

# Approaches to homozygosity mapping and exome sequencing for the identification of novel types of CDG

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**Abstract** In the past decade, the identification of most genes involved in Congenital Disorders of Glycosylation (CDG) (type I) was achieved by a combination of biochemical, cell biological and glycobiochemical investigations. This has been truly successful for CDG-I, because the candidate genes could be selected on the basis of the homology of the synthetic pathway of the dolichol linked oligosaccharide in human and yeast. On the contrary, only a few CDG-II defects were elucidated, be it that some of the discoveries represent wonderful breakthroughs, like *e.g.* the identification of the COG defects. In general, many rare genetic defects have been identified by positional cloning. However, only a few types of CDG have effectively been elucidated by linkage analysis and so-called reverse genetics. The reason is that the families were relatively small and could—except for CDG-PMM2—not be pooled for analysis. Hence, a large number of CDG cases has long remained unsolved because the search for the culprit gene was very laborious, due to the heterogeneous phenotype and the myriad of candidate defects. This has changed when homozygosity mapping came of age, because it could be applied to small (consanguineous) families. Many novel CDG genes have been discovered in this way. But the best has yet to come: what we are currently witnessing, is an explosion of novel CDG defects, thanks to exome sequencing:

seven novel types were published over a period of only two years. It is expected that exome sequencing will soon become a diagnostic tool, that will continuously uncover new facets of this fascinating group of diseases.

**Keywords** Congenital disorders of glycosylation · Genetic testing · Disease identification · Homozygosity mapping · Autozygosity · Exome sequencing · Next generation sequencing · Time line

## Introduction

The massive parallel sequencing or next generation sequencing (NGS) technologies are revolutionizing genetic research and medicine in general, and accelerating the discovery of novel genetic defects in rare diseases in particular. The Congenital Disorders of Glycosylation (CDG) are a group of diseases that will greatly benefit from the use of these technologies. Actually, we are witnessing the process, as the first successful applications of exome sequencing in this field have already been published.

In this contribution, we will very briefly introduce the different genomic approaches and refer to publications that describe the techniques in much more detail. At the same time, we go back and compare the present achievements in genetics with those from the past—all on CDG, of course.

For research, it is quite obvious: the more patients we can ‘exome sequence’, the more cases we will solve. Interestingly, very soon one would not need specific skills to analyze exomes, this will become a piece of cake. Is this true? Maybe, but we would like to highlight some restrictions.

A major issue, observed by ourselves but described already by others, is the use of NGS for diagnostics. CDG is a perfect sample for the use of these technologies in offering ‘one-stop’ testing. However, it is today still not easy to

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choose between specific targeted enrichment approaches for (known) gene panels or whole exome sequencing.

### The technology in brief

Several techniques for massive parallel sequencing of DNA molecules have been developed in recent years [1, 2]. The earliest commercial platform was the Roche 454 or Genome Sequencer (GS) FLX machine, that uses micro bead technology and the pyrosequencing chemistry to sequence molecules in hundreds of thousands of microscopic wells in a 2.5×3 inch glass plate. The machine has the advantage of generating relatively long sequence reads (up to 1000 bp) but from a genomic point of view, the capacity of the machine is limited. Sequencing a human genome or exome on this platform is too costly, but the GS-FLX as well as the GS Junior platform offer possibilities for a dedicated (diagnostic) approach on (small) gene panels.

The other commonly available platforms generate short reads (up to 100 bp). The sequence is generated at a much lower cost per base. Given that the sequence capacity of these platforms is huge, they are best suited for exome sequencing (and other molecular applications, of course). The Illumina™ Genome Analyzer and HiSeq (and their little brother, the MiSeq) belong to this group, as well as the Life Technologies™ Solid™ series.

Novel machines, like the Ion Torrent's PGM™ or Proton™ Sequencers (commercialized by Life Technologies™), have to be situated somewhere in between. They are (relatively) low cost machines, but the capacity is lower than on the above-mentioned high-throughput machines. Other, 'single molecule sequencing' platforms have been proposed by Helicos (HeliScope™) and Pacific Biosciences™ (PACBIO RS), which again feature specific advantages and disadvantages. Currently, very few research or genetic centres have such machines. So we focus on the hype of the moment, which is sequence capture and exome sequencing, as these applications are run on the commonly available machines.

