Croissance et système des IGFs (insulin-like growth factors) : l'apport physiopathologique des maladies soumises à empreinte parentale

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Sorbonne Université

École doctorale 934 : physiologie, physiopathologie et thérapeutique

Centre de recherche Saint-Antoine – UMR 938

Système des IGF et croissance fœtale et postnatale

Croissance et système des IGFs (*insulin-like growth factors*) : l'apport physiopathologique des maladies soumises à empreinte parentale

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Thèse de doctorat présentée et soutenue publiquement 1er juillet 2019

Devant un jury composé de :

Pr Béatrice Dubern	Présidente du jury
Pr Juliane Léger	Rapporteure
Pr Jean-Marc Ricort	Rapporteur
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Je termine avec un pudique « merci » à toi, Gabriel, dans lequel tu sais qu'il faut mettre une reconnaissance infinie pour ton soutien et ta compréhension de chaque instant.

Abréviations

AKT : protéine kinase B

ALS : acid-labile subunit

CBS : CTCF binding site

CCDC8 : coiled-coil domain containing 8

CDKN1c : cyclin-dependant kinase inhibitor type 1

CpG : dinucleotide CG

CTCF : CCTC-binding factor

CUL7 : cullin 7

DLK1: delta like non-canonical notch ligand 1

DMR : differentially methylated regions

DNMT : DNA methyltransférase

DOHaD : Developmental Origins of Health and Disease

DPPA3: developmental pluripotency-associated protein 3

DS : déviation standard

EGF : epidermal growth factor

ERK : extracellular signal-regulated kinase

ELISA : dosage immuno-enzymatique

FGF : fibroblast growth factor

GH : hormone de croissance

GHR : récepteur de l'hormone de croissance

GHRH : GH releasing hormone

GNAS : G protein subunit alpha S

ICF : immuno-déficience, instabilité paracentromérique, dysmorphie faciale

ICR : imprinting center region

IGF : insulin-like growth factor

IGN : imprinted genes network

IGF1R : récepteur de type 1 des IGF

IGF2R ou M6PCI : récepteur du mannose 6-phosphate cation dépendant

IGFBP : insulin-like growth factor binding protein

iPPSD : inactivating PTH/PTHrp signaling disorders

IR : récepteur de l'insuline

IRS : insulin receptor substrate

K_i : constante de dissociation

KO : knock-out

LOM 11p15 : hypométhylation en *H19/IGF2*:IG-DMR

MAPK : mitogen-activated protein kinase
MBD : methyl CpG binding domain
MeCP : methyl CpG binding protein
MEG: maternally expressed gene
MIM : mendelian inheritance in man
miR : microARN
MLID : multiloci imprinting defects
mupd7 : disomie uniparentale maternelle du chromosome 7
NH-CSS : Netchine-Harbison clinical scoring system
NLRP : nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain
OBSL1 : obscurin like 1
PAG : petit pour l'âge gestationnel
PAPP-A : pregnancy associated plasma protein-a
PLAGL1 : pleomorphic adenoma gene 1
PRGF : platelet-derived growth factor
PHP : pseudohypoparathyroïdie
PI3K : phosphatidylinositol 3-kinase
PTEN : phosphatase and tensin homolog
PTP : phospho-tyrosine phosphatases
rGH : hormone de croissance recombinante
RIA : dosage radio-immunologique
SBW : syndrome de Beckwith-Wiedemann
SETDB1 : histone-lysine N-methyltransferase
siRNA : small interfering ARN
snoARN : small nucleolar ARN
SH2 : SRC-homology 2
SPW : syndrome de Prader-Willi
SRS : syndrome de Silver-Russell
TET3 : 10-11 translocation protein
TGFβ : transforming growth factor
TNDM : diabète néonatal transitoire
TRIM28 : tripartite motif-containing 28
TS : syndrome de Temple
ZFP57 : zinc finger protein 57

