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Management of Incidental Findings in the Era of Next-generation Sequencing

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Abstract: Next-generation sequencing (NGS) technologies allow for the generation of whole exome or whole genome sequencing data, which can be used to identify novel genetic alterations associated with defined phenotypes or to expedite discovery of functional variants for improved patient care. Because this robust technology has the ability to identify all mutations within a genome, incidental findings (IF)- genetic alterations associated with conditions or diseases unrelated to the patient's present condition for which current tests are being performed- may have important clinical ramifications. The current debate among genetic scientists and clinicians focuses on the following questions: 1) should any IF be disclosed to patients, and 2) which IF should be disclosed – actionable mutations, variants of unknown significance, or all IF? Policies for disclosure of IF are being developed for when and how to convey these findings and whether adults, minors, or individuals unable to provide consent have the right to refuse receipt of IF. In this review, we detail current NGS technology platforms, discuss pressing issues regarding disclosure of IF, and how IF are currently being handled in prenatal, pediatric, and adult patients.

Keywords: Incidental findings, Next-generation sequencing, Disclosure, ACMG.

INTRODUCTION

Over the past 150 years, the field of genetics has evolved from a rudimentary understanding of the units of inheritance, or genes, to the complete sequence of the human genome [1]. Landmark discoveries, such as the causal link between trisomy 21 and Down syndrome and *CFTR* mutations and cystic fibrosis, have heralded the field of molecular diagnostics, where gene, chromosomal, and biochemical tests allow genetic defects associated with human diseases to be identified in prenatal, pediatric, and adult settings.

Centuries ago, family history was used to identify heritable diseases. As early as 1757, familial aggregation of breast cancer was one of the indications that breast cancer may have a hereditary component [2]. Family history has since been complemented by technologies such as karyotype analysis to diagnose chromosomal disorders and gene testing to identify genetic carriers for diseases such as sickle cell anemia [3]. Today, genetic tests are widely used in reproductive medicine: 1) to determine whether prospective parents carry DNA variants that would increase risk of genetic diseases in their offspring, 2) for implantation of only embryos free from specific genetic conditions, and 3) in prenatal testing to provide parents with information about the genetic health of their unborn child. Diagnostic tests may be used to confirm clinical diagnosis based solely on patient symptoms, while predictive testing can be used to identify patients at increased risk of developing disease in the future, despite being asymptomatic at the time of testing [4]. Genetic testing can thus improve diagnosis, prevention, and treatment of hereditary conditions.

Previously, gene testing was performed on a single gene or a few genes with bidirectional Sanger sequencing, which was considered the gold standard for mutation detection [5]. Since Sanger sequencing cannot detect most structural alterations, other technologies were often necessary. Next-generation sequencing (NGS) can detect point mutations, insertion/deletion (in/del) polymorphisms, splice site variants, copy number alterations, and structural changes in a single experiment. While whole-genome (WGS) and whole-exome sequencing (WES) have been used in basic research for gene identification and genotype-phenotype correlations, WGS or WES may be used clinically to identify unknown or rare mutations not detected by single gene analysis or multigene targeted assays [6]. The use of NGS, while expediting the identification and delivery of genetic results to the patient, has the ability to identify numerous mutations within a genome, many of which are not related to the phenotype in question, but may have clinical ramifications. The genetics community is currently debating whether, when, and how to convey these incidental findings (IF) to the patient. In this review, we describe current NGS technologies and present information about how IF are handled in preconception and preimplantation, prenatal, pediatric, and adult patient populations.

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METHODS

This review summarizes literature available in PubMed from 2005-2015. We begin with the development of the first commercially available NGS platform in 2005 and track how the ethical concerns and recommendations for reporting IF have evolved in the ensuing decade. Search terms included next-generation sequencing, incidental findings, preconception, preimplantation, prenatal, pediatric, and adult.

NEXT-GENERATION SEQUENCING (NGS)

Technology Overview

In 1977, Sanger and colleagues developed the chain termination method for DNA sequencing that would become widely used over the next two decades [7]. The chain termination or Sanger sequencing method incorporates a mixture of 2′-deoxynucleotides (dNTPs) and radio- or fluorescently-labeled 2′,3′-dideoxynucleotides (ddNTPs) during template synthesis, resulting in DNA fragments truncated at every base pair. Originally, these radiolabeled fragments were synthesized in four separate reactions, one for each nucleotide (ddATP, ddCTP, ddGTP and ddTTP), electrophoresed in individual lanes on polyacrylamide gels, and the sequence was read manually. Advances in enzymology, fluorescent dyes, detection, and capillary electrophoresis allowed for development of automated sequencers, which increased read lengths and the number of samples that could be sequenced in a single run [8]. The Human Genome Project was completed using Sanger sequencing methods at a cost of $2.7 billion using single 96-capillary systems that could produce ~0.5 Mb of sequence/day [9]. NGS technology with massively parallel processing greatly increased throughput and lowered the cost per base [10], making NGS platforms (Table **1**) ideal for sequencing large amounts of DNA for whole genome, exome, transcriptome, and metagenome analysis.

The basic steps of all NGS technologies involve formation of a library which requires: 1) fragmentation of DNA (genomic, reverse-transcribed, or immunoprecipitated) to 150-750 bp fragments, 2) ligation of DNA adaptors at both ends of each fragment (barcoded DNA sequence can also be added to allow for multiplexing of samples), and 3) PCR amplification with primers to attach fragments to a solid surface (flow cell) or adaptor-complimentary oligonucleotides to attach to beads [9, 11]. These fragment libraries then undergo sequencing reactions in parallel to generate millions of sequencing reads, usually a few hundred base pairs in length, which are then aligned to a reference sequence [12].

The shorter read lengths generated with NGS technology does make *de novo* sequencing as well as aligning repetitive regions to a reference sequence more challenging. Certain DNA variants, including translocations and in/dels as well as palindromic regions, can also be more difficult to sequence with NGS compared to Sanger sequencing [11]. Paired-end NGS can increase interpretation of the sequence, ensuring sufficient sequencing coverage is critical to generating accurate results. Sequencing coverage refers to the number of times on average a single base is read, thus 30X depth of coverage equates to sequencing each base pair an average of 30 times. Depth of coverage will need to be increased as the frequency of a variant decreases in a population of interest. Current recommendations from the ENCODE (Encyclopedia Of DNA Elements) Consortium include 30-80X coverage for DNA re-sequencing, 100x for *de novo* sequencing, 10-30x for SNP analysis or rearrangement detection, 100-200x for WES, and 100x for ChiP-Seq.