Needless to say that the technology—as well as the costs—are changing very rapidly, and a review cannot be up to date. So we will stick to the principles instead.

For massive parallel sequencing, the genomic DNA is fragmented (sonicated) into small pieces (up to a few hundred base pairs). Adapters and sequencing primers are ligated to the ends of the fragments and fragments containing adapters are enriched by PCR. Templates are then attached to either microscopic beads or to the flow cell where they will be clonally amplified and sequenced. The specific treatment of the samples depends on the sequencing platform. Also, the (individual patient) samples receive unique sequence tags for identification and retrieval after the parallel processing. For targeted sequencing, the regions of interest are isolated from the rest of

the genomic fragments by hybridization of the DNA fragments to oligonucleotides either linked to microchips (array based hybridization) or magnetic beads (solution based hybridization). After stringent hybridization washes, the captured DNA fragments are eluted and sequenced. Three major exome sequencing platforms are currently available, from Agilent Technologies, Illumina™ and Nimblegen (Roche) [3]. Both the density of their probes and the regions they target differ. Nimblegen uses overlapping baits and covers miRNAs better than the other two platforms. Agilent uses adjacent baits and more extensively covers the Ensembl genes, whilst Illumina uses non-adjacent baits and better covers untranslated regions (UTRs).

More important—and cumbersome for the time being—are the data processing and analysis. First, the raw data are processed to sequence reads. This process includes quality measures, to remove 'bad' sequences. For mutation detection, it is important to watch this process: a thorough cleaning of the sequences may remove specific mutations (*e.g.*, deletions of several base pairs), as was exemplified in the early days of GS FLX sequencing (personal observation). Second, the reads are aligned to a reference genome. Several alignment tools exist, and this process is very well established now; it is not a major hurdle except that it takes tremendous computer power and time for the calculations. The result is a large file with the mapped sequences. Third comes the variant calling and filtering. This is a delicate step. Again, based on quality criteria, variants will either be included or discarded. The latter would be sequencing artifacts that typically occur in a small proportion of overlapping sequence reads. It is clear that this process has to be finely tuned, and has to be tailored to the sequencing platform, to avoid both false positive and false negative results. The fourth step is the bioinformatic annotation of the variants. The software will identify the individual variants, and consult databases to correctly annotate them. In this process, sequence variants are translated to protein variants (*i.e.*, mutations) and frequency data from dbSNP, the 1000 Genome Project (1000GP) and other databases are collected.

Finally, a biological interpretation of the mutations is necessary [4]. This is where the scientist gets back into business: the identification of a causal mutation is based on predictions of the pathogenic nature of a mutation, as well as on the possible implication of the gene in the phenotype, and of course on the genetic fit with the disease. Clearly, at this stage, one has to apply filters in different ways, depending on the expected inheritance pattern of the disease (autosomal dominant, recessive, X-linked or de novo) and the family structure. It is also at this stage that several patients and families can be combined, on the hypothesis that they share the same disease.

Specifically for recessive diseases—which constitute the largest group amongst the CDG [5]—one would first focus on homozygous mutations if there is evidence for consanguinity

in the family; if not, compound heterozygous mutations would be equally of interest. The availability of parents and affected or unaffected siblings increases the efficacy of this process, but evidently also increases the sequencing cost. Importantly, even for a rare recessive disease, one should not exclude a variant because it is present in population databases like dbSNP and 1000GP. Ku and coworkers have rightly underlined that the presence of a given variant in 1 of 500 control genomes is compatible with a recessive phenotype with an incidence of 1 in a million live births ( $500 \times 500 \times 4$ ) [6]. We have made such observations when dealing with novel CDG genes (unpublished data). *Vice versa*, perfectly harmless polymorphisms can be unique, so the fact that a variant has not been reported, does not mean it is causative.

