Supplementary methods

Sequencing

UZ Gent: The MagCore Automated Nucleic Acid Extractor (RBC Bioscience, New Taipei City, Taiwan) was used for extracting genomic DNA from blood treated with Ethylenediaminetetraacetic acid (EDTA). Genomic DNA was enriched with different methods/panels over time: BeGECSv1 panel was captured with a custom SureSelectXT Low Input kit (design ID S3225453; Agilent Technologies), BeGECSv2 panel was first captured with a combination of HyperExome v1 probes and HyperChoice custom probes (design ID IRN1000045815) (1:1) (Roche Diagnostics, Basel, Swiss) and later on, the HyperExome v2 probes were used. On each sample, genotyping of single-nucleotide variants (SNPs) was performed using the Human Sample ID Kit (Pxlence, Ghent, Belgium) to prevent sample mix-up. Each library was quantified using the Fluoroskan microplate fluorometer (Thermo Scientific, Waltham, MA, USA), followed by pooling and quantification of the final pool by quantitative PCR (qPCR) using the LightCycler 480 system (Roche Diagnostics, Basel, Swiss), and subsequent sequencing using massively parallel sequencing (MPS) technology on the NovaSeq 6000 or NovaSeq X Plus platform (Illumina, San Diego, CA, USA). At least 90% of the targeted genes had a minimum coverage of 20x. (Likely) pathogenic variants with low quality scores are confirmed using an independent method: PCR amplification followed by Sanger sequencing.

UZ Leuven: The analysis was performed by massively parallel sequencing (MPS) of an exome panel (Exome CustomV2; Twist Bioscience) on NovaSeq 6000 or NovaSeq X Plus (Illumina).

H.U.B: Genomic DNA (extracted by Maxwell, Promega) was enriched with different methods/panels over time: BeGECSv1 panel (1234 genes) was first captured by SeqCap EZ Choice XL Nimblegen Roche (référence interne: V6) followed by sequencing on a Novaseq 6000 platform (Illumina, San Diego, CA). BeGECSv1 panel (1234 genes) was then captured by Twist Comprehensive Exome Panel (TWIST Bioscience, USA) followed by sequencing on a Novaseq 6000 platform (Illumina, San Diego, CA). BeGECSv2 panel (1703 genes), is captured by Twist Human Exome 2.0 Plus Comprehensive Exome Spike-in to which a custom panel of probes was added to cover additional or poorly covered regions

(Twist Bioscience), followed by sequencing on a Novaseq X Plus. At least 90% of the targeted genes had a minimum coverage of 30×.

Multiplex Ligation-dependent Probe Amplification (MLPA) for DMD, SMN, and CYP21A2.

For CYP21A2 analysis, SALSA MLPA P050-D1 kit (MRC-Holland, Amsterdam, the Netherlands) was used to detect exon-level deletions and duplications. For SMN analysis, the SALSA MLPA P021 SMA kit (MRC-Holland, Amsterdam, the Netherlands) was used, to determine copy number variations in the SMN1 and SMN2 genes with exception for H.U.B as they perform Real-time PCR analysis of the copy number of exon 7 of the SMN1 gene (Zentech “Targeted qPCR SMA” kit). For DMD, the SALSA MLPA P034 (DMD mix 1) and P035 (DMD mix 2) probemixes (MRC-Holland, Amsterdam, the Netherlands) were used. MLPA analyses for SMN, CYP21A2, and DMD were performed exclusively on female samples; when the female was identified as a carrier for SMA or CYP21A2, the partner’s sample was subsequently analysed. For CYP21A2, analysis of the partner consisted of MLPA and allele-specific PCR with sequence analysis.

Reporting policy CYP21A2

In May 2023, the BeGECS working group decided to report only the most frequently observed pathogenic CYP21A2 variants associated with classical CAH: the intron 2 splice mutation, 8 bp deletion in exon 3, p.(Ile173Asn), exon 6 mutation cluster, p.(Leu308Phefs6), p.(Gln319Ter), and p.(Arg357Trp), as described by Baumgartner-Parzer et al. (32).

