

Genomic testing leads clinical care in neonatal diabetes: a new paradigm

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ABSTRACT (300 words)

Background

Traditional genetic testing focusses on analysis of one or a few genes according to clinical features; this approach is changing as improved sequencing methods enable simultaneous analysis of multiple genes. Neonatal diabetes is the presenting feature of many discrete clinical phenotypes defined by different genetic aetiologies. Genetic subtype determines treatment, with improved glycaemic control on sulfonylurea therapy for most patients with potassium channel mutations. We investigated the impact of early, comprehensive testing of all known genetic causes of neonatal diabetes.

Methods

We studied a cohort of 1020 patients with neonatal diabetes diagnosed <6 months referred from 79 countries between 2000-2013. Mutations were identified by comprehensive genetic testing including next-generation sequencing of 21 neonatal diabetes genes.

Findings

A genetic diagnosis was obtained in 840 patients (82%). Mutations in the potassium channel genes are the most common cause (n=390), but occur less frequently in consanguineous families (12% vs 46%, $p<0.0001$). Median duration of diabetes at the time of genetic testing decreased from >4 years before 2005 to <3 months after 2012. Earlier referral for genetic testing influenced the clinical phenotype. In patients with genetically diagnosed Wolcott-Rallison syndrome, just 12% of those tested within 3 months from diagnosis had syndromic diabetes vs 82% of those referred later (>4 years, $p<0.0001$) in whom skeletal/liver involvement was common. Similarly, for patients with transient neonatal diabetes only 10% of those tested early (<3 months) had remitted compared to 100% ($p<0.0001$) of the later referrals.

Interpretation

Patients are now referred for genetic testing closer to their presentation with neonatal diabetes. Comprehensive testing of all aetiologies identifies causal mutations in >80% of cases. The genetic result predicts optimal diabetes treatment and development of related features. This represents a new paradigm for clinical care with genetic diagnosis preceding development of clinical features and guiding clinical management.

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INTRODUCTION

The traditional genetic testing approach is based on selection of a gene or genes which are tested according to the clinical phenotype. This places an emphasis on the recognition of clinical syndromes and results in genetic testing being mainly confirmatory. The advent of next-generation sequencing has the potential to revolutionize the approach to diagnostic genetic testing as both exome sequencing and custom gene-panel tests for specific disorders analyse all genes known to cause a genetically heterogeneous condition in a single test (Figure 1).

Neonatal diabetes diagnosed before 6 months is a rare (approximate incidence 1:100,000 live births)^{1,2}, genetically heterogeneous disease, typically caused by mutation(s) in a single gene. This is in contrast with diabetes diagnosed after 6 months which is most likely to be Type 1 diabetes with an autoimmune aetiology^{3,4}. There are 22 known genetic causes of neonatal diabetes which identify different clinical subtypes of the disease, including isolated permanent neonatal diabetes, transient neonatal diabetes and complex syndromes where neonatal diabetes is often the presenting feature (e.g. Wolcott-Rallison Syndrome). Traditional genetic testing for neonatal diabetes requires accurate clinical information regarding the patient's phenotype to allow selection of a small number of genes to test. Since neonatal diabetes is generally the presenting feature of the disease, this approach is limited by the clinical information available at the time of referral and relies on timely clinical updates on the subsequent development of additional features from the referring clinicians.

The most common cause of neonatal diabetes are mutations in the potassium channel subunit genes *ABCC8* and *KCNJ11*⁵⁻⁷. Patients with neonatal diabetes caused by a potassium channel gene mutation are sensitive to sulfonylurea treatment and therefore their clinical management can be improved by replacing insulin with oral agents⁸⁻¹¹. This highlights the importance of an early genetic diagnosis in neonatal diabetes and international guidelines suggest immediate referral for genetic testing as soon as a clinical diagnosis of neonatal diabetes is made¹².

Three targeted next-generation sequencing assays have been developed for genetic testing of monogenic diabetes¹³⁻¹⁵, including our panel which includes all the 21 known neonatal diabetes genes¹⁴. A methylation assay is required to detect 6q24 abnormalities. We used the targeted next-generation sequencing assay to test all the genetically undiagnosed patients in a large, international cohort of 1020 neonatal diabetes patients with the objective of evaluating the impact of early, comprehensive genetic testing in this disease.

METHODS

Patient Cohort

Genetic testing was performed on 1020 patients (571 males, 449 females) diagnosed with diabetes before 6 months who were referred to the Exeter Molecular Genetics laboratory from 79 countries between January 2000 and August 2013. We excluded from the study 85 patients for whom there was insufficient DNA available for comprehensive testing (Supplementary Figure 1). Clinical information was provided by the referring clinicians from clinical notes via completion of a neonatal diabetes request form (available at www.diabetesgenes.org). Genetic testing for neonatal diabetes was offered free of charge to patients. The study was conducted in accordance with the Declaration of Helsinki principles with informed parental consent given on behalf of children.

The median age at diagnosis of diabetes was 6 weeks (IQR (expressed as Q1-Q3)=1-12 weeks), the median birth weight was 2,460g (median standard deviation score (SDS)=-1.69 (-2.6—0.8), median centile=4.73 (0.53-22.8)).

We defined patients born to consanguineous parents as those whose parents were first or second cousins (n=215), or those from countries with a high prevalence (>20%) of consanguineous unions¹⁶ (Supplementary Table 1) where genome-wide SNP typing showed genomic homozygosity of $\geq 1.56\%$ ¹⁷ as previously described¹⁸ (n=15) (Supplementary Figure 1). Chi-square test was used to assess the differences between the consanguineous and non-consanguineous groups.

Molecular genetic testing

All patients were tested until a causative mutation was identified by Sanger sequencing or targeted next-generation sequencing¹⁴ for all 21 known neonatal diabetes genes or by methylation analysis for chromosome 6q24 abnormalities.

The current testing pipeline is summarised in Supplementary Figure 2. Initial analysis consisted of rapid (<2 weeks) Sanger sequencing of *KCNJ11*, *ABCC8*, and *INS*¹⁹ with testing for chromosome 6q24 abnormalities by methylation analysis²⁰ in those patients with a clinical diagnosis of transient neonatal diabetes or those patients aged <6 months at the time of testing. Since 2012 this initial testing is followed by comprehensive testing of all other genes by targeted next-generation sequencing assay. Prior to 2012 additional genes were sequenced by Sanger sequencing according to clinical features or as part of a cohort analysis when a new gene was discovered and the phenotype needed to be defined.

In all patients for whom a genetic diagnosis had not been identified by this initial testing, a custom targeted next-generation sequencing panel¹⁴ was used to sequence all 21 known neonatal diabetes genes (see Supplementary Methods). This assay uses the Agilent SureSelect in solution capture system. Bait density and replication were adjusted from Agilent v1 exome capture data to achieve more even coverage over the targeted genes. A minimum coverage of 30 reads per base for the coding region +/-50bp was achieved for 98.5% of bases. Partial/whole gene deletions or duplications were identified by relative read depth coverage as previously described¹⁴. The mutations identified by targeted next-generation sequencing were confirmed by Sanger sequencing or MLPA.

We used the bioinformatic tool ALAMUT (Interactive Biosoftware, Rouen, France) to predict the effect of novel variants. Microsatellite analysis of parent/proband trios using the PowerPlex 16 kit (Promega, Southampton, UK) was used to confirm biological relationships in probands with apparently *de novo* mutations.

253 of the 1020 patients have been included in previous publications, numbers and references are reported in Supplementary Table 2.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the manuscript. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Molecular characterisation of neonatal diabetes

The genetic cause of neonatal diabetes was identified in 840/1020 patients (82% of cohort). These included mutations in all 21 known neonatal diabetes genes (details in Figure 2 and Supplementary Table 3) and chromosome 6q24 methylation abnormalities (n=113). The proportion of patients for whom a genetic diagnosis could not be identified was similar in patients born to unrelated parents (non-consanguineous) and related parents (consanguineous) (18% vs 15%, $p=0.27$, $df=1$), indicating that it is likely that both dominant and recessive causes of neonatal diabetes are still undiscovered (Figure 2, Supplementary Figure 1).

Parental consanguinity made a striking difference to the mode of inheritance of neonatal diabetes (Figure 2). The major difference was that, as expected, recessive causes were common (81%, 158/195) in the offspring of consanguineous parents but unusual (13%, 81/645) when the parents were not consanguineous ($p<0.0001$, $df=1$). Dominant heterozygous mutations were more common (71%, 457/645) in the offspring of non-consanguineous parents and the majority of them were spontaneous (68%, 216/320 when both parents were available for testing).

The major genetic aetiology differed depending on whether the parents were related. Mutations in *KCNJ11*⁵ and *ABCC8*^{6,7} account for 46% of cases in the non-consanguineous cohort, but only 12% in the consanguineous group ($p<0.0001$, $df=1$). This is an important diagnosis as glycaemic control can be dramatically improved for most of these patients by transferring from insulin injections to high dose sulfonylurea tablets^{9,11}. In patients born to consanguineous parents the most common cause (24%) of neonatal diabetes is a homozygous mutation in the *EIF2AK3* gene causing Wolcott-Rallison syndrome²¹.