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1 ÉTAT DES CONNAISSANCES

La physiopathologie de la restriction de croissance fœtale n'est pas parfaitement connue. Les anomalies vasculaires placentaires ou les infections au cours de la vie fœtale sont des causes fréquentes et bien décrites de restriction de croissance fœtale. Le rôle de l'état nutritionnel maternel ou de l'insuline dans la croissance et le développement fœtal sont également établis (Sferruzzi-Perri *et al.* 2017; Mitanchez & Chavatte-Palmer 2018). Ainsi, la carence protéique (principalement en acides aminés essentiels) influe négativement sur la croissance fœtale, tandis que l'hyperinsulinémie secondaire à une insulinorésistance (diabète gestationnel) a un effet d'accélération de la croissance fœtale (Buchanan *et al.* 2012). Enfin, le système hormone de croissance-*insulin like growth factors* (GH-IGFs) joue un rôle essentiel tant lors de la croissance anténatale que postnatale, et cela a été bien étudié dans différents modèles animaux ainsi qu'en physiologie humaine (Liu *et al.* 1993; Sferruzzi-Perri *et al.* 2017; Burton & Jauniaux 2018).

De plus, l'existence de conséquences à long terme d'une restriction de croissance fœtale en termes de morbi-mortalité, essentiellement métabolique et cardiovasculaire (Developmental Origins of Health and Disease : DOHaD), justifie l'intérêt croissant porté à la compréhension de la régulation de la croissance à cette période clé (Barker *et al.* 1989, 1993; Leger *et al.* 1997). Cette « programmation » anténatale et ses conséquences sont notamment affectées dans certaines maladies rares de la croissance et du développement, leur conférant ainsi le statut de modèle d'étude des mécanismes physiopathologiques essentiels à la compréhension de cette programmation (Kappeler *et al.* 2017).

La régulation de la croissance fœtale fait intervenir différents facteurs environnementaux, génétiques et hormonaux dont les mécanismes ne sont pas tous élucidés. De plus, il existe une influence de ces différents facteurs les uns sur les autres (Finken *et al.* 2018). Les interrogations qui ont guidé mon travail de recherche ont porté sur des problématiques autour de la compréhension des régulations hormonales (par le système des IGFs) de la croissance fœtale, ainsi que les mécanismes épigénétiques (liés à l'empreinte parentale) régulant ces facteurs endocriniens.

1.1 Système des IGFs

La croissance fœtale est sous la dépendance de deux effecteurs essentiels que sont IGF-I et IGF-II (Fowden 1995; Gicquel & Le Bouc 2006). Leur activité est principalement modulée par leurs récepteurs et leurs protéines de liaison. Les expériences d'invalidation des différents éléments de ce système dans des modèles murins, ainsi que les pathologies humaines altérant certains acteurs du système des IGFs, sont à l'origine d'une restriction de croissance fœtale (Liu *et al.* 1993; David *et al.* 2011).

1.1.1 Physiologie du système des IGFs

1.1.1.1 IGF-I

La sécrétion hépatocytaire de l'IGF-I est soumise à l'action de l'hormone de croissance (*growth hormone,* GH) hypophysaire *via* son récepteur membranaire GHR, elle-même sous la dépendance de la sécrétion hypothalamique de la *GH releasing hormone* (GHRH, qui stimule la sécrétion de GH) et de la somatostatine (qui inhibe la sécrétion de GH) (Wilkins & D'Ercole 1985; Le Bouc *et al.* 2003; Ranke & Wit 2018).

Le gène *IGF1* est situé sur le chromosome 12 (q23.2), il est constitué de 6 exons (Ullrich *et al.* 1984; Le Bouc *et al.* 1986). Les expériences d'invalidation d'invalidation *knock-out* (KO) murins pour *Igf1* ont mis en évidence un poids de naissance diminué d'environ 60% par rapport aux contrôles, tandis que les souris KO pour *Igf1r* avaient une croissance fœtale restreinte d'environ 45%. Les double KO de ces deux gènes entraînaient une restriction de croissance fœtale de 45% (Liu *et al.* 1993).