Available Platforms

The first commercially available NGS platform, the 454 pyrosequencing system (now owned by Roche), was released in 2005 [13]. This system can generate 700 Mb of data every 23 hours via long read lengths (~1000 bp), making it desirable for *de novo* sequencing or highly variable sequences. Although the GS FLX Titanium+ has a 99.9% accuracy rate [9, 10, 14], these sequencers will no longer be commercially available in 2016 [15].

The SOLiD sequencing system (Life Technologies) released in 2007 generates libraries by PCR on beads. Clonal bead populations are then covalently attached to FlowChips, and sequencing is performed by ligation with the correct fluorescently-labeled dNTP out-competing the other nucleotides. All templates are then separated with a new sequencing primer located at position n-1 from the original primer. This primer resetting occurs five times for each reaction, allowing each base pair to be sequenced twice, resulting in a sequencing accuracy of 99.99% [16]. Attributes that make the SOLiD platform ideal for variant detection and transcriptome sequencing include: throughput of ~160 Gb/run, a high accuracy rate at coverages >30x completion of an entire run in ~1 week, and a cost of $0.13/million bases [10, 17]. However, short fragment read lengths and difficulties sequencing palindromic areas are shortcomings of the SOLiD platform [11, 18].

The Ion Personal Genome Machine (PGM; Life Technologies) is designed for targeted sequencing of amplicons or small genomes, which significantly decreases run times. The Ion PGM uses a semiconductor for sequencing with no fluorescence or camera. During a sequencing reaction, a proton is released when a nucleotide is added to a DNA strand, and the ion sensor detects the resulting pH change. Specific nucleotides are flooded onto the microwell containing the DNA, if the nucleotide is not incorporated no voltage will be detected, if multiple nucleotides are added, the voltage will increase in magnitude [10]. The Ion PGM utilizes chips of varying sizes: the 314 chip yields 30-50 Mb in 2.3 hours, the 316 chip yields 300-500 Mb in 3 hours, and the 318 chip yields 600 Mb-1 Gb in 4.4 hours using 200 bp reads. These chips are capable of reads up to 400 bp, with a 99.99% consensus accuracy rate and a cost of $1000/Gb. A larger scale sequencing machine, the Ion Proton, can be used for higher throughput applications, producing up to 10 Gb of data in 200 bp reads in 2-4 hours [19, 20].

The Illumina portfolio of NGS platforms (HiSeq, NextSeq, and MiSeq) utilizes the sequencing by synthesis methodology (Fig. **1**), in which DNA is fragmented and ligated with adaptors at both ends. The resulting fragments are then bound to the surface of a flow cell in a random configuration. Bridge amplification, in which the fragments bend over and attach to an adjacent adaptor forming a loop or "bridge", followed by double-stranded DNA amplification, forms clusters of amplified DNA fragments across the

Table 1. Comparing NGS Platforms[a].

	Library Amplification	Sequencing Reaction Chemistry	Maximum Read Length (bp[b])	Maximum Throughput per Run (total bp[b])	Accuracy (%)	Strengths of Platform	Weakness of Platform
Roche 454 GS FLX Titanium XL+	Emulsion PCR	Pyro-sequencing	1,000	700 Mb[c]	99.9	Suited for *de novo* sequencing & highly variable sequence	Cost of reagents
ABI SOLiD	Emulsion PCR	Ligation	75	160 Gb[d]	99.99	Variant detection & transcriptome sequencing	Short fragment read lengths
Illumina HiSeq 2500	Bridge amplification	Reverse terminator	2 x 125	50-1,000 Gb[d]	98	Whole genome sequencing, largest throughput	All samples on one flow cell must have same read length
Illumina MiSeq	Bridge amplification	Reverse terminator	2 x 300	0.3-15 Gb[d]	99	Targeted or small genome sequencing; short run times	Lower # of total reads than other platforms
Illumina HiSeq X Five or Ten	Bridge amplification	Reverse terminator	2 x 150	900-1,800 Gb[d]	98	Population studies, fast turnaround time, low cost	Not economical for small studies
Ion Torrent PGM	Emulsion PCR	Ion sequencing	400	1 Gb[d]	99.99	Targeted sequence, amplicons, or small genomes	Lower # of total reads than other platforms
Complete Genomics Nanoball Sequencer	PCR on nanoballs	Ligation	70	20-60 Gb[d]	99.9	Lower cost than buying instrument and reagents outright	Only offered as a service; short fragment read lengths
Pacific Biosciences RS II	NA	Single molecule real-time	20,000	20 kb on 150,000 ZMWs[e]	95	No amplification required; long read lengths	Lower accuracy rate
Oxford Nanopore MinION	NA	Single molecule real-time	5,000	150 Mb[c]	~85	Small size of platform; portability; long read lengths	Not yet commercially available; low accuracy rate

[a] All available NGS platforms are not represented. [b] Base pairs, [c] Megabases, [d] Gigabases, [e] Zero-mode waveguides.

flow cell. Four fluorescently labeled reversible terminators, DNA polymerase, and primers are then used to sequence the millions of clusters in parallel. A single labeled dNTP with a chemically blocked 3'-OH is added in each cycle to terminate polymerization, the fluorescence is imaged to determine the base added, the 3' blocking group is removed, and the next cycle is initiated [21, 22]. The three Illumina sequencing platforms handle different sequencing needs; for example, targeted or small genome sequencing can be performed using the MiSeq, which has a maximum read length of 2 x 300 bp, with 25 million reads on a flow cell. Run time for the MiSeq ranges from 5-55 hours with an output of 0.3-15 Gb. For mid-range sequencing, the NextSeq 500 can generate 30-120 Gb in 15-26 hours with 400 million reads per flow cell and a maximum read length of 2 x 150 bp. The HiSeq is used for large-scale sequencing because it can process two flow cells at once, resulting in an output range of 50-1000 Gb in 1-6 days. Two billion reads are possible per flow cell with a maximum read length of 2 x 125 bp. The HiSeq currently has one of the largest throughputs available in NGS systems. The cost of an Illumina sequencing run is ~$0.07/million bases at an accuracy rate of 98% [10]. In January 2014, Illumina introduced the HiSeq X Ten system, a compilation of ten ultra-high throughput sequencers marketed for large population studies. This new system can generate 1.6-1.8 Tb in less than 3 days at a cost of < $1000 per genome [23, 24].

With NGS rapidly evolving, numerous companies are developing new platforms utilizing novel chemistries, such as nanoball technology (Complete Genomics), single-molecule real-time (SMRT) sequencing (Pacific Biosciences), and the nanopore method (Oxford Nanopore).