Mutations in the *INS* gene were present at a similar proportion in the two groups (11% in non-consanguineous vs 10% in consanguineous, $p=0.89$, $df=1$) but the mechanism underlying the disease is fundamentally different. In the non-consanguineous group most *INS* gene mutations are heterozygous (90%, 77/86) and affect the structure of the preproinsulin protein²². Mouse models and *in vitro* studies suggest that these mutations result in altered protein folding causing severe endoplasmic reticulum stress and ultimately beta-cell destruction. Among patients born to consanguineous parents *INS* mutations are predominantly (75%, 18/24) homozygous loss of function changes that impair the synthesis of insulin²³.

Clinical features associated with specific molecular aetiologies of neonatal diabetes

The different genetic causes of neonatal diabetes identified so far have a range of both pancreatic and extra-pancreatic phenotypes (Figure 3). The specific subtypes have been described in detail elsewhere and are summarised in Supplementary Table 4.

i) Implications for diabetes treatment.

The pancreatic phenotypes include transient neonatal diabetes (6q24, *ABCC8*, *KCNJ11*, *INS*, *HNF1B*, *SLC2A2*, *ZFP57* subtypes), where the diabetes resolves (n=219), permanent diabetes responding to sulfonylurea treatment (*KCNJ11* and *ABCC8*, n=299), permanent insulin treated diabetes (*INS*, *GCK*, *EIF2AK3*, *FOXP3*, *GLIS3*, *NEUROD1*, *NEUROG3*, *NKX2-2*, *MXN1*, *IER3IP1*, *RFX6*, and some cases with *GATA6*, *GATA4* and *PDX1* mutations, n=265) and developmental disorders of the exocrine pancreas (*GATA6*, *PTF1A*, *PDX1* and *GATA4*) requiring pancreatic enzyme replacement in addition to insulin treatment (n=50). Diabetes caused by *SLC19A2* gene mutations can, in some cases, be successfully treated with thiamine (n=7). This means that identifying the genetic aetiology defines the treatment requirements for the endocrine and exocrine pancreatic function.

ii) Implications for extra-pancreatic features

Specific extra-pancreatic features are associated with different genetic subtypes of neonatal diabetes, with neurological features being the most common (n=184). Mutations in 9 genes (*ABCC8* (22% of cases), *KCNJ11* (29% of cases), *EIF2AK3*, *SLC19A2*, *IER3IP1*, *PTF1A*, *NEUROD1*, *MXN1*, *NKX2-2*) cause neonatal diabetes with neurological abnormalities. These additional features generally become evident in infancy, after diagnosis of neonatal diabetes; therefore early genetic diagnosis in these patients predicts future development of neurological complications.

Referral trend

The 1020 neonatal diabetes patients in our cohort were tested over a 14 year period from 79 referring countries. Genetic testing was offered free of charge to all these patients. We investigated the differences in referral rate and age at referral in this time period.

The median time from the diagnosis of diabetes to referral for genetic testing has shown a marked decrease from over 4 years (240 weeks, IQR=218-4099) in 2004 to less than 3 months (10 weeks, IQR=3-23) since 2012 (Supplementary Figure 3). The number of referrals per year has been steady over the last 10 years at 80-100 per year (Supplementary Figure 4) even though there has been a shift from prevalent cases to incident cases as shown in the median duration of diabetes at referral.

Impact of early genetic diagnosis

We investigated the effect of decreased time from diagnosis to referral for genetic testing to see if this resulted in patients having a genetic diagnosis before they developed typical clinical features of their genetic aetiology.

We evaluated the impact of early genetic testing in the 210 patients with a genetic diagnosis of transient neonatal diabetes caused by 6q24 methylation defects or potassium channel gene mutations (Figure 4A). The likelihood that the patient's diabetes had remitted before referral was dependent upon the time from diagnosis to genetic testing: only 10% (10/101) of patients tested early (<3 months from diagnosis) had remitted by the time of genetic testing but 100% (60/60) of late referrals (>48 months after diagnosis) had entered remission when genetic testing was performed ($p<0.0001$, $df=1$).

Recessive mutations in *EIF2AK3*, that confirm a genetic diagnosis of Wolcott-Rallison Syndrome, were found in 76 patients. Cardinal features of Wolcott-Rallison syndrome are neonatal diabetes, skeletal dysplasia and liver dysfunction. The non-diabetes features were present only in 12% (3/26) of patients with early referral (<3 months from diagnosis of diabetes) but were found in most patients (83%, 15/18) with late referral (>48 months from diagnosis of diabetes, $p<0.0001$, $df=1$) (Figure 4B).

Specific mutations in *KCNJ11* and *ABCC8* cause a syndromic form of neonatal diabetes characterised by severe development delay and neurological features (DEND - Developmental delay, Epilepsy and Neonatal Diabetes - and iDEND syndrome)^{5,7}. The most common of these mutations is the *KCNJ11* p.Val59Met which was detected in 26 patients in our cohort. Among these patients, neurological features were not present in any of those with early referral (0/7 referred <3 months from diagnosis) but were found in 100% of patients with late referral (14/14 referred >48 months from diagnosis of diabetes, $p<0.0001$, $df=1$) (Figure 4C).

DISCUSSION

Patients with neonatal diabetes are now referred for genetic testing close to their presentation with hyperglycaemia, often before other features have developed or are recognized. Comprehensive testing (including next-generation sequencing) of the 22 known genetic aetiologies identifies causal mutations in >80% of cases. An early genetic diagnosis predicts optimal diabetes treatment and allows anticipation of the development of related features.

Neonatal diabetes is therefore a good example of how early comprehensive genetic testing including next-generation sequencing can improve clinical management of a genetically heterogeneous disease. This will likely have implications for many other genetically heterogeneous disorders such as hereditary hearing loss²⁴, congenital muscular dystrophy²⁵, and inherited retinal diseases²⁶. Gene panels for early genetic diagnosis of these conditions have already been developed and are currently utilised to identify subsets of patients who are eligible for gene therapy²⁶. The paradigm shift in genetic testing has likely occurred earlier for neonatal diabetes as a consequence of the availability of a free, comprehensive test and, importantly, because of the possibility of treatment change for almost 40% of patients.

Implications for diabetes

Neonatal diabetes is a clinically and genetically heterogeneous disease, with 22 known genetic causes. Different genetic defects define the clinical subtypes of neonatal diabetes with important implications for clinical management and treatment (Figure 3, Supplementary Table 4). Approximately 40% of patients in our cohort have a mutation in a potassium channel subunit gene (*ABCC8* and *KCNJ11*) and most achieve improved glycaemic control upon transfer from insulin therapy to high dose sulfonylureas^{9,11}.

210/1020 patients in our cohort have a genetic diagnosis of transient neonatal diabetes caused by methylation abnormalities resulting in overexpression of the paternally inherited allele of genes at the 6q24 locus²⁷⁻²⁹ or mutations in *ABCC8* or *KCNJ11*³⁰. Patients with transient neonatal diabetes due to a potassium channel mutation tend to be diagnosed and remit later than patients with 6q24 methylation abnormalities (median age at diagnosis = 4 vs 0 weeks, median age at remission = 35 vs 13 weeks)^{7,31,32}. With the decrease in the age at referral in our cohort, 50% (105/210) of transient neonatal diabetes patients were referred before diabetes remission. Of the 48% of patients (101/210) referred less than 3 months from diagnosis 90% of them received a genetic diagnosis of transient neonatal diabetes before diabetes remission (Figure 4A).

These data illustrate a radical change in the role of genetic testing in neonatal diabetes where for the majority of patients the genetic test now predicts whether the diabetes will be transient or permanent, in addition to guiding treatment decisions (Figure 3).

Implications for extra-pancreatic features

Neonatal diabetes is a clinical feature of 16 syndromes caused by mutations in 17 genes and in most of them it is the presenting feature. Our data show that in the last 10 years the median time from clinical diagnosis to referral for genetic testing has fallen from over 4 years to less than 3 months (Supplementary Figure 3), meaning that most patients now have genetic testing before development of the additional clinical features that characterise the syndrome. We evaluated the impact of early genetic testing in the most common of these syndromes, Wolcott-Rallison syndrome. Wolcott-Rallison syndrome is caused by biallelic mutations in *EIF2AK3*, a gene known to be important for regulation of endoplasmic reticulum stress²¹. A clinical diagnosis of Wolcott-Rallison syndrome requires the presence of insulin-dependent diabetes and skeletal dysplasia and/or liver dysfunction (reviewed by Julier *et al*³³). While most of these patients are diagnosed with diabetes in the first 6 months of life, skeletal dysplasia is not evident until the infant is 1 or 2 years of age and liver dysfunction generally manifests during intercurrent illness as recurrent episodes of acute liver failure which can present at any time after the neonatal diabetes. 61% of patients (46/76) in our cohort received a genetic diagnosis of Wolcott-Rallison syndrome before developing either skeletal dysplasia or liver failure. This proportion was even higher (89%) for patients with early referral (less than 3 months from diagnosis) (Figure 4B). An early diagnosis of Wolcott-Rallison Syndrome is important to ensure rapid management of episodes of acute liver failure, which is a life threatening complication in these patients.

