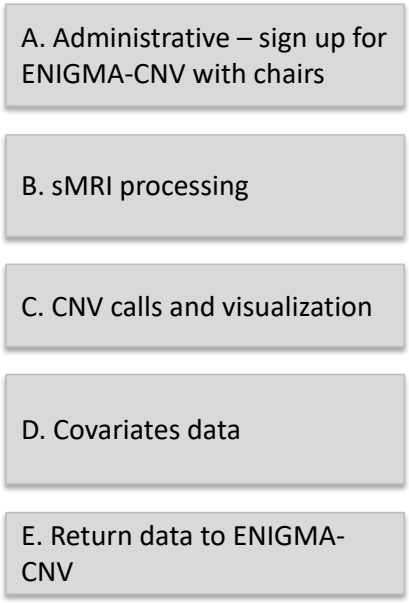

Figure 1: Overview, joining ENIGMA-CNV

Note: The ENIGMA-CNV working group is happy to help with both imaging processing and CNV calling and visualization if provided with raw data. Please contact us.

A. Administrative - sign up for ENIGMA-CNV

Please sign up for the ENIGMA-CNV e-mailing list to:
enigmacnvhelpdesk@gmail.com and i.e.sonderby@medisin.uio.no.

Please indicate this in your e-mail:

- cohort name
- names of individuals involved,
- e-mail addresses,
- roles [PI, main contact point, analysis of either imaging/CNV-data].

B. Structural imaging data processing

Please follow the protocol "ENIGMA-CNV, sMRI protocol, v2.0.pdf".

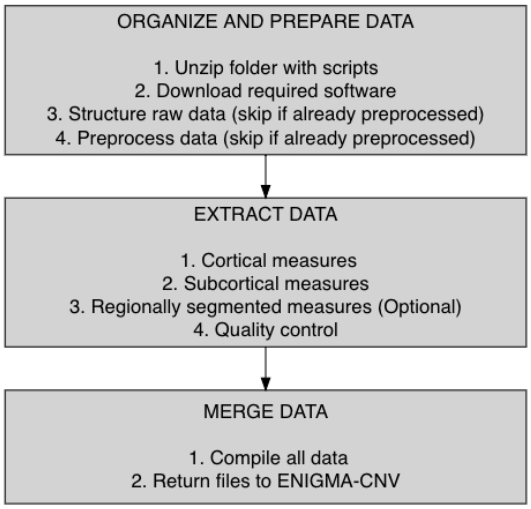

Figure 2: Overview, sMRI imaging data processing

C. CNVs - calling and visualization

Overview:
This part allows you to call raw CNVs and filter them with the commonly used CNV caller, PennCNV.
Likewise, all CNVs of Interest as well as CNVs >50 kb are visualized.

We advice that you skim through the entire protocol before starting to get an overview/not do repeated work.

Please address any questions to:
enigmacnvhelpdesk@gmail.com

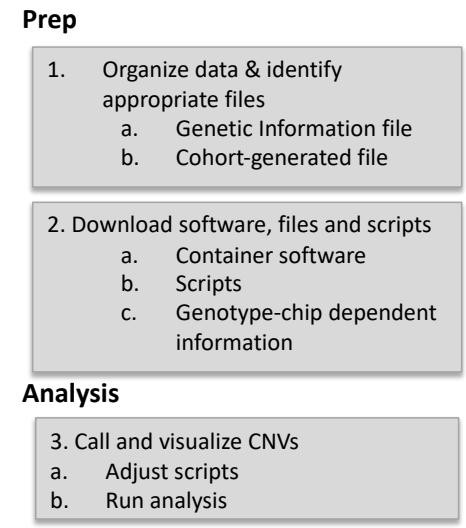

Figure 3: Overview, CNV processing

1. Organize your data and identify appropriate files for CNV calling

Create a folder called "ENIGMA-CNV_Analysis/" where you wish your output and software for the ENIGMA-CNV protocol to be. Henceforward referred to as "the Analysis-folder".