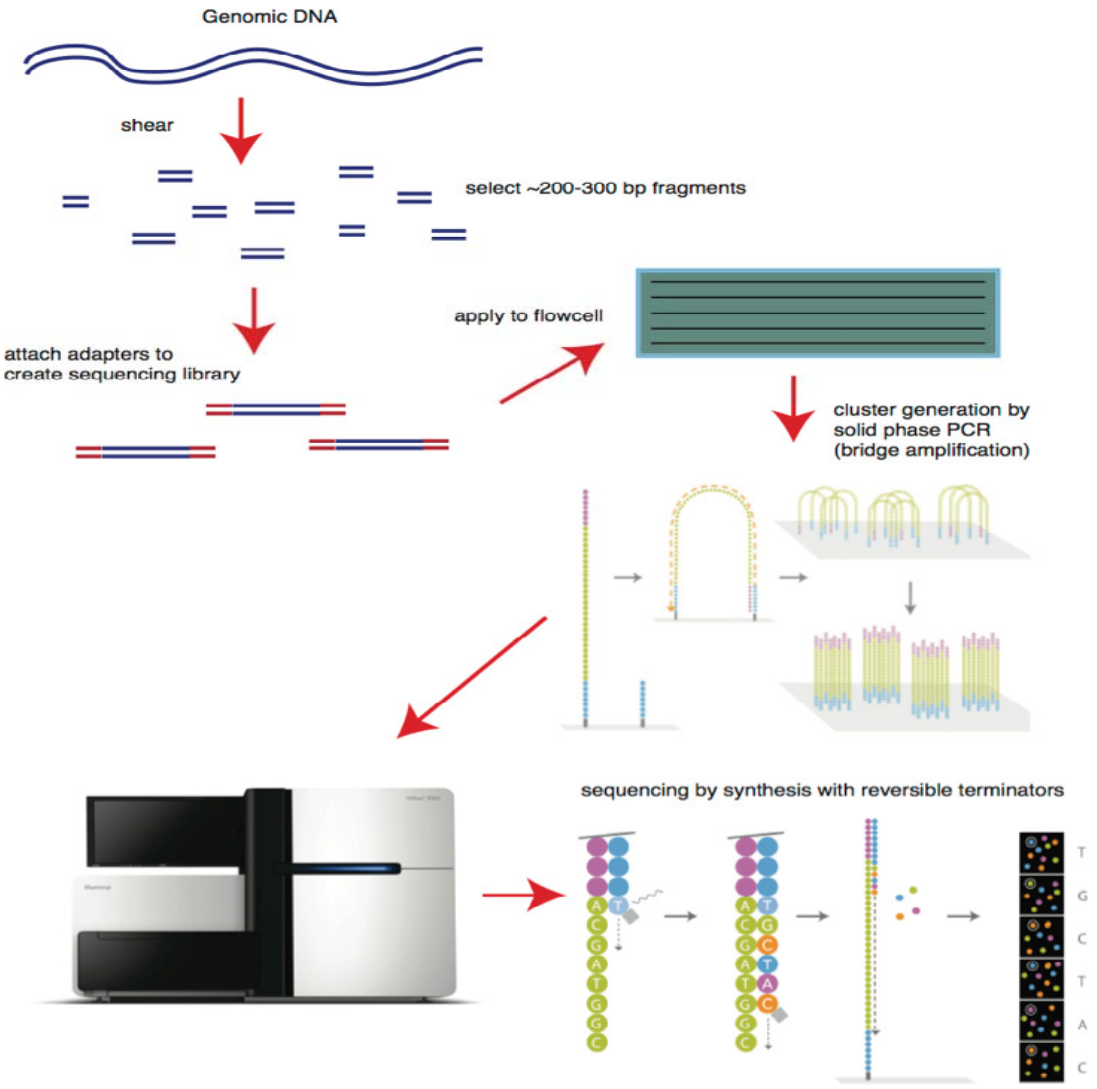

Fig. (1). Sequencing by synthesis method on the Illumina Hi-Seq. DNA is fragmented and labeled at both ends with appropriate adaptors. Once the fragments are randomly attached to the flow cell, cluster generation is performed using bridge amplification, and massively parallel sequencing is completed using reverse terminator chemistry [116].

Nanoball technology, which is currently offered only as a service including sequencing data analysis /management, has a high throughput of 20-60 Gb/run; however, the maximum read length of only 70 bp makes alignment challenging [11, 25]. SMRT sequencing has been referred to as "third-generation sequencing" because PCR amplification is not required and the zero-mode waveguide technology has the advantage of read lengths >20,000 bp, ability to detect chemical modification of bases such as methylation, and shorter preparation and sequencing run times; however, the error rate for SMRT sequencing is relatively high at ~5-12% [20, 24, 26]. The SMRT platform is commercially available along with the SMRT Analysis Software suite. Pacific Biosciences also offers a list of third party institutions that can sequence and analyze samples utilizing the PacBio® RS II. Nanopore technology utilizes changes in ionic current within a nanometer-width hole to determine DNA sequences as nucleotides are cleaved within the pore [26]. The MinION, made available to registered users in 2014, can generate 150 Mb per run averaging 5,000 bases at 1 bp/nanosecond, although accuracy is low, ranging from ~25-85% [27, 28]. Although not yet commercially available, users of the MinION™ Access Programme (MAP) are exploring its functions and its interpretation software MinKNOW™, completing base calling in real time accessing a cloud.

Applications of NGS

NGS can be utilized for a wide array of applications such as WGS, WES, transcriptomics, targeted panel sequencing, DNA methylation studies, and metagenomics (Table **2**). WGS provides sequence coverage of the entire genome including exons, introns, and regulatory regions and has been used to detect *de novo* disease variants and mosaicism in newborn screening and pathogen research [24, 29, 30]. WES and targeted sequencing are the major platforms currently used in the clinical arena. WES covers only the protein-

Table 2. NGS Applications.