Activating mutations in *ABCC8* and *KCNJ11* are associated with a variable spectrum of phenotypes according to genotype³⁴: milder mutations cause transient neonatal diabetes, whilst mutations which severely affect the potassium channels' ability to respond to ATP levels cause permanent neonatal diabetes associated with neurological/developmental features (iDEND and DEND)^{5,7,30} (Figure 3, Supplementary Table 4). The most common of these mutations is the *KCNJ11* p.Val59Met which was identified in 26 patients in our cohort. Among these patients, 100% of those referred for genetic testing less than 12 months after clinical diagnosis of diabetes had genetic testing before development of additional neurological features (Figure 4C). This is extremely important since previous studies have shown that high dose sulfonylurea therapy can improve neurological symptoms in patients with iDEND and DEND^{35,36}. In these cases an early genetic diagnosis provides clinicians with valuable information for the patients' clinical management, indicating the possibility of treatment change and awareness of the development of neurological features.

The awareness of future development of additional clinical features as a consequence of the genetic diagnosis has important implications for patients with other neonatal diabetes-associated syndromes, such as IPEX syndrome (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked). IPEX syndrome is a severe multi-organ autoimmune disease caused by a mutation in *FOXP3*³⁷. Onset is usually within the first months of life, with one of the 3 cardinal features: severe enteropathy, eczema or diabetes. Timely management of the symptoms is essential for survival. An early genetic diagnosis of IPEX syndrome is critically important for clinical management and treatment, since early allogeneic hematopoietic stem cell transplant (HSCT) has been shown to be the intervention leading to the best outcome (reviewed by Barzaghi *et al*³⁸).

Conclusions

In conclusion, our study describes the paradigm shift occurring in clinical practice once genetic testing is the initial investigation. Traditionally, genetic testing was employed to confirm a clinical diagnosis based on disease course or a cluster of clinical features. Now early comprehensive genetic testing gives a diagnosis before the development of specific features (Figure 1). The future of care in neonatal diabetes will increasingly rely on the results of genetic testing with the genetic diagnosis not only informing a clinician of the likely course and best treatment for the diabetes but also predicting development of additional clinical features. This represents a new paradigm with genetic testing defining, rather than just confirming, the clinical diagnosis.

Contributors

ATH and SE designed the study. EDF did the molecular and statistical analysis and wrote the manuscript. SEF, JALH, DJGM, IKT and SE analysed the molecular and clinical data and reviewed the manuscript. HLA did the bioinformatic analysis and reviewed the manuscript. ATH analysed the clinical data and reviewed the manuscript.

Declaration of Interests

The authors declare no conflict of interests.

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Panel: Research in context

Systematic review

We searched PubMed using MeSH terms "Neonatal Diabetes" OR "PNDM" OR "TNDM" AND "genetic testing" OR "sequencing" OR "mutation" for articles published before January 1st, 2014 describing genetic testing in neonatal diabetes. None of the articles identified by this search evaluated the results and impact of comprehensive genetic testing in patients with neonatal diabetes. There were 2 studies describing the development of targeted next generation sequencing approaches for genetic testing in neonatal diabetes^{13,15} but they only described their use in relatively small validation studies. The recent large study of 174 neonatal diabetes patients by Busiah *et al*³² reported the analysis of the 4 commonest of the 22 known genetic causes. Multiple studies have described the improvement in glycaemic control occurring in patients with a *KCNJ11* or *ABCC8* mutation transferring from insulin to sulfonylurea therapy eg⁷⁻¹⁰. There was no report of whether the progress in genetic testing has resulted in reduced time from diagnosis of diabetes to referral for genetic analysis over the past decade or the effect early genetic diagnosis might have on the clinical management of patients. Therefore we performed comprehensive testing in a large cohort of patients with neonatal diabetes and examined how an early genetic diagnosis may impact on clinical care.

Interpretation

We performed comprehensive genetic testing in the largest cohort of neonatal diabetes patients described to date (n=1020) from 79 countries over a 14 year period. In this study we provide an accurate estimation of the molecular contribution and clinical features of the 22 known genetic causes of neonatal diabetes. We report that patients are now referred for genetic testing soon after diagnosis, often when they only have isolated diabetes. Early comprehensive genetic testing allows identification of the underlying genetic defect in 82% of patients. The genetic diagnosis will inform clinicians on the probable course and optimal management of the patient's diabetes and the likely future development of additional clinical features. To our knowledge this is the

first study describing a new paradigm for clinical care in neonatal diabetes with genetic diagnosis often preceding development of clinical features and guiding clinical management.

Figure legends

Figure 1. The paradigm shift of genetic testing. Schematic representation of the steps involved in genetic testing in the pre and post next-generation sequencing era.

Figure 2. Different genetic causes of neonatal diabetes in patients born to non-consanguineous and consanguineous parents. Comparison of genetic causes of neonatal diabetes in non-consanguineous (n=790) and consanguineous groups (n=230). Consanguinity is defined by parents being second cousins or more closely related or by the presence of $\geq 1.56\%$ total homozygosity¹⁷. Genes mutated in $< 2.5\%$ of patients in both cohorts were grouped in the 'Other' category (Supplementary Table 3).

Figure 3. A genetic diagnosis guides clinical management. Schematic representation of genetic causes of neonatal diabetes and the implications of this genetic diagnosis. N indicates the number of patients identified with mutations in each of the genes in the 1020 neonatal diabetes patient cohort. Solid arrows indicate implications for the majority of mutations in the genes. Dashed arrows indicate the implications for specific mutations.

Figure 4. Genetic diagnosis precedes development of additional clinical features defining the neonatal diabetes subtype. **A. Impact of early genetic diagnosis in transient neonatal diabetes caused by 6q24 methylation defects or potassium channel gene mutations.** Bar chart representing clinical features at the time of genetic testing for neonatal diabetes. Yellow = diabetes, blue = diabetes remitted. **B. The impact of age at genetic testing on whether patients have non-diabetes features of Wolcott-Rallison Syndrome at the time of referral for genetic testing.** Bar chart representing clinical features at the time of genetic testing for neonatal diabetes. Yellow = diabetes only, solid blue = diabetes and either skeletal abnormalities or liver dysfunction, diagonal blue lines = diabetes, skeletal abnormalities and liver dysfunction. **C. The impact of age at genetic testing on whether patients with a *KCNJ11* p.Val59Met mutation have neurological features at the time of referral for genetic testing.** Bar chart representing clinical features at the time of genetic testing for neonatal diabetes. Yellow = diabetes only, solid blue = diabetes and neurological features.