OBS – note that if you have data from more than one type of chip, please run the protocol separately for each different chip set

Please place all generated files in the Analysis-folder.

a. Genetic information files

CNVs are called based on data from genotyping chips:
Illumina: The Illumina Final Report or LRR/BAF-files
Affymetrix: CEL-files or LRR/BAF-files

Illumina - *IlluminaFinalReport.txt/LRR-BAF-files*

-the Illumina Final report file of your Dataset/samples with, as a minimum:

SNP Name Sample ID B Allele Freq Log R Ratio.

Note: Allele 1 - Forward (or Allele 1 - Top), Allele 2 - Forward (or Allele 2 - Top), X & Y are requested by e.g. the Psychiatric Genetics consortium for use in the iPattern CNV caller. They are not used for this protocol but may be relevant for other CNV endeavors. Overall, having Illumina idat files (for loading in Genomestudio) may come in handy if wanting to call CNVs with a different algorithm than PennCNV.

The Illumina report file can be generated directly from the BeadStudio project files, go to GenomeStudio, click the "Analysis" menu, select "Report", then select "Final

Report", then make sure to drag the "Log R Ratio" and "B Allele Freq" field from the "Available Fields" to "Displayed Fields" so that these two signal intensity measures are exported to the final report file. Removing all other fields like GType, GC score, X Raw etc from the "Displayed Fields" can speed up the process and in addition decreases file size.

Format of Illumina final report file:

[Header]
BSGT Version 3.2.23
Processing Date 10/31/2008 11:42 AM
Content sample.bpm
Num SNPs 45707
Total SNPs 45707
Num Samples 48
Total Samples 192
[Data]

SNP Name	Sample ID	B Allele Freq	Log R Ratio
rs1000000	KS2231000715	1.0000	-0.0558
rs1000002	KS2231000715	1.0000	-0.0422

Etc…

OBS – file may not contain the first 8 lines

Note – a previously extracted Illumina Final Report with (as minimum) the mentioned columns is also perfect.

Please place the *IlluminaFinalReport.txt*-file in the Analysis-folder.

Alternatively (to the *IlluminaFinalReport.txt*-file), LRR-BAF files produced with the PennCNV command '`split_illumina_report.pl`' can be used directly. This saves time and computation (see the script and adapt). Please note that if these files are present in several subfolders, the script might run into issues – please contact the helpdesk for help if this is the case.

Format of (tab-delimited) LRR-BAF file [Name: SubjID1]:

Name	SubjID1.Log R Ratio	SubjID1.B Allele Freq
rs1000000	-0.0038	0.0161
rs1000002	0.0073	0.9943
rs10000023	-0.0307	0.0026

etc…

Genetic information files – Affymetrix CEL files

Affymetrix CEL files acquires an additional step for conversion to LRR/BAF-files. Already generated LRR/BAF-files can be used directly.

If you need to convert Affymetrix CEL files to LRR-BAF-files, perform step 1 of the protocol at: http://penncnv.openbioinformatics.org/en/latest/user-guide/affy/. ENIGMA-CNV can provide a 'helper script' ("*ENIGMA-CNV_AffyPrep_Protocol_v2.sh*") for performing this step – please contact the helpdesk.

SNP Position File

For generation of the PFB-file (population frequency file, see later), you need to provide a SNP-position file.

The SNP-position-file is a tab-delimited file with the positions of the SNPs on your chip, containing at least these columns:

Name	Chromosome	Position
rs1000000	12	126890980
rs1000002	3	183635768
rs10000023	4	95733906

These are, for instance, present in the SNP-map-file (.map) generated together with your IlluminaReport or the Illumina manifest file [.bpm]. Note that the header needs to have the exact headlines above to be used.

The PFB-file is specific to genome-version. First wave of ENIGMA-CNV CNVs was based on the hg18 genome build. Most genotyping chips are now released with hg19 or hg38 coordinates. In order of preference, we now aim for: hg38, hg19 and hg18. You need to take note of the genome version of this file as this info is needed for CNV calling.

Please place the SNPPosition-file in the Analysis-folder.

b. Cohort-generated files

Sex - *SexFile.txt*

-please produce a tab-delimited file specifying SampleID (as in the lluminaFinalReport-file) and sex in the format:

SampleID	sex
SampleID1	female
SampleID2	male
etc…	

[please note that sex MUST be in the format "female" or "male"; if missing sex-info, just leave the individual out of the list]. This sex-info is also needed for the covariate-file below.
If sex is missing for some individuals, please note the number down in the CNV calling script.