Application	Source of Input DNA	Identified Output
Whole genome	Genomic DNA	Complete genome sequence
Whole exome	Protein-coding genomic DNA	Sequence for all coding regions
Targeted gene panels	Protein-coding genomic DNA	Enriched sequence for genes of interest
RNA-seq (can be targeted)	Reverse transcribed DNA	Whole transcriptome or specific RNA sequence
miRNA-seq	Reverse transcribed DNA	microRNA sequence
CAGE-seq	Reverse transcribed 5' cap-targeted RNA	Transcription start sites
DNA methylome-seq	Bisulfite-treated DNA	DNA methylation sites
ChIP-seq	Immunoprecipitated DNA	Protein-DNA interactions, transcription factor binding sites
RIP-seq (NET-seq)	Reverse transcribed DNA from immunoprecipitated RNA	RNA binding proteins
DNase-seq	DNase-digested chromatin DNA	Genomic regions vulnerable to DNase
FAIRE-seq	Open/accessible chromatin DNA	Accessible chromatin, regulatory regions
MNase-seq	Nucleosome-associated DNA	Nucleosome positions in genomic DNA
Hi-C/5C-seq	Captured chromosome conformations	Chromosome interactions, spatial orientation of chromosomes
Metagenomics	Microbial DNA populations	Bacterial and viral genomes

Table adapted from Table 2 of Rizzo JM *et al.* [11].

coding regions of the genome, which consists of ~2% of the entire genome and 85% of known disease-causing polymorphisms [24]. Conversely, targeted NGS panels focus on genes or regions known to be involved in certain diseases or phenotypes of interest.

RNA sequencing (RNAseq) or transcriptome sequencing is used to sequence all coding and non-coding RNA molecules. RNAseq can identify polymorphisms in coding regions, expression levels of genes, and gene rearrangements such as fusion genes, which are often more active than normal genes and are expressed to a greater extent in cancer [31]. RNAseq can be conducted on single cells to reveal the vast molecular heterogeneity among cell populations. As with DNA applications, panels are available for RNA analysis. Tiling arrays target specific portions of the transcriptome, increasing coverage at higher depth, and allowing analysis of very rare transcripts and isoforms [24].

Patterns of DNA methylation can be observed using bisulfate-seq or DNA methylome-seq techniques. Bisulfite conversion transforms unmethylated cytosine into uracil, while methylated cytosine is protected from conversion. Since methylation of cytosine residues in DNA regulates gene expression in both normal and dysfunctional cells, NGS is useful for detecting genome-wide methylation changes associated with functional changes in cancer, heart disease and other human diseases.

Clinical Utility of NGS

NGS is becoming increasingly common in clinical medicine as technology improves, costs decline, and education of medical professionals increases. Many insurance companies still do not cover most NGS-based testing [32], which greatly hinders testing volumes, but as personalized and translational medicine become more mainstream, more tests are being offered. The National Center for Biotechnology Information Genetic Testing Registry currently lists 31 centers that offer clinical NGS, 13 of which are located in the United States. GeneDx was the first laboratory to offer NGS clinically to test for mutations associated with cardiomyopathy in 2008. Emory Genetics Laboratory was the first academic laboratory to offer a clinical service to detect X-linked intellectual disability, congenital muscular dystrophy, and congenital disorders of glycosylation [33]. In November 2013, Illumina became the first company to receive FDA approval for the MiSeqDx sequencer and its cystic fibrosis NGS panel [15], both of which can be used by any clinical laboratory. Illumina also offers a TruGenome clinical sequencing service for rare genetic diseases, predisposition screening for adult-onset conditions, and complete WGS. Turnaround time for screening is ~90 days, while WGS takes ~45 days at a cost ranging from $5,000-$17,500 [34]. Gene by Gene is currently the only company offering direct-to-consumer WGS and WES [35]. Using Illumina HiSeq technology, Gene by Gene offers 70x whole-exome coverage for $1,295/sample within a 10 week timeframe, while WGS (30x coverage) is $7,395/sample with a 10-14 week turnaround time [36]. Ambry Genetics offers WES through their ExomeNext test at a price of $5,800 for up to three individuals (trio testing) with a turnaround time of 8-12 weeks [37]. Ambry Genetics also offers many specific sequencing panels for hereditary cancers and cardiovascular disease. Several academic institutions also offer clinical NGS services to physicians for patient care: Medical College of Wisconsin

Developmental and Neurogenetics Laboratory, Mount Sinai Genetic Testing Laboratory, Baylor Whole Genome Laboratory, UCLA Medical Genetics Clinic, Children's Hospital of Philadelphia, Emory Genetics Laboratory, and Washington University in St. Louis Genomics and Pathology Services [38]. For patients with undiagnosed conditions, the National Institutes of Health (NIH) has an Undiagnosed Diseases Program, allowing patients with physician referrals to receive NGS if admitted into the program [39]. Another resource for patients with undiagnosed conditions is the Rare Genomics Institute, an international non-profit organization founded to provide NGS services, physicians, and genetic counselors to patients and families with rare conditions [40]. As further research identifies causative genomic variants for human diseases, additional NGS panels will be developed by commercial, academic, and reference laboratories.

INCIDENTAL FINDINGS

Despite the growing offering of NGS tests to patients and consumers, issues including clinical interpretation of data, utility in patient treatment, and ethical obligations to return IF that may arise from sequencing an entire exome or genome remain unresolved. Effectively translating large amounts of genomic data into a concise report that physicians can accurately interpret and convey to patients is challenging [30]. Many variants found when analyzing an entire genome are of unknown significance and may or may not be causative. IF also pose significant ethical problems; for example, if there is no treatment for a condition detected through NGS, should the incidental results be revealed? When testing children, what are the obligations for disclosing findings that do not currently pose a risk but which may manifest in adulthood, especially since the child being tested cannot consent? Below we describe the ethical concerns of disclosing IF when testing embryos, children, and adults and discuss current policies of organizations such as the American College of Medical Genetics and Genomics (ACMG) regarding IF.

IF are not unique to genetics. In 1951, a manuscript based on exploratory laparotomy surgery described the complexities of managing IF in clinical practice [41, 42]. Determining whether and when to report IF has received a great deal of attention in imaging studies. For example, emergency sonography may yield IF significant enough to alter a patient's diagnosis and outcome [43], while imaging modalities such as computed tomography or magnetic resonance imaging have been effective in identifying clinically significant cardiac abnormalities [44, 45]. IF ranging from brain atrophy to clinically serious lesions are typically seen in ~45% of magnetic resonance imaging (MRI) scans [46]. Overall questions stemming from these findings including whether, when, and how patients and research participants, including minors or individuals with diminished capacity, should be informed about IF are relevant to both researchers and clinicians, however, requirements of Institutional Review Boards (IRB) or medical liability differ between research and clinical settings, thus each will be addressed separately.