### The power of exome sequencing

Clearly, this technology offers the chance to identify a novel disease gene in a single patient or family (see [6–12] for seminal papers and reviews). There are a lot of examples already in the literature (a few of them, relating to CDG, are given below). However, in real life, success is not guaranteed. Three major limitations apply to exome sequencing, obviously: 1. The target is limited to exons (and the flanking intronic sequences), thus deep intronic mutations cannot be identified, while (single or multiple) exon deletions would also be missed. 2. Not all exons are successfully targeted, actually some 5 to 10 % (depending on the arrays and the sequence depth) will not be recovered (this is a technical feature, relating to the GC content and other physico-chemical properties that interfere with successful hybridization and sequencing). 3. Exome capture is based on the current annotation of the human genome, and some genes may still be missing. So your gene of interest may, unfortunately, not be there.

In order to illustrate this, we calculated the coverage of 65 genes (representing 983 exons), definitely or possibly related to CDG, in 26 exomes. The data that were used, were derived from a series in which we used one specific exome sequencing kit (Nimblegen exome capturing kit version 2) for sequencing on one of the main platforms (Illumina sequencing on a HiSeq2000) with a mean coverage of 50–80X. Almost 80 % of the exons were completely covered across the 26 exomes while 13 % of the exons were missing in all 26 exomes. The remaining 7 % of exons were partly covered. Generally either the first or last exons were missing, as illustrated in Fig. 1. Hence, specific exon or gene coverage is not always good when doing exome sequencing even when the mean coverage is high. It is important to mention that an accurate SNP detection relies, among other factors, on the coverage. Unfortunately, we do not (yet) have targeted capture arrays to make coverage comparisons. Nevertheless, exome sequencing is a great research tool.

### Great benefit for research

It is beyond the scope of this article to give an overview of all the possible applications of next generation sequencing, or to list all achievements of whole exome or genome sequencing. But what has been accomplished so far in the field of CDG may well serve to illustrate the power of the novel tools.

The first reported exome in CDG led to the identification of the *PIGV* gene [13]. The interesting feature in this paper is that the investigators have combined old and novel principles: the exome was sequenced, but the data was interrogated with homozygosity as the tool to reduce the number of variants and identify the causal gene. A prime example of the fact that CDG genes will not—or no longer—exclusively emerge from dedicated CDG research, is the identification of the *DHDDS* gene, responsible for a recessive form of retinitis pigmentosa [14, 15]. SRD5A-CDG is an example of the concurrent identification of novel defects through cell biological and genomic approaches. The genetic defect, first published by Cantagrel *et al.* [16] using a successful mapping approach, was (re)discovered by Kahrizi *et al.* [17] through exome sequencing. This will happen increasingly, and we have run into similar situations [18]. It is an argument in favor of the use of targeted assays before transferring samples into the genomic research pool.

The most striking example of the power of these novel technologies is in the work of Ropers and co-workers [19]. Among 50 unselected cases (families) with intellectual disability (ID) that were ‘solved’ using autozygosity mapping and/or exome sequencing, two happen to be CDG cases: one has a *PMM2* deficiency (and must thus definitely show other clinical features related to *PMM2*-CDG, apart from the ID), and the other revealed a *MAN1B1* defect, a novel type of CDG [20]. The great advantage is that, indeed, single cases can be solved without any prior clinical or biological work-up. However, the authors of this publication rightly point out that an approach like this could lead to the wrong hit, *i.e.*, a gene or mutation not causally related to the disease. This would of course dramatically jeopardize carrier testing and prenatal diagnosis in such a family. This observation calls for prudence and for a clear distinction between research and diagnostics (see below).

Nevertheless, the evolution is clear: NGS applications will boost the discovery of novel CDG genes in the next few years. It will allow the identification of glycosylation defects without any background knowledge of the pathophysiology. This is not novel though: it is a feature of linkage analysis as well. For instance, the latter led to the identification of defects in subunits of the OST complex that would have escaped any direct approach [21, 22].

The question is to which extent we only read about the lucky strikes. A number of cases will remain unsolved, and

we will probably only solve them at the end of a long road. The challenge will be to solve every single case. In our first series of exomes, we identified mutations in a known CDG gene in 3 families, mutations in novel candidate genes in 2 families and no apparent causative defect in 5 families (unpublished data). We believe that the different approaches of positional cloning, homozygosity mapping and total genome sequencing will have to be combined to solve the most elusive cases.