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Figure 1

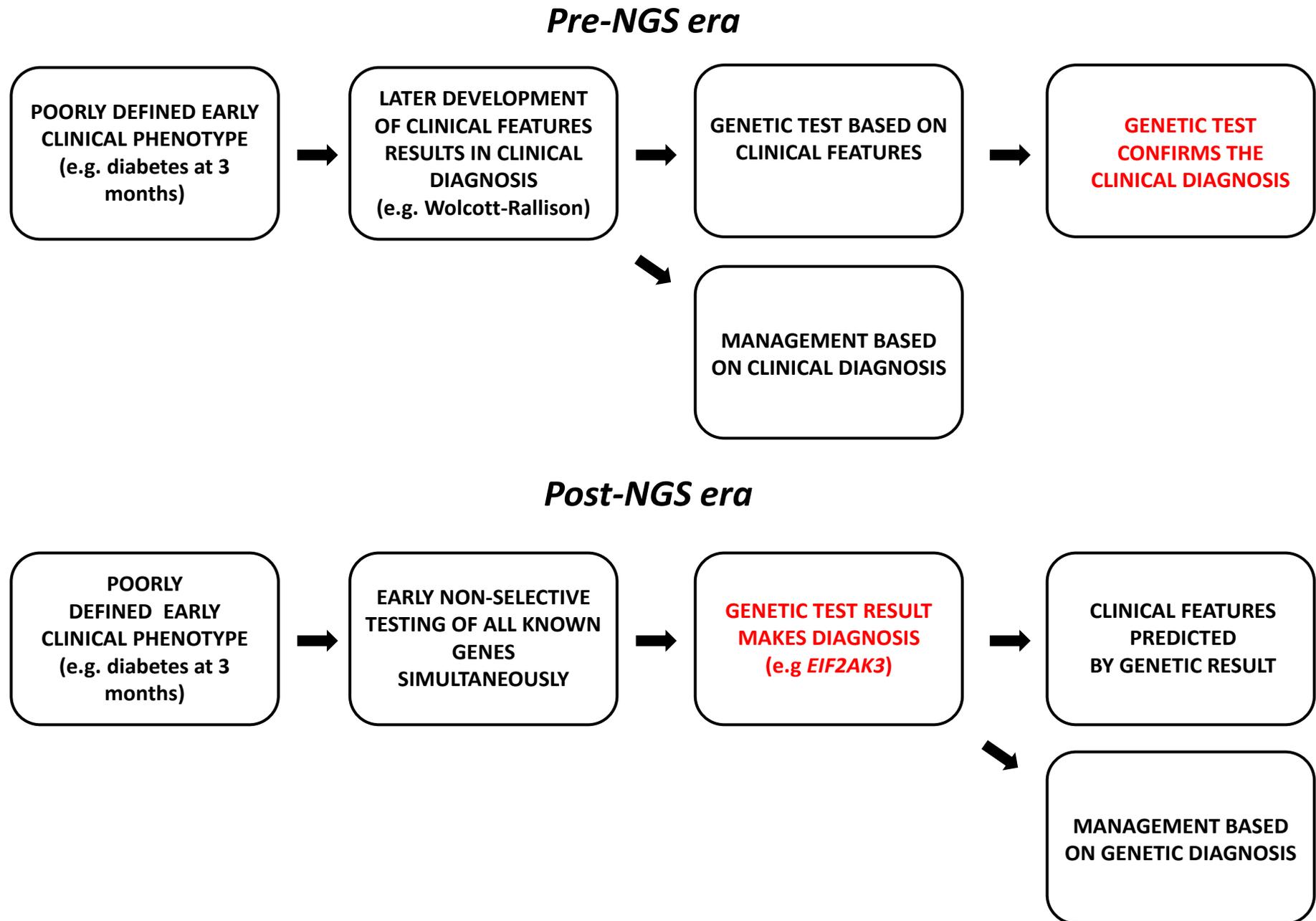


Figure 2

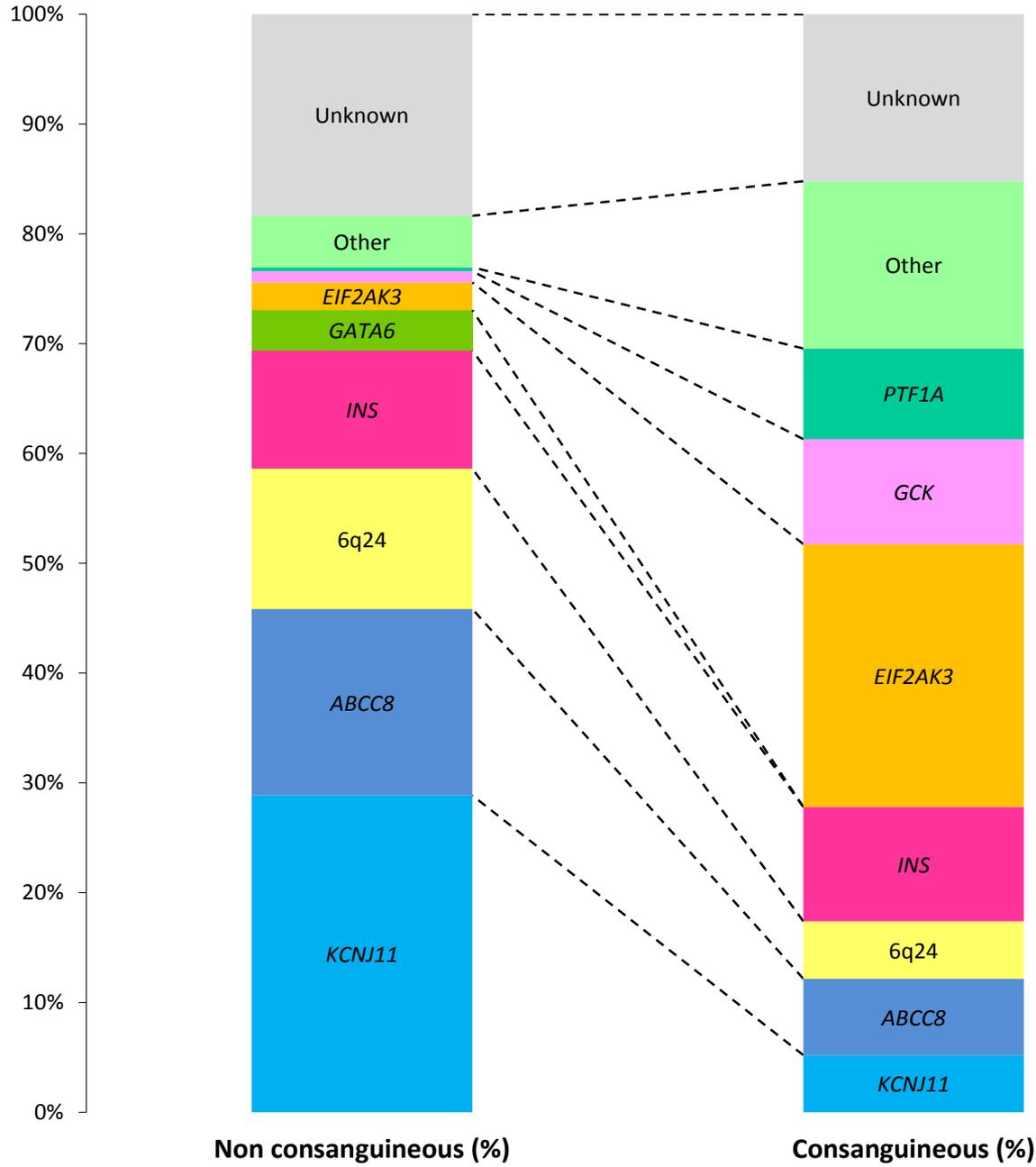
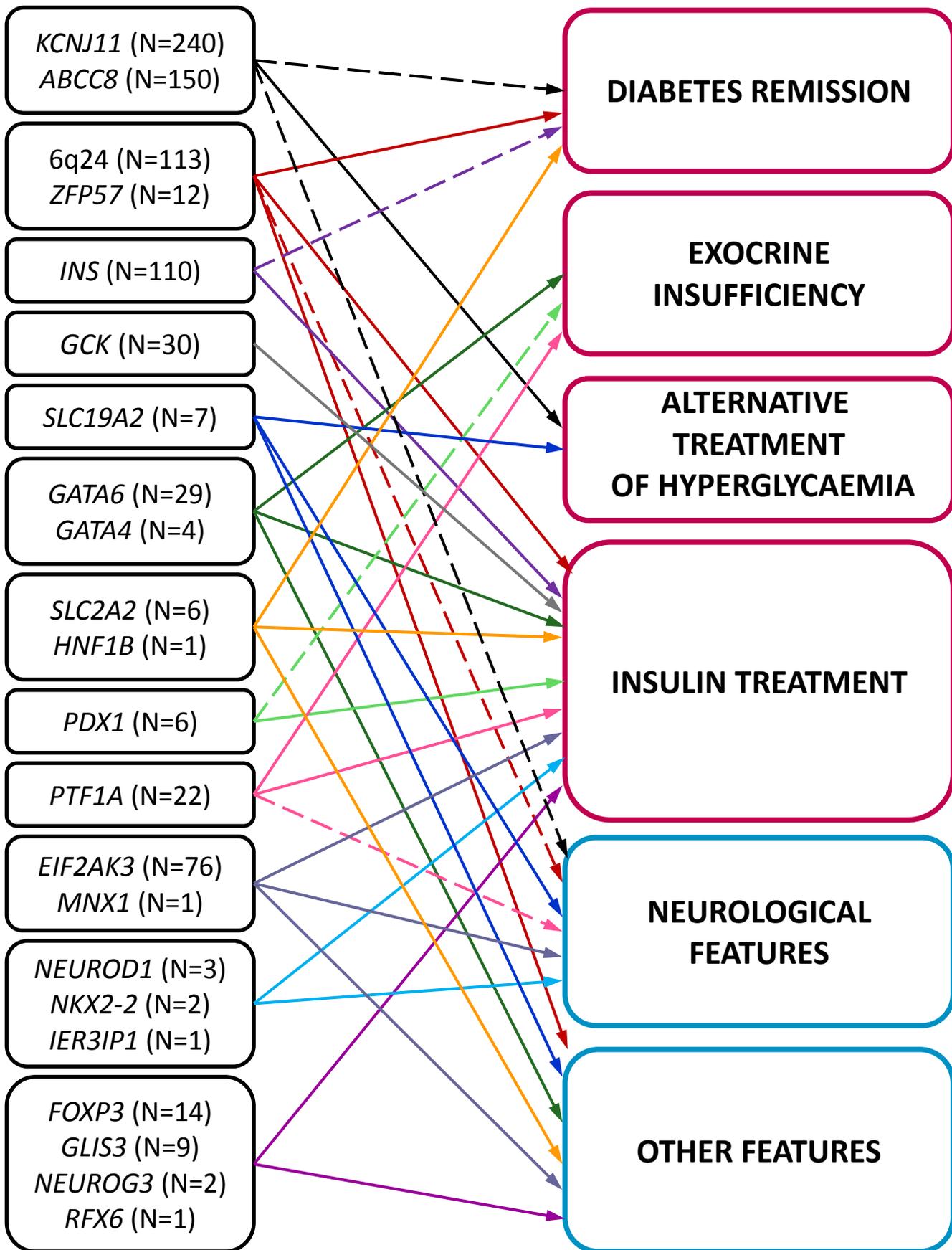