Genetic Testing in the Research Setting

The genetics community has a history of protecting patients from possible harm by not disclosing genetic results [47]. During the era of positional cloning, genetic research was heavily dependent on pedigree analysis; however, non-paternity was evident in ~10% of research subjects [48]. While nondisclosure of non-paternity among research subjects may avoid possible psychological or legal harm, non-disclosure may significantly complicate clinical genetic counseling [49]. The 1992 Conference on Ethical and Legal Aspects in Pedigree Research suggested that patients engaging in pedigree research should be warned about the potential for economic, social, or psychological harm. Other landmark activities include: 1) a 1994 NIH conference focusing on whether investigators are obliged to disclose clinically relevant findings when studying archived tissues [50], 2) a report from the National Bioethics Advisory Commission in 1999 recommending that genetic results should only be disclosed to study participants in exceptional circumstances, and only if the results have been validated, have significant impact on the subject's health, and if treatment is readily available [51], and 3) passage of the Genetic Information Nondiscrimination Act in 2008 to provide federal protection from genetic discrimination in health insurance and employment.

The desire to protect patients from possible harm by not disclosing genetic information must be balanced by the patient's autonomy, including the right to know personal information [52]. In the research setting, three approaches have been proposed regarding the type of results that should be disclosed: 1) genetic results are never disclosed to study participants, 2) all genetic results, including IF, are provided to individuals who consent to such disclosure, or 3) provision of results only with known clinical significance [53]. Other questions about disclosure of IF in the research setting include when, how, and to whom results should be disclosed [47]. These decisions will be made with input from and under the auspices of the host institute's IRB. Currently there no national-level standards for how IF are handled in genomic research, and recent research suggests that the majority of researchers and IRBs have little experience disclosing IF, although there is consensus that the protocol for disclosing IF must be included in the informed consent process [54, 55]. Recommendations from the National Heart, Lung and Blood Institute (NHLBI) advise that the informed consent process include a description of the protocol for returning IF which contains an option to opt out of receiving IF [56]. Because genetic information is constantly evolving, the status of DNA variants may change over time, such as a rare mutation eventually classified as pathogenic after functional studies have been completed. The NHLBI guidelines suggest once the research protocol is terminated, genetic results will no longer be available to participants; however, within the clinical community, periodic reanalysis and provision of updated results to patients may be appropriate [57] Finally, future-use of biological specimens including whether participants wish to be re-contacted in the event IF are discovered by secondary researchers, must be discussed at the time of consent.

Other issues that must be considered in the research setting include the responsibility of the researcher, who may have limited resources and expertise, to actively search for IF. If a researcher does identify IF of potential interest, consultation with an outside clinician and/or validation of the results in a CLIA-approved laboratory may be necessary.

Finally, IF can be classified as: 1) those providing a strong net benefit that could avert a life-threatening condition if appropriately treated, 2) those with possible net benefit such as those that may be helpful in reproductive decision making, and 3) those with unlikely net benefit that are not associated with serious conditions and should therefore not be reported [52].

Genetic Testing in the Clinic

Incorporating NGS into clinical practice has been slower than in the research setting. WGS may be used for diagnosis of Mendelian disorders, to individualize cancer treatment through molecular characterization of tumors, to enhance family planning by determining carrier status of prospective parents, and to generate pharmacogenomic data to optimize choice and dosage of medications [58]. Disclosure of genetic results in the clinical setting has important health implications, thus patients undergoing WGS or WES should receive pre-test counseling, be fully informed of potential harms, and be guaranteed that their personal information will remain confidential [59]. A recent study of 200 patients who underwent diagnostic exome sequencing found that 94% of patients chose to receive IF [60]. Given that each person may have > 4 million variants including 50-100 associated with human disease, it is critical that a reasonable and useful system for determining which IF to report is developed [61].

A number of methods to identify which IF should be reported have been published. In 2011, a three-tiered system was proposed to classify genes by clinical utility or actionability, clinical validity, or potential to cause harm. After reviewing the results, only known or likely disease-causing mutations would be reported [62, 63]. National Human Genome Research Institute sponsored groups, such as the Electronic Medical Records and Genomics Network and the Clinical Sequencing Exploratory Research Consortium, are developing protocols for reporting IF [64, 65]. Similarly, the Centers for Disease Control and Prevention Office of Public Health Genomics have established the Evaluation in Genomic Applications in Practice and Prevention Working Group to develop recommendations for using genetic data in clinical practice [61, 66]. The American College of Pathologists recommends that any clinical laboratory providing genetic testing services have a policy in place describing how and when IF will be returned [67].

In 2013, the American College of Medical Genetics and Genomics (ACMG) released a policy statement for reporting IF in the clinical setting. Under these recommendations, genetic testing results for all patients will be reported to the ordering clinician who is responsible for explaining the meaning and context of specific variants to patients (Table **3**) [68]. Patients who do not wish to receive IF would have to forgo clinical sequencing. The ACMG recommendations apply to children as well as adults, with the justification that reporting IF for adult-onset diseases to children and their parents provides greater benefit than harm, although this seems to contradict the ACMG's own policy, reaffirmed in 2013 in conjunction with the American Academy of Pediatrics (AAP) that predictive testing for adult-onset diseases should not be offered to children [69]. Reporting IF for other types of testing such as preconception, prenatal, or newborn sequencing was not addressed in this policy statement.

The ACMG recommendations have been criticized for not allowing patients to express a preference for receiving IF, which overrides the concept of patient autonomy and may violate the patient's ability to provide informed consent and refuse unwanted medical tests [70]. As a consequence, in November 2014, ACMG released updated recommendations for reporting IF that includes an option to opt out of analysis of genes deemed important and actionable by ACMG [71]. Other criticisms suggest that genetic variants for which preventive measures or treatments are available should be considered diagnostic rather than IF. In addition, identification and evaluation of each variant is time consuming, there may be significant costs for appropriate genetic counseling, and there is insufficient data to support the clinical utility of the ACMG recommendations to date [72, 73].

Despite the controversial nature of IF, use of NGS is increasing at a dramatic rate. Below we present how NGS and IF have been used in preconception/preimplantation, prenatal, pediatric, and adult populations, including the specific ethical concerns relevant to each patient population (Table **4**).

IF in Preconception and Preimplantation Screening

For many patients, the decision to undergo genetic testing that is predictive of possible outcomes of pregnancy (reproductive genetic testing) is driven by family history of a particular disorder, where analysis of one or a few genes would be sufficient. The ability of NGS to multiplex samples and assess hundreds of genes in a single run reduces the cost and time while maintaining accuracy, thus making NGS an attractive option. Recently, a targeted panel of 448 genes associated with severe recessive childhood disorders was developed for use as a preconception screening test. An initial pilot study examining 104 individuals found an average of 2.8 recessive mutations per person [74]. This test could be useful for community-based screening to identify carriers of mutations in *HEXA*, β-globin, and *CFTR* that cause Tay-Sachs, sickle-cell anemia, and cystic fibrosis in Ashkenazi, African American, or Caucasian populations, respectively. Identification of carrier status would allow prospective couples to consider options such as remaining childfree, adopting, or undergoing *in vitro* fertilization (IVF) with preimplantation genetic diagnosis (PGD).