Interestingly, by collecting genomic data from different patients into a larger database, we may also identify bigenic or multigenic causes of the disease. Those have been particularly hard to tackle with classical genetics. So far, we have not seen any such cases in CDG, but we believe they must exist. Modifier genes may also be identified and they may be potential targets for supportive therapies.

### The history—and the future—of genetics in CDG

The power of genetics lies in the fact that nothing needs to be known about the pathophysiology of the disease for the identification of a novel genetic defect. Hence, this is the ideal way to identify completely new aspects of the disease, well beyond the glycosylation pathway itself. It is a feature that is intrinsic to the current genomic approaches, as well.

In Fig. 2, a schematic overview is given of the ‘genetic’ versus ‘biochemical’ achievements in CDG. By way of illustration, we went from the original linkage analysis approaches—and successes—to the latest NGS breakthroughs. NGS actually represents a third wave of genetic achievements in disease gene discovery.

The first wave had been heralded by the use of positional cloning for the identification of candidate genes. This has worked essentially only once for the typical CDG, for the mapping and cloning of the *PMM2* gene [23, 24]. The reason is that either very large families (which is unlikely for recessive diseases) or many small families with patients with the same disease are necessary for this type of analysis. Hence, strikingly little has been achieved—genetically speaking—for the identification of defects of N-glycosylation through linkage analysis between 1996 and 2010. The 20 or so novel defects of N-glycosylation that were discovered between these dates, have repeatedly emerged from biochemical, glycobiological and cell biological research, which was incredibly effective in pinpointing the candidate genes, that were eventually sequenced. This series of discoveries includes fantastic breakthroughs, like the systematic elucidation of the defects in the assembly of the N-glycans in the endoplasmic reticulum—purely based on the conservation of the LLO pathway between humans and yeast [25–39], the discovery of the first defect in

**Fig. 1** Proportion of exons covered at 2, 10, 20, 30 and 50 X for four CDG genes, illustrating the power and limitations of exome sequencing. For the *ALG8* gene (*top panel*): all the exons are 100 % covered. For the *SRD53A* gene (*second panel*): all exons are 100 % covered, except the first and last ones. For the *ALG1* gene (*third panel*): sequence data are missing for exons 8 and 10 whilst the first and last exons are very poorly covered. For the *FUT1* gene (*bottom panel*): exons 8 and 10 are poorly covered. Proportions of exon coverage were averaged over 26 individuals whose exome was captured with the Nimblegen exome capturing kit and sequenced on HiSeq2000 using paired-end sequencing (reads of 76 bp)

the dolichol synthesis [40], the sophisticated identification of the COG defects [41–46], etc. The identification of the causative gene in Peters Plus syndrome was a lucky, genetic strike: the patient carried a large deletion on one allele, encompassing the *B3GALTL* gene and detectable by genomic arrays (comparative genome hybridization or CGH) [47].

When homozygosity and autozygosity mapping became accessible and affordable, largely thanks to the availability of genotyping arrays, novel genes were identified based on this approach. Autozygosity refers to the state of a genetic variation in which the two alleles in an individual are homozygous as a result of being inherited from a common ancestor. Basically, small consanguineous families could now be used to identify regions of homozygosity (autozygosity), which would then harbor the candidate genes. The *ATP6V0A2* defect has been identified using this approach [48], as well as a dozen other novel N- and/or O-glycosylation defects (Fig. 2).