Figure 3

Implications for pancreatic phenotype



Development of extra-pancreatic features

Figure 4A

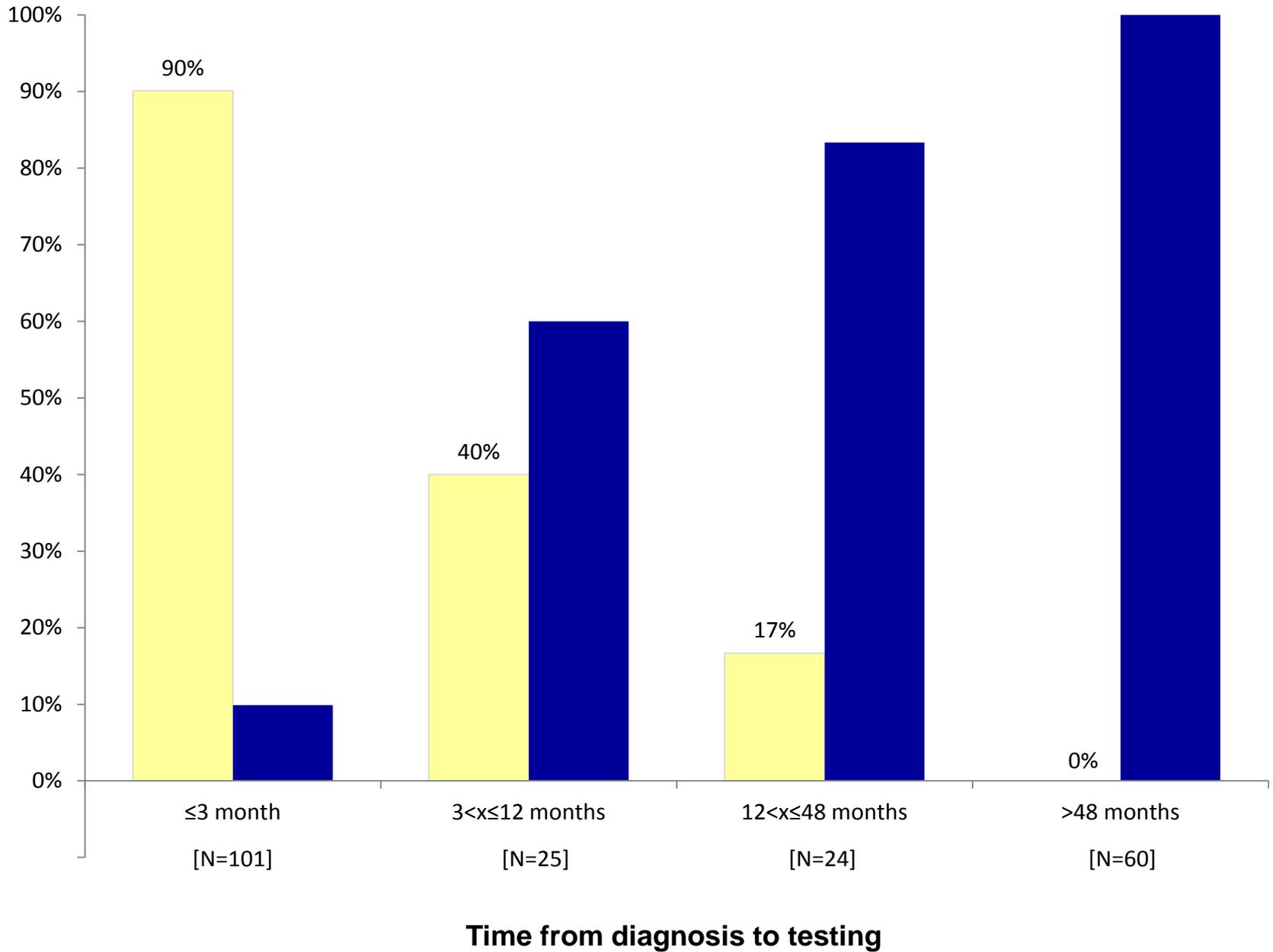


Figure 4B

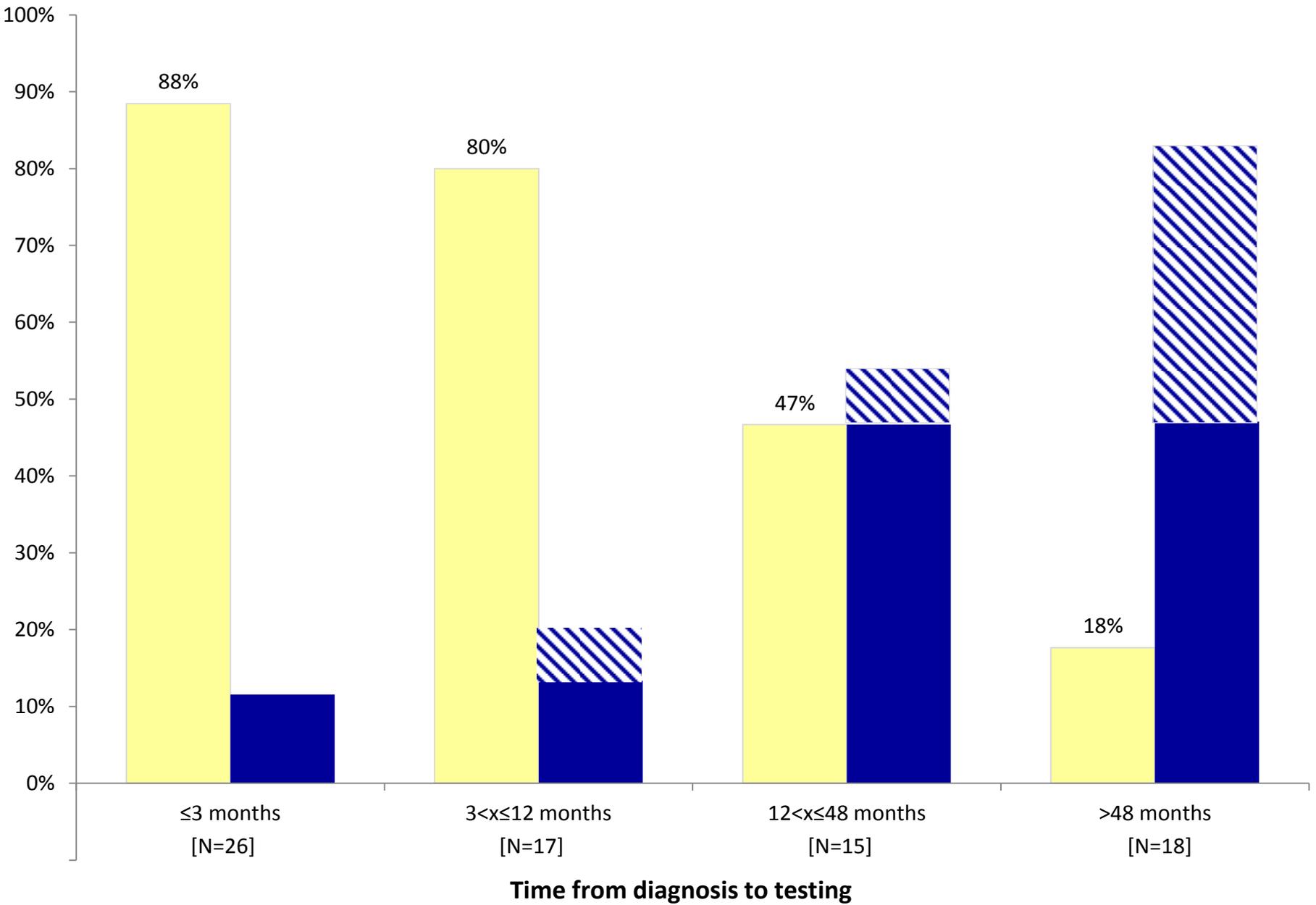
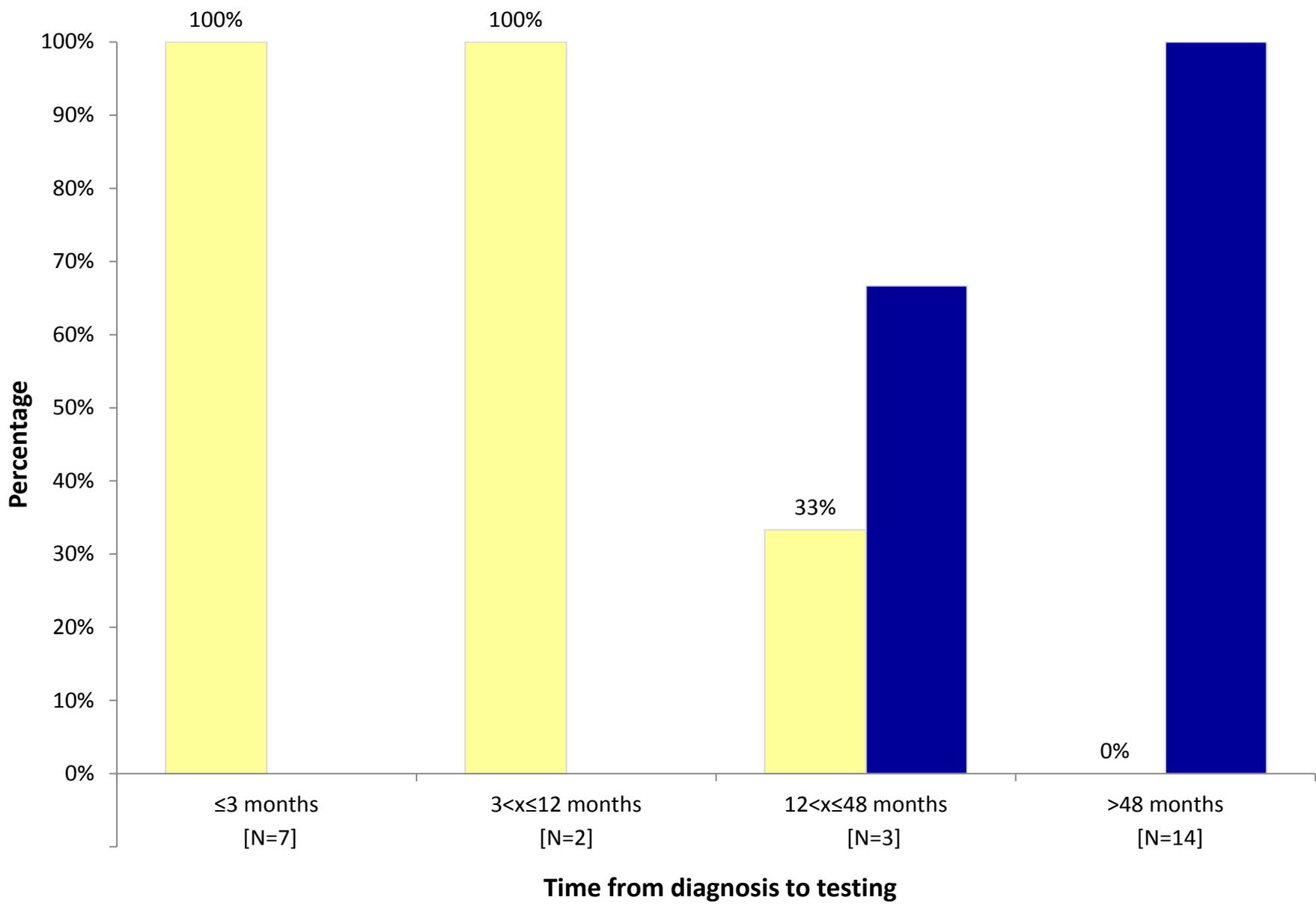