Although the 448 gene panel is a targeted screen designed to identify deleterious mutations enriched in specific populations, unexpected findings may occur. Carrying a single mutation for an autosomal recessive syndrome will not cause disease, and if parents do not carry the same mutation, 50% of their offspring may be carriers but none of their children will have the disorder. Although there would be no need to pursue prevention or treatment strategies and disclosure of all mutations would be of minimal utility, failure to disclose all mutations could provide a false sense of security should either parent have additional reproductive partners. In addition, caution must be used in disclosing results associated with genetic ancestry as unexpected findings may lead to alterations in personal, familial, or community identity [75].

PGD was originally performed to detect single gene disorders in one blastomere cell from cleavage-stage embryos

Table 3. Genes reportable as incidental findings[a].

Phenotype	Age of Onset	Gene	Inheritance
Hereditary breast and ovarian cancer	Adult	*BRCA1*	Autosomal Dominant
		BRCA2	
Li-Fraumeni syndrome	Child/Adult	*TP53*	Autosomal Dominant
Peutz-Jeghers syndrome	Child/Adult	*STK11*	Autosomal Dominant
Lynch syndrome	Adult	*MLH1*	Autosomal Dominant
		MSH2	
		MSH6	
		PMS2	
Familial adenomatous polyposis	Child/Adult	*APC*	Autosomal Dominant
MYH-associated polyposis	Adult	*MUTYH*	Autosomal Recessive
Von Hippel-Lindau syndrome	Child/Adult	*VHL*	Autosomal Dominant
Multiple endocrine neoplasia type 1	Child/Adult	*MEN1*	Autosomal Dominant
Multiple endocrine neoplasia type 2	Child/Adult	*RET*	Autosomal Dominant
Familial medullary thyroid cancer	Child/Adult	*RET*	Autosomal Dominant
PTEN hamartoma tumor syndrome	Child/Adult	*PTEN*	Autosomal Dominant
Retinoblastoma	Child	*RB1*	Autosomal Dominant
Hereditary paraganglioma	Child/Adult	*SDHD*	Autosomal Dominant
		SDHAF2	
		SDHC	
		SDHB	
Tuberous sclerosis complex	Child	*TSC1*	Autosomal Dominant
		TSC2	
WT1-related Wilms tumor	Child	*WT1*	Autosomal Dominant
Neurofibromatosis type 2	Child/Adult	*NF2*	Autosomal Dominant
Ehlers-Danlos syndrome	Child/Adult	*COL3A1*	Autosomal Dominant
Marfan syndrome, Loeys-Dietz syndromes	Child/Adult	*FBN1*	Autosomal Dominant
		TGFBR1	
		TGFBR2	
		SMAD3	
		ACTA2	
		MYLK	
		MYH11	

(Table 3) contd….

Phenotype	Age of Onset	Gene	Inheritance
Hypertrophic cardiomyopathy	Child/Adult	*MYBPC3*	Autosomal Dominant
		MYH7	
		TNNT2	
		TNNI3	
		TPM3	
		MYL3	
		ACTC1	
		PRKAG2	
		GLA	X-Linked
		MYL2	Autosomal Dominant
		LMNA	
Catecholaminergic polymorphic ventricular tachycardia		*RYR2*	Autosomal Dominant
Arrhythmogenic right-ventricular cardiomyopathy	Child/Adult	*PKP2*	Autosomal Dominant
		DSP	
		DSC2	
		TMEM43	
		DSG2	
Romano-Ward long QT syndrome	Child/Adult	*KCNQ1*	Autosomal Dominant
		KCNH2	
		SCN5A	
Familial hypercholesterolemia	Child/Adult	*LDLR*	Semidominant
		APOB	Semidominant
		PCSK9	Autosomal Dominant
Malignant hyperthermia susceptibility	Child/Adult	*RYR1*	Autosomal Dominant
		CACNA1S	

[a] Reported by the American College of Medical Genetics and Genomics. [68]

Abbreviations: MYH, mutY Homolog (E. coli); PTEN, phosphatase and tensin homolog; WT1, Wilm's tumor suppressor gene 1; QT, Q wave and T wave of the heart's electrical cycle.

Table 4. Ethical Considerations Summary of IF in Different Testing Populations.

Testing Population	Ethical Considerations of IF
Preconception/ Preimplantation	• Not releasing IF of a single autosomal recessive mutation may have ramifications to a parent if they have additional reproductive parterres that also carry the mutation. • Disclosing IF of genetic ancestry could lead to psychological harm [75]. • Disclosure of IF may allow parents to gain knowledge of deleterious genetic conditions & undergo additional screening for siblings and themselves. • No policies currently in place for releasing IF.

(Table 4) contd….

Testing Population	Ethical Considerations of IF
Prenatal	• Return of IF can determine continuation or termination of a pregnancy so must be cautious & may need a re-testing policy in place. • Autonomy of fetus can conflict with the beneficence responsibility of parents [117]. • Misinterpretation of IF in prenatal testing has been shown to occur, causing unnecessary termination [89]. • No policies currently in place for releasing IF.
Pediatric	• ACMG, AAP, and ASHG recommend releasing IF in children only when necessary for treatable diseases, prevention, or to slow onset [93]. • Disclosure of IF for adult onset conditions is only appropriate when there is a clear benefit to the parent or child [94]. • When there is no clear benefit, IF should not be released because of psychological harm and violation of the child's autonomy [94]. • Child and parent should be made aware of IF possibility before testing & whether/what kind of IF they want disclosed [96]. • Regardless of the parent/child consent of IF disclosure, if actionable IF is found, it should be disclosed. • Actionable IF should be confirmed by additional testing before disclosure. • Clinical genetic counselor should return IF & recommend follow-up care [94].
Adult	• ACMG recommends return of IF of 56 genes (Table 3). • Patients must be made aware of IF possibility during consent & decide what kinds of IF they want disclosed [96]. • Variants of unknown significance can occur frequently and need to be addressed by the testing laboratory before consent as to whether they will be disclosed. • In tumor/normal sample testing, ACMG recommends releasing IF found in normal tissue if covered in its list of 56 genes [109] • Actionable IF should be confirmed by additional testing before disclosure. • Physician knowledge of NGS findings, including IF, is often lacking causing concern of improper interpretation and treatment.

generated through IVF, which are susceptible to aneuploidy [76-78]. Techniques such as fluorescence *in situ* hybridization and array comparative genomic hybridization were initially used to determine which embryos were euploid, increasing the odds of a successful pregnancy [79]. Pilot studies have shown that NGS results are accurate [76, 80-82] and can be completed within 15 hours, avoiding the need for cryopreservation. In addition, use of NGS allows for detection of multiple genetic abnormalities including aneuploidy, mutations associated with single gene disorders, mitochondrial copy number alterations, and chromosomal imbalances, which may affect the viability of the embryo and have detrimental health effects on the offspring [81].