Place the file in the Analysis-folder.

Individuals to remove - *RemoveFile.txt*

Please produce a text-file with the individuals you want to remove (if any) in the analysis.
Each row has the SampleID [and ONLY this ID] specified as in the IlluminaReportFile

SampleID
SampleID1
SampleID2

The *RemoveFile.txt* should contain individuals that you know are sample mixups or subjects that for instance withdrew from the study so that these are kept out of the analysis. Please note that these are the ONLY initial exclusion measures we wish you to apply as some CNVs are so low in frequency that we want to be as inclusive as possible.

We request that you keep both duplicates and related individuals (and indicate these in the duplication/relatedness file below) since these will be helpful in QC as well as in analysis.

Place the file in the Analysis-folder

Individuals to keep - *KeepFile.txt*

Needed if you have files without imaging in your IlluminaFinalReport-file (or for others reasons just want to keep some individuals in the analysis)
-produce a text-file with the individuals you want to keep in the analysis
Each row has the SampleID [and ONLY this ID] specified as in the IlluminaReportFile:

SampleID
SampleID1
SampleID2

Place the file in the Analysis-folder

Duplication/relatedness *DupsRelatives.txt*

Please produce a tab-delimited list of duplicate individuals and related individuals:

SampleID1	**SampleID2**	**PI_HAT**	**Relation**
SampleID10	SampleID24	0.5	Mother_child
etc…			

-where *SampleID1* and *SampleID2* are the related individuals, *PI_HAT* the proportion of the genomic variation shared IBD and *Relation* (if known or suspected - e.g. duplicate, sibling, parent/child, uncle/nephew, grandparent/grandchild, halfsibling, cousin if known). If Pihat is unknown, keep Pihat as "_", but instead indicate Relation. Please only include PI_HAT>0.125.
Pihat can be calculated with the plink –genome command (https://zzz.bwh.harvard.edu/plink/ibdibs.shtml).

Place the file in the Analysis-folder.

2. Download necessary software, files and scripts

Please place all scripts and files downloaded in the Analysis-folder.

a. Container software (independent of dataset)

To minimize the impact of differences in computer hardware and software between sites, we developed a containerized pipeline - all required software is included in this container and it runs independent of hardware.

We adopted two container softwares

1. Docker – for all systems on computer with internet access
b. Singularity – for unix only and for systems without internet-access

The software in the enigma-cnv container is:

- PennCNV v1.0.5, used for CNV calling
- R 3.3.1 + relevant R packages including iPsychCNV for visualization

1. Docker software

You first need to install Docker:
https://docs.docker.com/get-docker/
Please follow the instructions to download docker for your particular platform.

Docker container (*enigma-cnv???*) – download and install

After download of docker software, please download the enigma-cnv:latest container by writing in the terminal
`docker pull bayramalex/enigma-cnv`[1]

2. Singularity software

You first need to install Singularity (sylabs.io)[2] along with some required dependencies (if already installed on your system (v3.0 or later), proceed to b.).

Please follow the instructions on the page here:
https://sylabs.io/guides/3.0/user-guide/installation.html
to install Singularity version 3.0 or later (latest stable release at time of writing is v3.8.5.)[3]. If you need admin rights to install, please consult your IT representative.

Note that a Linux based operating system is required. For users without a Linux-system, there are a few alternatives (not recommended, see footnote[4]) - please consider using docker (above) instead. Likewise, if unable to install 3.0 due to an old machine, see footnote[5].

Note, storage required to install:
Singularity: ~140MiB disk space (once compiled and installed)
Container: 1.3 GB

Singularity container (*enigma-cnv.sif*[6]) – download and install

Download with singularity already installed

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[1] Also available here:
https://github.com/comorment/gwas/blob/main/containers/enigma-cnv/Dockerfile

[2] Singularity is an open-source program that allows the 'containerization' of software packages, their dependences and libraries to build entire pipelines that are cross-platform compatible.

[3] This may be a slightly more user-friendly instruction: https://singularity-tutorial.github.io/01-installation/

[4] A less (non-)supported MacOS/Windows solution does exist here: https://sylabs.io/guides/3.7/admin-guide/installation.html#installation-on-windows-or-mac. Please note that we do not recommend the latter - we did not test this and it may not be suitable to run all analysis defined. Alternatively, we recommend installing Linux via dual-boot or as a virtual machine.