Multiplex-PCR for GJB6

Two recurrent deletions in the GJB6 gene (232 kb and 309 kb) were assessed using an allele-specific multiplex PCR assay with primers published in Del Castillo et al. 2005, J Med Genetics 42:588-894, followed by sizing of the PCF fragments by microchip-elektrophoresis.

Repeat-primed PCR for FMR1

CGG trinucleotide repeat expansions in the 5′ untranslated region (5′ UTR) of the FMR1 gene were detected using a PCR-based assay using the AmplideX® PCR/CE FMR1 kit (Asuragen, Inc., Austin, TX,

USA). Intermediate alleles, defined as 45–54 CGG repeats, were previously included in the screening report; however, following a 2025 working group decision, these alleles are no longer routinely reported due to their limited clinical significance. Abnormal results were classified as premutation alleles (55–200 repeats) or full mutations (>200 repeats). When indicated by the repeat size (<70 repeats), AGG interruptions were analysed to refine the estimated risk of further expansion.

CFTR

To assess cystic fibrosis risk, the poly-T tract and adjacent TG repeats in intron 9 of the CFTR gene were analysed, as these elements influence exon 10 splicing and can result in non-functional mRNA and reduced protein expression.

Reporting policy CFTR

In the absence of the R117H variant in cis, only the TG(12–13)T5 allele—when in trans with a pathogenic variant—has been associated with cystic fibrosis, and only in rare cases (33). Consequently, as of 21 December 2023, the BeGECS working group no longer reports the TG11T5 allele when R117H is not present in cis.

Variant filtering, interpretation, and reporting

UZ Gent: The first 444 couples were tested with the initial version of the panel, which included 1,248 genes; thereafter, an updated panel comprising 1,704 genes was used (version 2). A biallelic filter was not applied initially but was subsequently implemented because of its advantages. Three variant filtering strategies were implemented, differing in allele frequency thresholds, variant impact categories (low, medium, high), and the application of the bi-allelic filter. Variant impact was interpreted using the Variant Effect Predictor (VEP) and GEMINI. For the nine AR genes with the highest population allele frequencies, a maximum allele frequency of 0.15 was applied without a bi-allelic filter. For the remaining AR genes, a threshold of 0.05 was applied, with the bi-allelic filter used for medium- and high-impact variants, and for all variants if a known pathogenic low-impact variant was present in the gene. For XL recessive genes, a threshold of 0.02 was applied without a bi-allelic filter. Variant

classification was performed according to ACMG guidelines using an in-house developed tool, and only likely pathogenic and pathogenic variants (classes 4 and 5) were reported.

UZ Leuven: Variant annotation was performed using RefSeq v214, genome build GRCh38 (gcap_22_10), and Alissa Interpret (v5.4.1; Agilent) with a version-specific configuration of the available annotation sources. Variant interpretation was performed in accordance with the ACMG guidelines [Richards et al., 2015, Genetics in Medicine, 17(5)]. Following couple analysis only carrier statuses meeting specific criteria were reported: genes associated with an autosomal recessive disorder in which both partners are carriers of a variant, or genes associated with an X-linked disorder in which the female partner is a carrier. In both cases, only likely pathogenic (class 4) and pathogenic (class 5) variants were reported.

H.U.B: The first 52 couples were tested with the initial version of the panel BeGECSv1; thereafter, they were analysed with panel BeGECSv2. The variants are interpreted using the Highlander software (http://sites.uclouvain.be/highlander/). Differents filters were used: a bi-allelic filter, an XL genes filter and specific filters for RNA and genes know to have variants at higher frequency in the population. The interpretation takes into account the frequency of variants in the general population, their presence in patient databases, their putative effect on the protein (according to prediction software) and literature data. Only likely pathogenic and pathogenic variants (classes 4 and 5) were reported.