Figure 4C



Supplementary Note

Genomic testing leads clinical care in neonatal diabetes: a new paradigm

De Franco E¹, Flanagan SE¹, Houghton JAL¹, Lango Allen H¹, Mackay DJG^{2,3}, Temple IK^{3,4}, Ellard S¹ and Hattersley AT¹

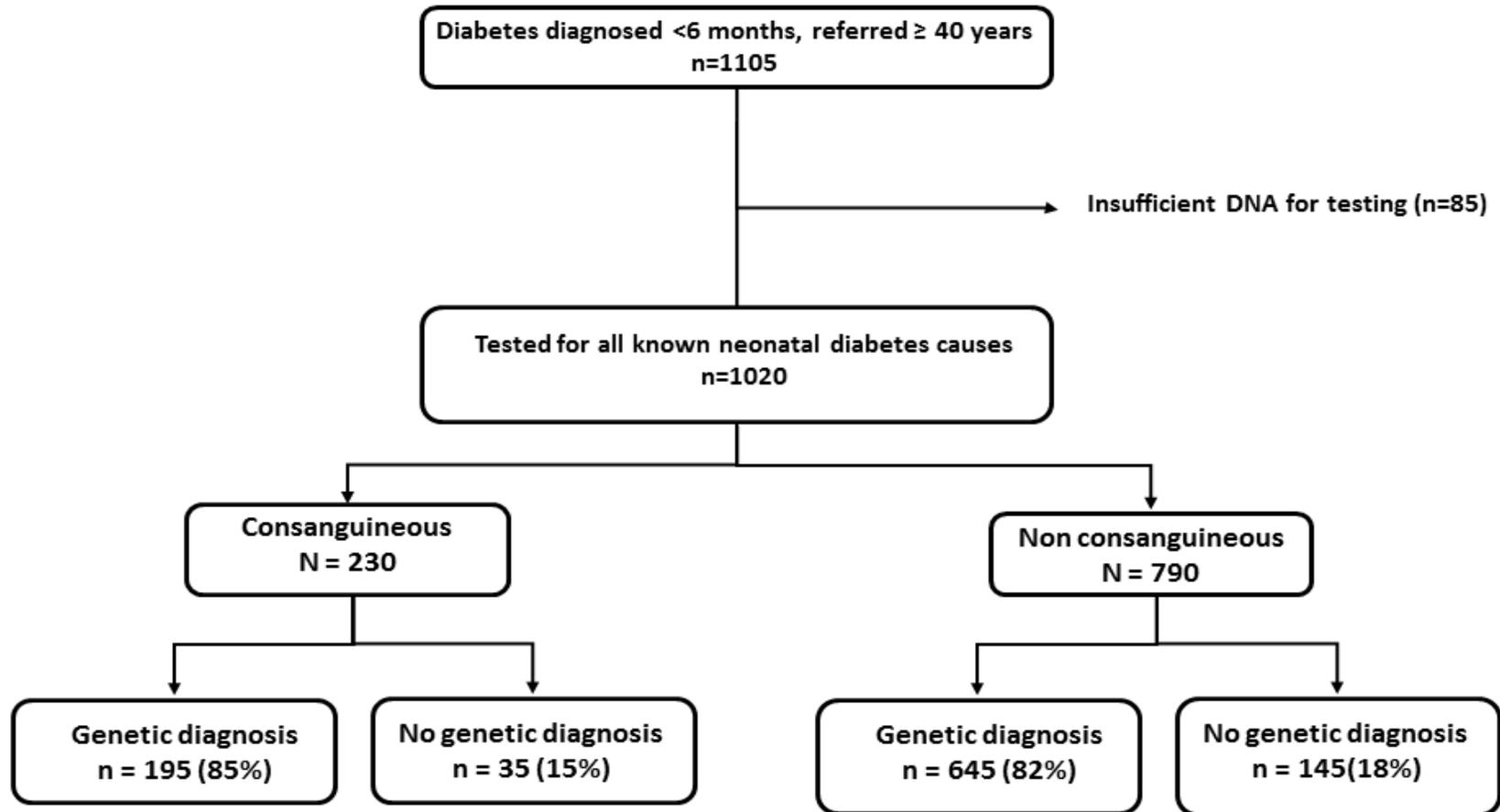
¹Institute of Biomedical and Clinical Science, University of Exeter Medical School, EX2 5DW, UK