[5] On some older systems and kernels, Singularity v3.x may not work. If that is the case you can install Singularity v2 – however, please note that the container has not been tested with V2 although it may work.

[6] If you wish to modify/expand the container, the docker can be found here:
https://github.com/comorment/gwas/blob/main/containers/enigma-cnv/Dockerfile

```
singularity build enigma-cnv.sif docker://bayramalex/enigma-cnv:latest
```
enigma-cnv.sif (the name of the container) will be downloaded to your $PWD.

Download without singularity installed
(e.g. if you need to import into a system without internet-access and need to download first):
https://github.com/ENIGMA-git/ENIGMA-CNV/blob/main/CNVCalling/containers/enigma-cnv.sif

Place the singularity container in the Analysis-folder.

b. Scripts for running analysis

Please download theses scripts from github:
https://github.com/ENIGMA-git/ENIGMA-CNV/CNVCalling/scripts/

ENIGMA_CNV_CNVProtocol_v2_singularity.sh or
ENIGMA_CNV_CNVProtocol_v2_docker.sh [choose either singularity or docker-script]
ENIGMA-CNV_visualize_v1.R
compile_pfb_new.pl

c. Genotyping-chip-dependent files

As mentioned, genetic files and data are specific to genome-version. First wave of ENIGMA-CNV was based on the hg18 genome build. Most genotyping chips are now released with hg38 coordinates. In order of preference, we now aim for: hg38, hg19 and hg18.

In addition to the correct genome version, appropriate files according to your genotyping chip must be used:
-*The PFB-file* (population frequency of B-allele file) - supplies the PFB information for each marker, and can give the chromosome coordinate information to PennCNV for CNV calling.
-*The GCMODEL file* - specifies the GC content of the 1Mb genomic region surrounding each marker (500kb each side) (calculated using the UCSC GC annotation file).
-*the HMM-file* - tells the program what would be the expected signal intensity values for different copy number state, and what is the expected transition probability for different copy number states (For more information, see here: http://penncnv.openbioinformatics.org/en/latest/user-guide/input/)

Generating your own PFB-file

For cohorts with more than 300 individuals with good quality genotyping data (for a suggestion good data, see footnote[7]), we advise that you create your own PFB-file and GC-model files – this procedure is part of the script.

[7] We suggest to follow the guidelines of the ENIGMA genetics imputation protocol, i.e:
-filter out individuals genotype call rate <0.95 (and SNPs with Minor Allele Frequency < 0.01 and Hardy--Weinberg Equilibrium < 1x10--6).

If you have less than 300 individuals in your cohort, you need to use a generic PFB- and GCmodel file. Please confer with the ENIGMA-CNV working group with enigmacnvhelpdesk@gmail.com for advice.

The HMM-file

There are several HMM-files:
hhall.hmm – can be used for all Illumina arrays (path, container: */opt/PennCNV-1.0.5/lib/*) except:
exome.hmm[8] – more appropriate for Illumina Infinium HumanExome BeadChip and Infinium HumanCoreExome-24
affygw6.hmm - Affymetrix Genome-Wide Human SNP Array 6.0 and SV (path, container: *(/opt/PennCNV-1.0.5/affy/libgw6/)*
exome.hmm needs to be downloaded from the ENIGMA-CNV github repository (Present at: https://github.com/ENIGMA-git/ENIGMA-CNV/CNVCalling/PFBGCMODELHMM/)– the others are present in the containers.

CNV of interest file based on genotyping chip

We visualize a selected set of CNVs - both with set and non-set boundaries.

For this we need one of the following files:
CNVsofInterest_ENIGMA-CNV_hg18.csv
CNVsofInterest_ENIGMA-CNV_hg19.csv
CNVsofInterest_ENIGMA-CNV_hg38.csv
This list of CNVs is compiled from the paper from UK biobank: Kendall et al., 2017 (doi: 10.1016/j.biopsych.2016.08.014)[9] with a few additions.
Present at: https://github.com/ENIGMA-git/ENIGMA-CNV/CNVCalling/CNVsofInterests/

Download according to your genome version and put in your Analysis-folder.