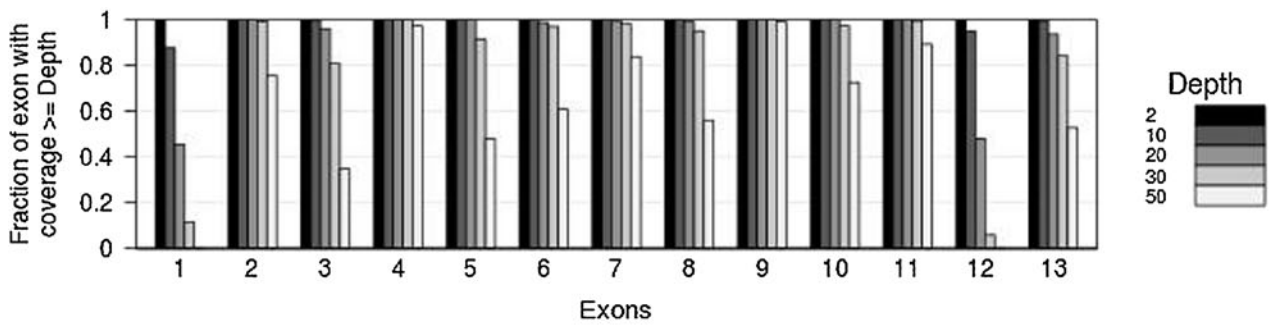
Interestingly, one study illustrates the power of the latter approach, and instantly also underlines one of the limitations of exome sequencing. In a small nuclear family with 2 affected siblings, a deep intronic mutation in the *TMEM165* (*TPARL*) gene was identified through a combination of homozygosity mapping and expression profiling [49]. Such a defect can never be identified through exome sequencing.

We anticipate that the search for novel types of CDG will become increasingly competitive, as was exemplified by the *DHDDS* publications that were published almost back to back [14, 15]. Clearly, the field will no longer be exclusive to research groups with an experience in CDG. Anyone can now reveal a novel CDG gene, solely on the basis of an exome sequence. However, as was pointed out by Ku *et al.*: “a definitive genetic diagnosis cannot be established solely on the basis of a newly identified mutation; further screening of additional cases is invariably required” [6]. We agree but would like to slightly amend the statement: a substantial biochemical and cell biological analysis of the genetic deficiency could also convincingly make the case. Hence, we look forward to the publication of beautiful examples of neatly elaborated, novel types of CDG.

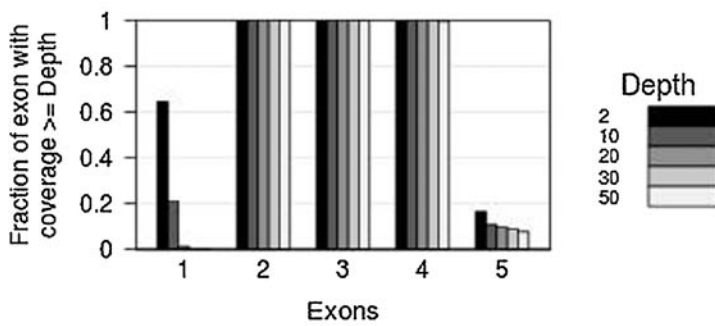
Here, we have duty as members of a network on CDG. The DNA samples of unsolved CDG-I and CDG-II patients (CDG-Ix and CDG-IIx) that have been collected over the years, have to be tested for the novel genes on each new hit.



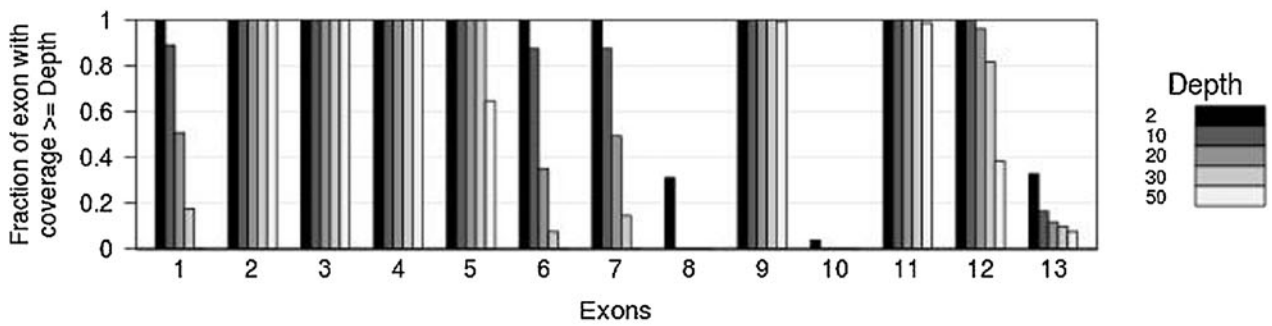
**ALG8\_NM\_024079**  
**chr11 Strand - 77811987 - 77850699**