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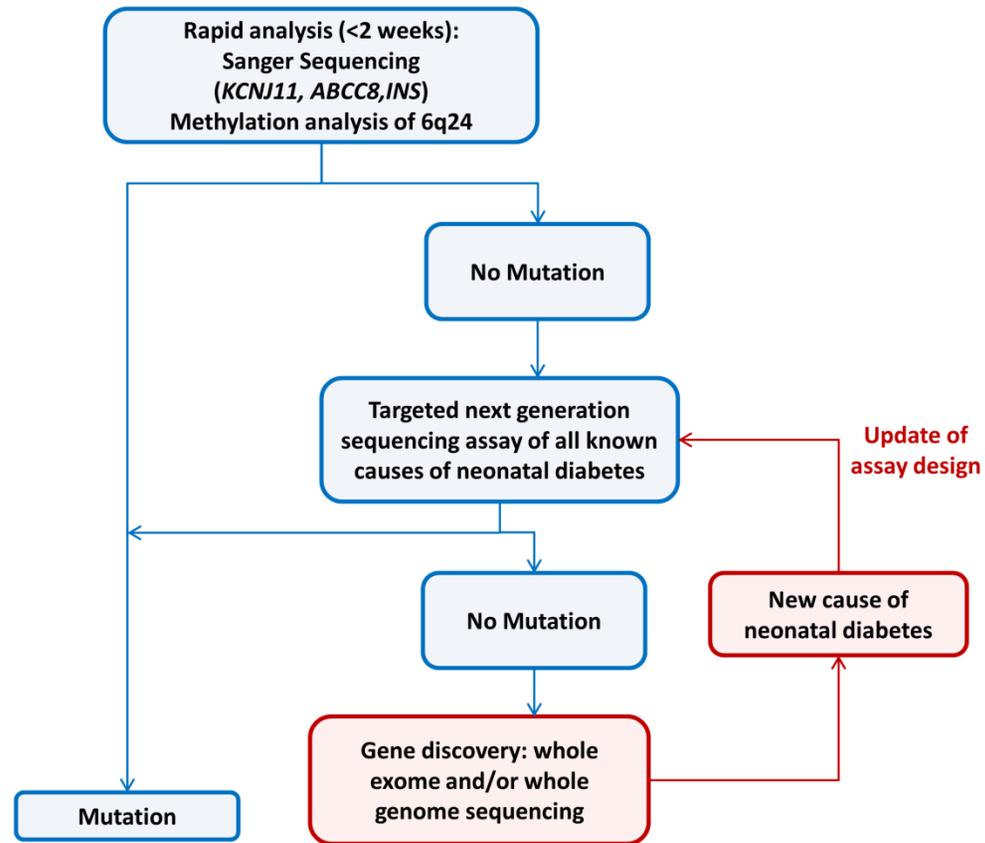
³Human Genetics and Genomic Medicine, Faculty of Medicine, University of Southampton, Southampton, UK

⁴Wessex Regional Genetics, Salisbury Foundation Trust and University Hospital Southampton NHS Trust, Salisbury, UK

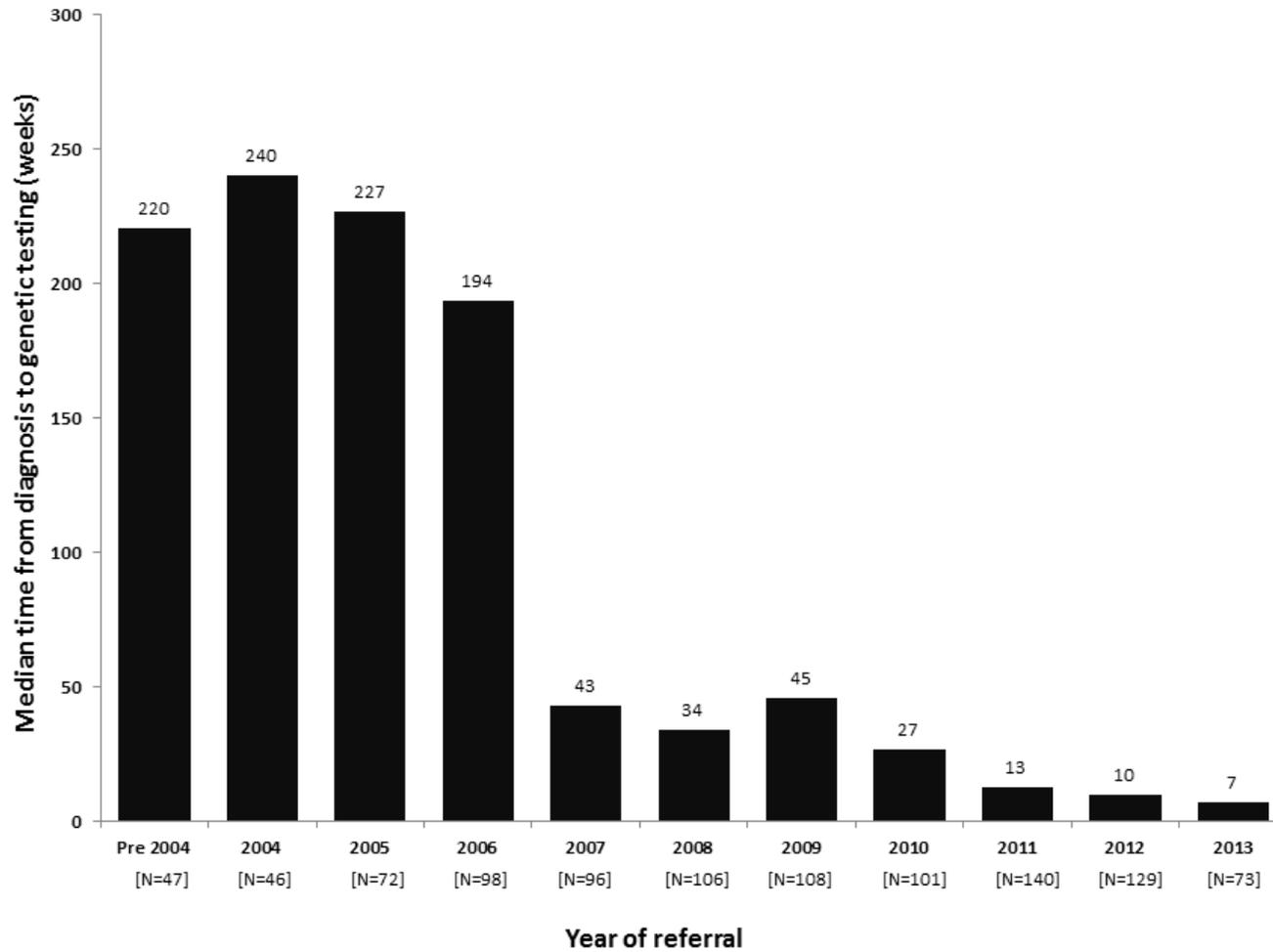
Supplementary Figure 1. Schematic representation of the neonatal diabetes cohort. Analysis of genetic aetiologies excluded patients for whom there was insufficient DNA for comprehensive testing (n=85).



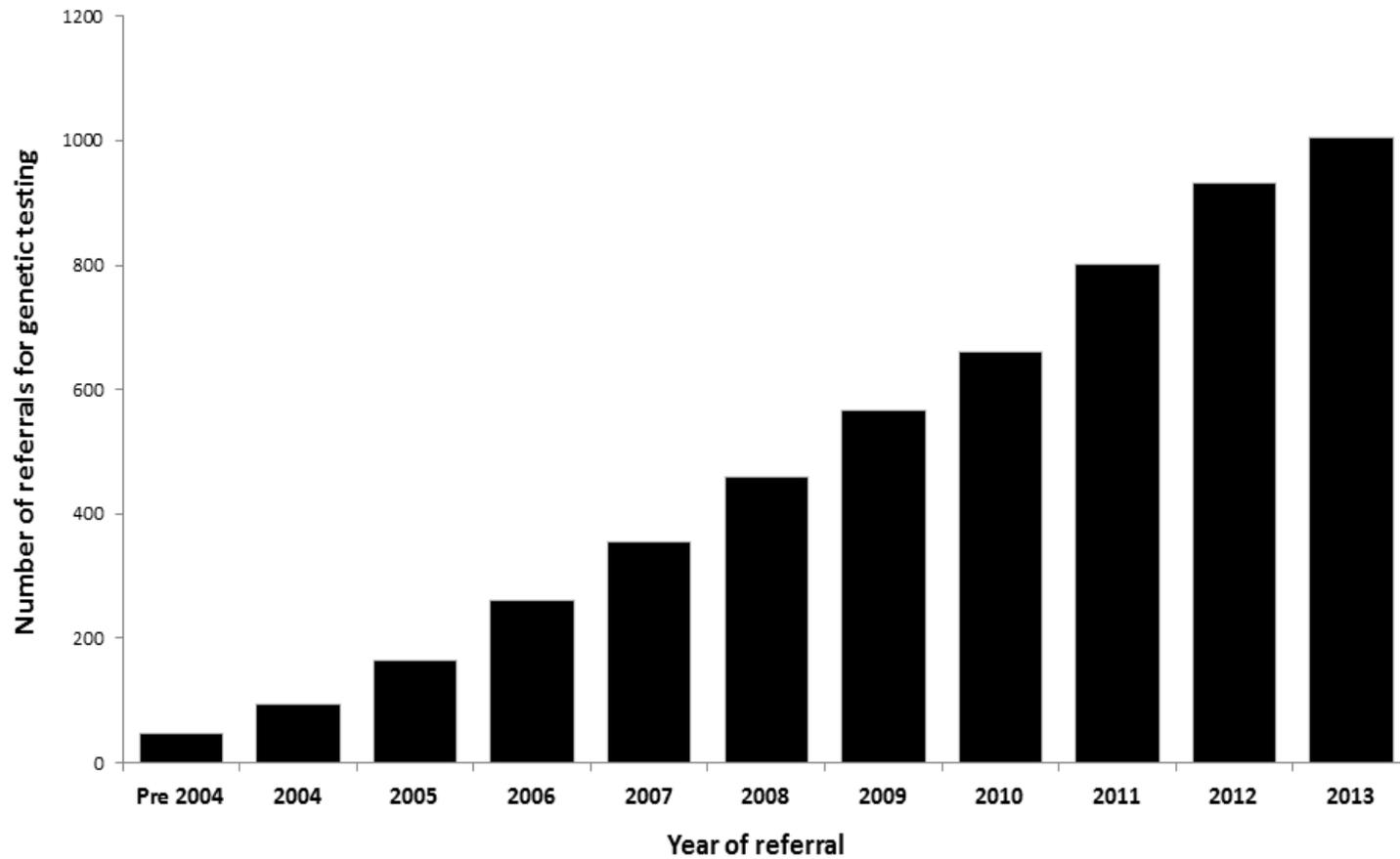
Supplementary Figure 2: Current genetic testing pipeline for neonatal diabetes referrals. Blue outline: diagnostic pipeline. Red outline: gene discovery pipeline



Supplementary Figure 3. Fall of the median time from diagnosis to referral for genetic testing over time. Bar chart representing the median time from clinical diagnosis of diabetes to referral for genetic testing between 01/01/2000 and 31/08/2013 (N=1016, age at diagnosis not available for N=4).