Filtering genomic regions files

Several genomic regions are known to harbor spurious CNV calls that might represent cell-line artifacts.

Therefore, we remove CNVs in certain genomic regions overlapping >50%:
-centromeric regions
-telomeric regions
-segmental duplication regions
-immunoglobulin regions

You can find the corresponding files here: https://github.com/ENIGMA-git/ENIGMA-CNV/CNVCalling/filtergenomeregions/, e.g.:

This can be done with plink, e.g.: plink ----bfile $datafileraw ----hwe 1e--6 ----geno 0.05 ----maf 0.01 ----noweb ----make--bed ----out ${datafileraw}_filtered). For reference, please see: the ENIGMA genetics imputation protocol, http://enigma.ini.usc.edu/protocols/genetics-protocols/.
[8] Derived from this article: Szatkiewicz et al (2013): Detecting large copy number variants using exome genotyping arrays in a large Swedish schizophrenia sample. Mol Psychiatry, 18(11):1178-84]
[9] Originally in hg19 – lifted over to hg18 and hg38 with UCSC liftover tool (https://genome.ucsc.edu/cgi-bin/hgLiftOver).

telo_hg38.txt
immune_hg38.txt
segmentaldups_hg38.txt
centro_hg38.txt

Download according to your genome version and put in your Analysis-folder.

3. Call and visualize CNVs

a. Adjust the script

a. Please rename the script *ENIGMA_CNV_CNVProtocol_v2.sh* to *${Dataset}_ENIGMA_CNV_CNVProtocol_v2.sh* [i.e. the name of your file with your dataset name [as stated below] as prefix]

b. edit the script by changing to your dataset-specific files as directed in section 0a [labeled "USER-INPUT needed] in the script.

d. Regarding deidentification – the protocol has an option to add an additional step of deidentification to the IDs – this leaves a key in the hands of the cohort submitting data. Adding this can give additional complications (if trying to track back to data). Please only tick this if required by your IRB-approvals.

b. Run the analysis

Run the ${Dataset}_ENIGMA_CNV_CNVProtocol_v2.sh script in the terminal:

```
bash ./${Dataset}_ENIGMA_CNV_CNVProtocol_v2.sh
```

After the run is done, please check the checklist found in ${Dataset}_visualize/${Dataset}_checklist.txt to see if things seem to make sense.

The CNV calling data is now ready for transfer (see E. below).

D. Covariate files

The covariate files are produced by manually constructing a covariate-file and obtaining ancestry covariates following the standard protocols of ENIGMA genetics.

General covariates

Please create a csv-file called *${Dataset]_Covar_ENIGMACNV.csv*

The table will look like this:

SubjID	GeneticID	PID	MID	DiseaseType	AffectionStatus	Affectionstatus2	Age	Sex	ScannerSite
Subj1	A2035	A1003	A6008	Population	0	Healthy_bipolar	30.0	male	Oslo2
Subj2	D1000	NA	NA	Population	1	Bipolar	35.5	female	Oslo2

(use excel or your favorite spreadsheet program saving as a csv-file)

General NOTES on the columns

- Missing values should be coded as NA.
- Please include individuals, even if they are missing one or two covariates (say AffectionStatus) – the data might still be valuable. Just fill out the missing values as NA.

Specific NOTES on the columns

- **SubjID**: Must match the SubjID in the imaging files.
- **GeneticID:** The ID used in the ENIGMA-CNV-calling protocol ("ENIGMA-CNV_CNVCalling_Protocol_final.sh") prior to de-identification. If this is the same as the SubjID, fill in the column accordingly.
- **Sex**: Must be coded as follows: Male="male", Female= "female".
  Note sex-info is also needed for the CNV-calling above.
- **DiseaseType**: Type of cohort.
  ENIGMA-CNV receives both population cohorts (or volunteer-cohorts) and disease-cohorts (e.g. epilepsy, psychiatric disease, dementia).
  - For *population-studies,* please note the best fitting term (copy to all fields):
    - Twinstudy
    - Population
    - Volunteers
    - Familystudy
  - For *case-controls-studies,* please note the best fitting term (copy to all fields):
    - E.g bipolar, schizophrenia, ADHD, autism, dementia, stroke, psychosis, epilepsy…
- **AffectionStatus**: a binary indicator where Patient = 1 & Control = 0. If your cohort does not have patients, code = 0.
- **AffectionStatus2**
  - For *population-studies*: please code as NA in all columns
  - For *case-controls-studies*:
    - Please code patients with
      - a standardized version of the diagnosis per individual such as DSM or ICD (We are particularly interested in 'Mental, behavioural and neurodevelopmental disorders', corresponding to ICD-10 F01-99 and 'Diseases of the nervous system, corresponding to ICD-10 G00-G99.)
      - If lacking such detailed information, SCZ (schizophrenia), BD (bipolar disorder), ADHD, dementia, stroke or the likes will suffice.
    - Please code controls as:
      - **Healthy_psych, Healthy_epilepsy**, **Healthy_dementia** (controls have been ascertained/screened for lack of e.g. psychiatric diseases, epilepsy or dementia)