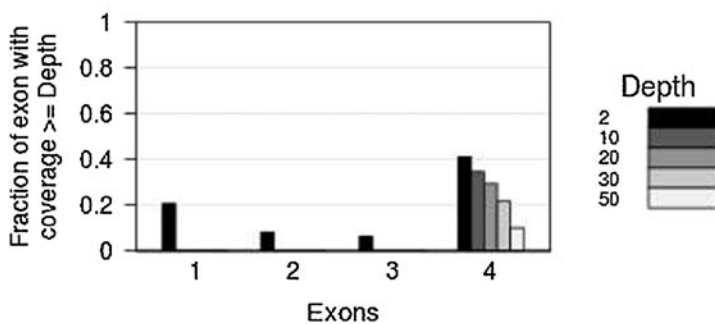
**SRD5A3\_NM\_024592**  
**chr4 Strand + 56212387 - 56239266**



**ALG1\_NM\_019109**  
**chr16 Strand + 5121809 - 5137380**



**FUT1\_NM\_000148**  
**chr19 Strand - 49251267 - 49258647**



This is a prior commitment that now has become a practical challenge.

### The blurred border between diagnostics and research

Massive parallel sequencing offers unique tools for the identification of the genetic defect in unsolved cases [6, 7, 50]. Also in our hands, exome sequencing has allowed us to solve old cases. Is this diagnostics or is this research?

Diagnostics differs from research in several ways. There are technical challenges to make the assays perform reproducibly for each new batch of patients. Because all the tools and kits that are currently provided are ‘research grade’, the assays have to be validated extensively before they are used in clinical (accredited) laboratories. This extends to the software for analysis and interpretation of the results. Not much of this is in order, currently.

For diagnostics, the general discussion for the moment is whether to go for targeted assays or to offer exome sequencing. There are pros and cons for both.

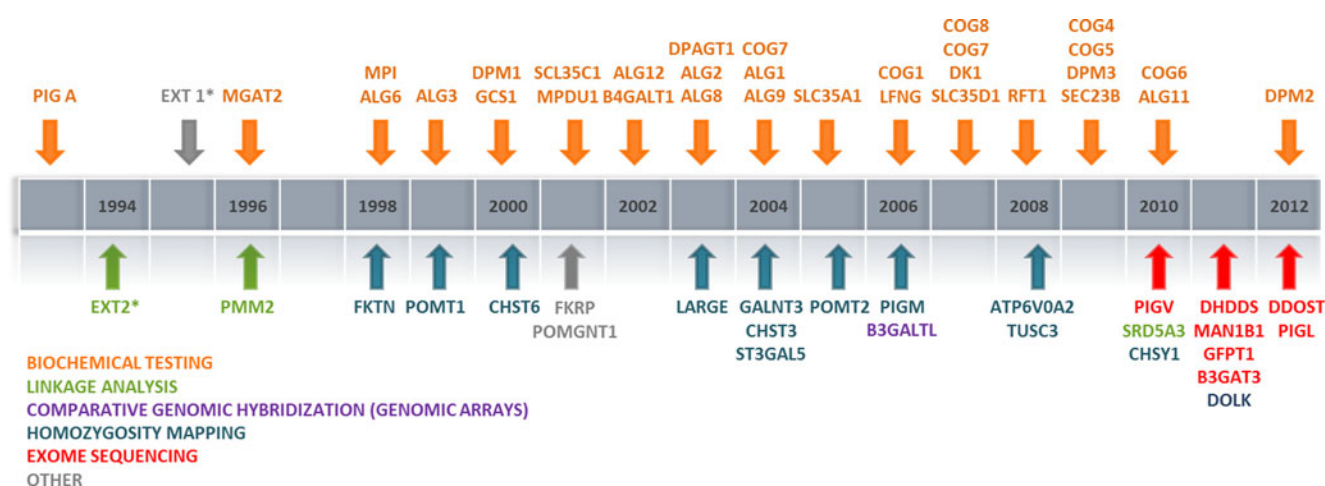
Jones *et al.* described a targeted approach for the rapid identification of (known) genetic defects in CDG patients [51]. This is a valuable approach that will speed up the diagnosis in a significant proportion of the CDG patients. Advantages are speed and high coverage of the target sequence, and relatively low cost. However, the same datasets could be obtained by exome sequencing in combination with filtering of the data for exactly those genes. An advantage is that the rest of the exome can be scanned if no causal defect is found in the selected genes. This is where the border between diagnostics and research would (again) be crossed. Disadvantages of the exome approach are the