Supplementary Figure 4. Increase in the total number of referrals over time. Bar chart representing the cumulative number of worldwide referrals from 01/01/2000 to 31/08/2013 (N=1020).



Supplementary Table 1. List of countries with high prevalence (>20%) of consanguineous unions¹ and number of referrals for neonatal diabetes testing to the Exeter Molecular Genetics laboratory

Country	Number of neonatal diabetes referrals
Bahrain	2
Bangladesh	10
Egypt	9
India	75
Iran	2
Iraq	1
Israel	6
Jordan	16
Kuwait	8
Lebanon	2
Libya	6
Morocco	8
Oman	8
Pakistan	9
Qatar	1
Saudi Arabia	36
Sudan	8
Syria	1
Tunisia	1
Turkey	83
United Arab Emirates	14

Supplementary Table 2. Number and references for 253 patients included in the cohort who have been included in previous publications by the Exeter team.

Gene	N of patients tested in Exeter and previously published	References
<i>ABCC8</i>	41	2-13
<i>EIF2AK3</i>	27	3, 14-17
<i>FOXP3</i>	5	18
<i>GATA4</i>	4	19
<i>GATA6</i>	25	20-22
<i>GCK</i>	28	23
<i>GLIS3</i>	4	15, 24
<i>HNF1B</i>	1	25
<i>IER3IP1</i>	0	
<i>INS</i>	40	26-28
<i>KCNJ11</i>	50	3, 4, 7, 9, 29-43
<i>MNX1</i>	1	44
<i>NEUROD1</i>	2	45
<i>NEUROG3</i>	1	46
<i>NKX2-2</i>	2	44
<i>PDX1</i>	3	47
<i>PTF1A</i>	11	22, 48
<i>RFX6</i>	1	49
<i>SLC19A2</i>	2	50
<i>SLC2A2</i>	5	51
Total	253	

Supplementary Table 3. Genetic causes of neonatal diabetes identified in 840 neonatal diabetes patients.

Genetic cause	Mode on inheritance	Non consanguineous N (%)	Consanguineous N (%)
6q24		101 (12.8%)	12 (5.2%)
ABCC8	Dominant	112 (14.2%)	3 (1.3%)
	Recessive	22 (2.8%)	13 (5.7%)
EIF2AK3	Recessive	20 (2.5%)	56 (24.3%)
FOXP3	X-linked	11 (1.4%)	3 (1.3%)
GATA4	Dominant	3 (0.4%)	1 (0.4%)
GATA6	Dominant	29 (3.7%)	0 (0.0%)
GCK	Recessive	8 (1.0%)	22 (9.6%)
GLIS3	Recessive	3 (0.4%)	6 (2.6%)
HNF1B	Dominant	2 (0.3%)	0 (0.0%)
IER3IP1	Recessive	0 (0.0%)	1 (0.4%)
INS	Dominant	77 (9.7%)	6 (2.6%)
	Recessive	9 (1.1%)	18 (7.8%)
KCNJ11	Dominant	228 (28.9%)	12 (5.2%)
MNX1	Recessive	0 (0.0%)	1 (0.4%)
NEUROD1	Recessive	1 (0.1%)	2 (0.9%)
NEUROG3	Recessive	2 (0.3%)	0 (0.0%)
NKX2-2	Recessive	0 (0.0%)	2 (0.9%)
PDX1	Recessive	2 (0.3%)	4 (1.7%)
PTF1A	Recessive	3 (0.4%)	19 (8.3%)
RFX6	Recessive	0 (0.0%)	1 (0.4%)
SLC19A2	Recessive	2 (0.3%)	5 (2.2%)
SLC2A2	Recessive	2 (0.3%)	4 (1.7%)
ZFP57	Recessive	8 (1.0%)	4 (1.7%)
Unknown		145 (18.4%)	35 (15.2%)
Total		790	230

Supplementary Table 4. Summary of the clinical features associated with the 22 neonatal diabetes subtypes. * indicates features associated to specific mutations.

Genetic cause	Neonatal Diabetes Phenotype	Diabetes Treatment	Exocrine insufficiency needing replacement therapy	Additional Features	References
6q24	Transient	Insulin	No	Intrauterine growth retardation, macroglossia, umbilical hernia, neurological features (rare)	52-55 56
ABCC8	Transient, Permanent	Sulfonylureas	No	Developmental delay with/without epilepsy* (22% of cases)	12, 57 56
EIF2AK3	Permanent	Insulin	No	Skeletal dysplasia, liver dysfunction, developmental delay	17, 58
FOXP3	Permanent	Insulin	No	Eczema, enteropathy, other autoimmune features	59
GATA4	Transient, Permanent	Insulin	Yes*	Congenital heart malformation	19, 60
GATA6	Transient (rare), Permanent	Insulin	Yes	Congenital heart malformation, neurological defects, hypothyroidism, gut and hepato-biliary malformation	21, 22
GCK	Permanent	Insulin	No		61-67
GLIS3	Permanent	Insulin	No	Congenital hypothyroidism, renal cysts	24, 68, 69
HNF1B	Transient	Insulin	No	Pancreatic hypoplasia, renal cysts	25, 70
IER3IP1	Permanent	Insulin	No	Microcephaly, epilepsy	71-73
INS	Transient, Permanent	Insulin	No		27, 28 56
KCNJ11	Transient, Permanent	Sulfonylureas	No	Developmental delay with/without epilepsy* (29% of cases)	35, 36 56
MNX1	Permanent	Insulin	No	Sacral agenesis, neurological defects	44
NEUROD1	Permanent	Insulin	No	Cerebellar hypoplasia, sensorineural deafness, visual impairment	45
NEUROG3	Permanent	Insulin	No	Congenital malabsorptive diarrhea	46

<i>NKX2-2</i>	Permanent	Insulin	No	Severe neurodevelopmental defects	44
<i>PDX1</i>	Permanent	Insulin	Yes*		47, 74-76
<i>PTF1A</i>	Permanent	Insulin	Yes	Cerebellar agenesis*	77, 78, 48
<i>RFX6</i>	Permanent	Insulin	No	Intestinal atresia and/or malrotation, gall-bladder agenesis	49, 79
<i>SLC19A2</i>	Permanent	Thiamine	No	Thiamine-responsive megaloblastic anemia, sensorineural deafness	50, 80-82
<i>SLC2A2</i>	Transient	Insulin	No	Hepato-renal glycogen accumulation, renal dysfunction, impaired utilization of glucose and galactose	51
<i>ZFP57</i>	Transient	Insulin	No	Intrauterine growth retardation, neurological features (rare)	83, 84

Supplementary Methods

Samples were fragmented using a Bioruptor (Diagenode, Liège, Belgium), indexed for multiplexing and hybridised (in pools of 12 samples) according to the manufacturer's instructions. Sequencing was performed with an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA) (48 samples per lane) and 100 bp paired end reads. The resulting reads were aligned with BWA and duplicates were removed with Picard. We then applied GATK indel realignment, and performed SNV and INDEL discovery and genotyping using GATK UnifiedGenotyper with standard hard filtering parameters according to GATK Best Practices recommendations⁸⁴. Variants were annotated with ANNOVAR and pathogenic mutations located within 50 bp upstream and 50 bp downstream of each exon were identified.

As previously described⁸⁵, for the 21 genes for which testing is available in the Exeter laboratory by Sanger sequencing, the average depth of coverage was over 250 reads and >99% of bases had a minimum read depth of 30. Two specific regions of low coverage (<20 reads) were observed across two ~300bp GC-rich regions in the exon 2 of *GATA6* and *GATA4*. In patients for whom these regions were not sufficiently covered and no pathogenic mutation was identified, Sanger sequencing of the specific exon 2 amplicons were carried out in patients with congenital features suggestive of a *GATA6/GATA4* mutation (e.g. low birth weight, exocrine insufficiency, congenital heart malformation). Two positive controls (for a known heterozygous deletion and a known insertion) were included in each 48 sample batch to verify the ability to detect deletions/insertions.

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