- **ScannerSite:** The name of your scanner site.

OBS: Make sure the ScannerSite name in *Covar_ENIGMACNV.csv* correlate with that/those put into n *ScannerInfoSheet.xlxs* (see sMRI protocol).

If you have previously run the ENIGMA genetics association protocol, you can find the info on sex and age in: *SubCortCovs_nopatients.csv* [methodA], *SubCortCovs_havepatients.csv* [method B], *SubCortCovs_related_nopatients.csv* [method C]

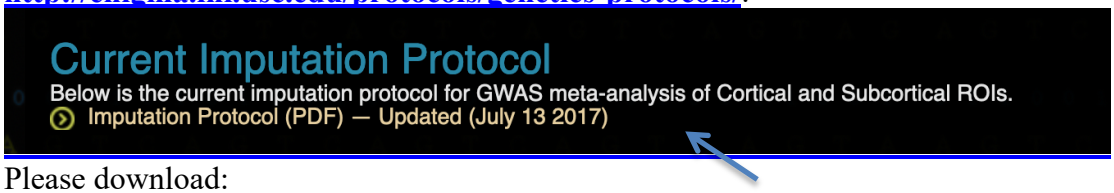
MDS-covariates

MDS-covariate-file: *HM3_b37mds2R.mds.csv* (version July 13, 2017), alternatively *HM3mds2R.mds.csv (*version July 27, 2012).

This file contains covariates for the ancestry of each subject in your cohort.

If you previously participated in ENIGMA-genetics (and followed the protocols of ENIGMA), you have this file already.

If not, go to the ENIGMA-imaging-protocol-homepage:
http://enigma.ini.usc.edu/protocols/genetics-protocols/:



Please download:
"Imputation Protocol (PDF) – Updated (July 13, 2017)".
Or use this link:
https://enigma.ini.usc.edu/wp-content/uploads/2020/02/ENIGMA-1KGP_p3v5-Cookbook_20170713.pdf

OBS: To obtain MDS-covariates, you only need to run the "Multi-dimensional Scaling (MDS) Protocol" part of the code in the ENIGMA imputation protocol, NOT the imputation part.

The output file, *HM3_b37mds2R.mds.csv*, is a spreadsheet containing the following columns:
Family ID (FID), individual ID (IID), 4 MDS components (C1, C2, C3 and C4), and PLINK's assigned solution code (SOL). If you have more MDS components available, please provide us with up to 20.

FID	IID	SOL	C1	C2	C3	C4
Fam1	Subj1	…	…	…	…	…
Fam2	Subj2	…	…	…	…	…

OBS: Please confirm that either the FID OR the IID in MDS file match the GeneticID or SubjID in the *${Dataset]_Covar_ENIGMACNV.csv*-file.

E. Return data to ENIGMA-CNV

Files to transfer:
${Dataset}_visualize.tar.gz [CNV-calling data]
${Dataset}_sMRI.tar.gz [sMRI calling data]
HM3_b37mds2R.mds.csv [MDS-data]
${Dataset]_Covar_ENIGMACNV.csv [covariates]

Please send the data to enigmacnvhelpdesk@gmail.com or request a secure transfer-link.

Data storage

The received data will be stored at the secure server TSD (https://www.uio.no/english/services/it/research/sensitive-data/) at University of Oslo in Oslo, Norway. Only registered users have access.