higher cost (although the cost of an exome is constantly decreasing, and may become as low as 500 EUR very soon) and the lower coverage of the target.

Actually, we believe that the CDG genes will probably be included in laboratory developed or commercial panels for intellectual disability (ID) testing. Hence, they will become part of large gene panels or gene sets that will be offered to screen patients with ID. The driver behind the development of such panels is the will to confirm the molecular diagnosis in all ID cases at the earliest possible age, while at the same time to deal with the extreme genetic heterogeneity of ID—which surpasses the genetic heterogeneity of CDG, even if CDG increasingly contributes to it.

### Practical and ethical issues

As research gets closer to diagnostics—and *vice versa*—three major features arise. First, patients and families have to be made aware about the thin line between those two. This situation may not be very novel, as CDG patients’ cells and DNA have already been previously included in research projects whereby clinically relevant results were reported back to the clinicians and the families. However, it might generally become a new standard now. Second, total exome approaches may also identify genetic defects that are not related to the phenotype and clinical request of the patient. These so-called ‘incidental findings’ may *e.g.* include the detection of mutations in known cancer-predisposing or late-onset neurological diseases. This is the major hurdle for moving from targeted assays to total exome or total genome approaches. Hence, the issue has to be discussed with the parents, prior to the analysis. Third, the informed



**Fig. 2** Discovery of CDG-genes by different approaches over time. Top row (orange arrows): genes identified by a biochemical approach. Bottom row (green, blue and red arrows): genes identified by genetic and genomic approaches. The years are included in the bar. All types of

CDG are recessive, except the ones indicated by the asterisk. The arrows and references correspond to the first publication (even if for some genes, different publications are available). [13–17, 19–48, 53–76]

consent is important. Actually, it is the only way to adequately deal with the first two features: the informed consent has to be taken and needs to indicate that the above issues have been discussed; it needs to stipulate that the parents have chosen to or not to hear about incidental findings. The parents also have to agree that the exome will only be scrutinized for CDG related variants, and that other clinically relevant variants will not be actively sought. This means that only ‘incidental findings’ that are easily revealed during the analysis should be reported, and that the laboratory or clinician cannot be sued afterwards for not having made a comprehensive analysis of features that did not relate to the clinical request, *i.e.*, to the question: which type of CDG or developmental disorder has this child?

The fact that research is getting closer to diagnostics may have one advantage. It happens—unfortunately—that novel gene defects and mutations that were detected in specific patients are published in literature without the families being informed. This would certainly occur when the research is performed on anonymized samples, whereby the link between patients and results is lost. In our CDG research, and within EUROGLYCANET [52], we have always followed a policy in which research data were returned to the referring clinicians and hence to the families, through the network and after clinical confirmation of the results. The latter should be maintained.

Can data from research be used for diagnostics? We have dealt with this above. Can ‘diagnostic exomes’ be transferred back to research. It seems obvious, but it is not. Again, the informed consent has to be such that the clinicians and families are aware that, at the end of a diagnostic phase, the samples may become the subject of research again.

## Conclusion

The NGS applications have created enormous possibilities for the identification of novel types of CDG. The greatest impact will be on the clinical diagnosis and the medical care for rare disease patients in general and CDG patients in particular [50]. Very soon, we will have tens of new genes. We will enter a phase where we will have to spend more time on basic research into the pathogenesis of those defects, than on the gene identification per se. But in the meantime, let’s ride the NGS wave as high as we can for the benefit of the patients, and for the benefit of science.

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