PGD identifies embryos with a normal genomic composition, which can be selected for implantation. Disclosure of IF may be useful to parents who elect PGD for reproductive assistance with no *a priori* knowledge of deleterious genetic conditions. IF may allow parents or siblings to undergo mutation screening and receive appropriate prevention or treatment strategies. Since ACMG recommendations for disclosing IF do not apply to preimplantation screening, policies governing use of NGS are needed.

IF in Prenatal Genetics

Prenatal diagnostics is performed on a fetus *in utero* and detection of deleterious mutations may result in termination of the pregnancy. Fetal cells can be obtained by invasive procedures, such as chorionic villus sampling at 11-14 weeks gestation or by amniocentesis after week 15, which increases risk of miscarriage [83]. Alternatively, fetal cell-free DNA (cfDNA) can be collected non-invasively from a maternal blood sample at ~7 weeks gestation and used for genetic testing [84]. However, due to the low quantity and short fragment length typical of fetal cfDNA, use in non-invasive prenatal diagnosis (NIPD) is currently limited. NIPD can be used to determine the gender of a fetus by detecting Y chromosomal material in a maternal background. Gender determination may be useful for screening X-linked conditions, which usually affect males. In addition, NIPD can detect aneuploidy and identify pregnancies at risk for single gene disorders such as dominant conditions inherited from the father, *de novo* mutations, or compound heterozygotes [85, 86]. The American College of Obstetricians and Gynecologists recommends that NIPD DNA be used in primary screening tests in at-risk women, aged 35 years or older, with abnormal ultrasound findings or a family history of aneuploidy/trisomy [87].

NGS is an attractive technology for use in NIPD; however, IF are a potential issue. Because IF in prenatal testing can influence the decision to continue or terminate a pregnancy, reporting IF must be considered carefully. For example, because a fetus is incapable of calculating its own best

interests, parents have the responsibility to provide consent for the fetus and the obligation to act with beneficence, protecting the best interests of the fetus. Receipt of IF, regardless of the possible severity, may, however, lead to termination of the pregnancy, rendering the mother's autonomy more important than beneficence towards the fetus [88] In addition, IF results may be misinterpreted. Early studies reporting sex chromosome anomalies led to a significant increase in pregnancy terminations even though many of these abnormalities are associated with good prognosis. Reporting all IF, including variants of unknown significance, may similarly lead to an increased number of elective terminations. Variable expressivity, as seen with neurofibromatosis type 1, cannot be predicted based on genotype alone, and a recent study using WES and WGS data from healthy individuals demonstrated that many DNA variants thought to be pathogenic were in fact benign [89]. Given that otherwise healthy children may be terminated unnecessarily based on IF, disclosure policies for prenatal testing must be precise and thorough.

IF in Pediatrics

Many of the same concerns involving IF in prenatal genetic testing are shared in the testing of children. NGS is primarily used in pediatrics to test for rare genetic disorders, intellectual disabilities, or autism spectrum disorders [90]. Children with rare diseases who have not been diagnosed via traditional means, such as blood tests, CT scans, MRI, or physical examination, are often referred for genetic testing. In the past few years, WES has been implemented more routinely to alleviate the number of genetic tests, overall cost, and stress on the child and family caused by customary single gene testing. Of the estimated 7,000-15,000 rare-disease -causing genes, > 3,500 were identified using traditional approaches such as linkage analysis. From 2009-2012, 182 additional rare-disease-causing genes were identified using WES [91]. The NIH Undiagnosed Diseases Program recently completed exome sequencing on 159 families (543 individuals), and identified 14 independent reportable IF in 8.8% of the families following the ACMG recommendations of screening for 56 genetic variants [92].

The original recommendations of the ACMG, mandating disclosure of a subset of IF andlater amended to allow patients to opt out of receiving IF, did not distinguish between adult and pediatric patients; in contrast, policies specific to genetic testing in children has been developed by a number of groups, including, paradoxically, the ACMG. The ACMG, AAP, and American Society of Human Genetics all recommend genetic testing and release of IF in children only when known treatments, preventive interventions, or ability to slow onset of disease or symptoms are available [93]. Disclosure of IF associated with increased risk of adult-onset diseases may be appropriate only when there is a clear benefit to the child and/or parents receiving the results [94]. For example, *BRCA1* mutations are not known to affect a child's health; however, there is increased risk for breast and ovarian cancer later in life. Thus, the consensus among genetic professionals is to reveal these findings because they may be medically actionable to the carrier parent [95]. For adult-onset diseases like Alzheimer's or Huntington's, disclosing IF is deemed inappropriate due to the potential for psychosocial harm to the child or parent with no treatment benefit [94]. The Presidential Commission for the Study of Bioethical Issues recommends that a clinician or researcher determine which types of IF would be appropriate to disclose before conducting testing and insure the child's best interests. During the informed consent process, parents and children (at an appropriate age and mental capacity) must be made aware of the possibility of IF and must decide whether they wish to receive IF, and if so, which type of IF [96]. Ethically, a clinical genetic counselor or trained professional should disclose IF and should aid the children and parents in understanding the implications of the findings and recommend appropriate follow-up care [94].

Other countries currently have few guidelines in place regarding IF in pediatric genetic testing. In 2010 prior to the widespread use of NGS, Canada released the Tri-Council Policy Statement 2, which obligated researchers to disclose IF. The Finding of Rare Disease Genes (FORGE) project, a consortium of 21 genetic centers across Canada, examined 264 childhood genetic disorders using WES. The FORGE policy was to report clinically actionable findings that affect children even if the parents/child opted not to receive incidental results [97, 98]. Researchers associated with FORGE and the Canadian Pediatric Cancer Genome Consortium did not feel a strong responsibility to look for meaningful incidental results, but felt patients should receive results whether they were incidental or primary findings [99]. The genome clinic at SickKids hospital in Ontario is developing a new clinical paradigm for individualized care and a prototype for implementing genomic medicine. The genome clinic reports all IF associated with a major childhood disorder and pharmacological variants of high-predictive value, but does not report pathogenic IF predictive of adult-onset disease unless consented to by the patient or parent [100]. The European Society of Human Genetics currently recommends that guidelines be established to define which IF should be returned when testing minors and that preventable or treatable health conditions be disclosed regardless of patient preference. In the United Kingdom, the Association of Genetic Nurses and Counsellors and Public Health Genomics Foundation both endorse the following: 1) the right of patients to receive or decline return of IF, 2) the position that children should not be tested for adult-onset conditions, and 3) use of clinical judgment to determine which IF to disclose [96]. In contrast, however, 47% of British adults believe that children should be able to be tested for adult-onset conditions, and 60% feel children should be tested for carrier status even in cases where the children are unable to decide for themselves at the time of testing [101].

IF in Adult Populations

Adults may undergo NGS testing to determine their predisposition for adult-onset hereditary diseases or to develop a personalized treatment regimen. Many laboratories now offer NGS carrier testing panels for numerous hereditary conditions including: 1) breast, colorectal, uterine, ovarian, and pancreatic cancers [102], 2) cardiovascular diseases such as cardiomyopathy, channelopathies, coronary artery disease, and aortic aneurysm [103], and 3) neurologic diseases such as Alzheimer's, multiple sclerosis, and epilepsy [104]. Although these targeted sequencing panels only examine spe-

cific genes, IF may result. Many genes linked to one type of cancer may be associated with other cancers or with other diseases: for example, a woman who undergoes carrier testing for breast cancer may have a variant in the *PALB2* gene, which may increase risk for both breast and pancreatic cancer [102]. A 2013 survey of 279 clinical genetic professionals in the US found that 96% agreed that adult patients should be made aware of clinically actionable IF [105]. However, variants of uncertain significance are detected 15-88% of patients [106] and represent a problem for clinicians and laboratories when reporting IF. As with other NGS testing, scientific and ethics boards recommend laboratories determine which types of IF will be disclosed to patients before testing, and that the informed consent process ensures patients understand the possibility IF will be detected and indicate which (if any) IF they wish to be receive [96]. In a recent study, six adult focus groups undergoing NGS at NIH for coronary artery disease differed in their perceptions of IF in genome sequencing. Some patients believed uncertainty associated with IF was expected and would improve with additional research, while others found the uncertainty unexpected, distressful, and therefore unreliable [107].

NGS may be useful for optimizing oncology treatment. High-throughput genomic sequencing allows for comprehensive analysis of tumors in a relatively rapid timeframe and with small biopsy sample sizes [108]. WGS, WES, and gene panel testing of tumor and normal samples can be used to identify actionable mutations in patients who may benefit from targeted treatments [109]. For example, in a recent WES study of DNA from 98 small cell lung carcinomas, 52 cases had at least one actionable mutation [110]. Other studies used NGS on lymphoma [111], gastroesophageal, hepatobillary, and colorectal tumor specimens to determine molecular targets for patient therapy [112]. The ACMG acknowledges that IF will be identified in tumor/normal testing and recommends that incidental variants found in normal tissue be reported to the patient if occurring in one of the 56 actionable genes [109]. Unfortunately oncologists are often unprepared to handle IF. Additional training and education of NGS is needed for oncologists as technology becomes more prevalent in clinical medicine.

DISCUSSION

Policies for disclosure of IF are complicated and controversial. While disclosure of IF in imaging studies provides clear benefit to patients, genomic IF generated through WES and WGS may or may not be useful in treatment and disease prevention. When disclosing IF the four principals of medical ethics (autonomy, beneficence, non-maleficence, and justice) must be considered. Important questions are: what is the proper balance between the physician's desire to provide optimal treatment and the patient's request to not receive IF? If disclosure of IF causes psychological or emotional distress to the patient, has the principal of do no harm been violated? How do differences in patient access to preventative treatments affect disclosure of IF? Does the possible benefit to living family members of disclosing IF postmortem override the autonomy of the deceased or violate the US HIPAA Privacy Rule [113-115]?

In addition to these ethical considerations, there are practical aspects to consider when disclosing IF. For example, when IF are disclosed in a research setting, who is responsible for covering the financial costs of additional genetic tests to validate IF in a clinically approved laboratory? Reporting IF requires that clinicians thoroughly understand IF and convey these results to patients who likely do not understand genetic phenomena such as complex inheritance, variable expressivity, and incomplete penetrance. Establishing genetics curricula in medical and nursing schools and training licensed genetics counselors will be increasingly necessary as the use of NGS-based testing expands. Establishing whether clinicians or researchers have legal liability for failing to recognize and provide IF with clinical utility to patients is important to protect both patient and provider [114, 115].

Quickly evolving NGS technologies may impact disclosure of IF. Although WES and WGS are frequently used to detect largely static DNA variants in genomic DNA, many NGS platforms are able to measure more dynamic genetic profiles, including patterns of DNA methylation, gene expression, and metagenomes, each of which may be associated with disease. Because these types of genomic profiles may be affected by the environment, risk of disease may be altered through changes in diet or use of pharmaceuticals. One must consider whether there is value in revealing IF detected with various types of NGS platforms available today and in the future.

LIST OF ABBREVIATIONS

AAP	=	American Academy of Pediatrics
ACMG	=	American College of Medical Genetics and Genomics
ASHG	=	American Society of Human Genetics
BRCA1	=	Breast cancer 1, early onset
cfDNA	=	Cell-free DNA
CFTR	=	Cystic fibrosis transmembrane conductance regulator
CLIA	=	Clinical Laboratory Improvement Amendments
CT	=	Computed tomography
dNTPs	=	Deoxyribonucleotide
ddNTPs	=	Dideoxyribonucleotide
ENCODE	=	Encyclopedia Of DNA Elements
FORGE	=	Finding of Rare Disease Genes
HEXA	=	Hexosaminidase A
IF	=	Incidental findings
In/del	=	Insertion/deletion
MRI	=	Magnetic resonance imaging
NGS	=	Next-generation sequencing
NIH	=	National Institutes of Health
NIPD	=	Non-invasive prenatal diagnosis
PALB2	=	Partner and localizer of BRCA2
PGD	=	Preimplantation genetic diagnosis

PGM = Personal Genome Machine

RNAseq = RNA sequencing

SMRT = Single-molecule real-time

ssDNA = Single stranded DNA

Tb = Terabase

WES = Whole exome sequencing

WGS = Whole genome sequencing

CONFLICT OF INTEREST

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