L'IGF-I est un peptide composé de 70 acides aminés et qui possède 50% d'homologie dans cette séquence d'acides aminés avec la pro-insuline (Clemmons 1989). Il se compose de quatre domaines, les domaines A et B qui partagent environ 60% d'homologie de séquence d'acides aminés entre eux, le domaine C de liaison entre les domaines A et B, et un domaine D, C-terminal, composé de huit acides aminés (figure 1) (Denley *et al.* 2005). La région critique pour la fixation d'IGF-I (et IGF-II) aux protéines de liaison correspond aux 16 premiers acides aminés du domaine B et est, de ce fait, non conservée dans la structure de l'insuline (Bayne *et al.* 1988).

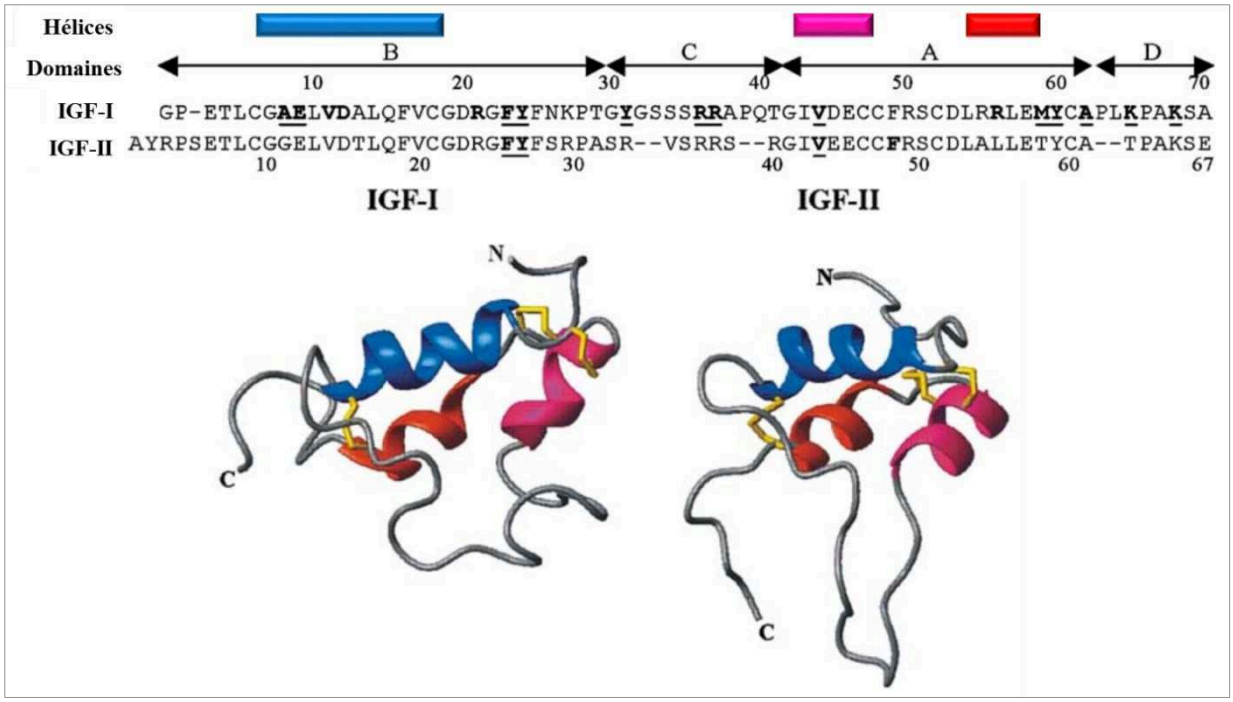
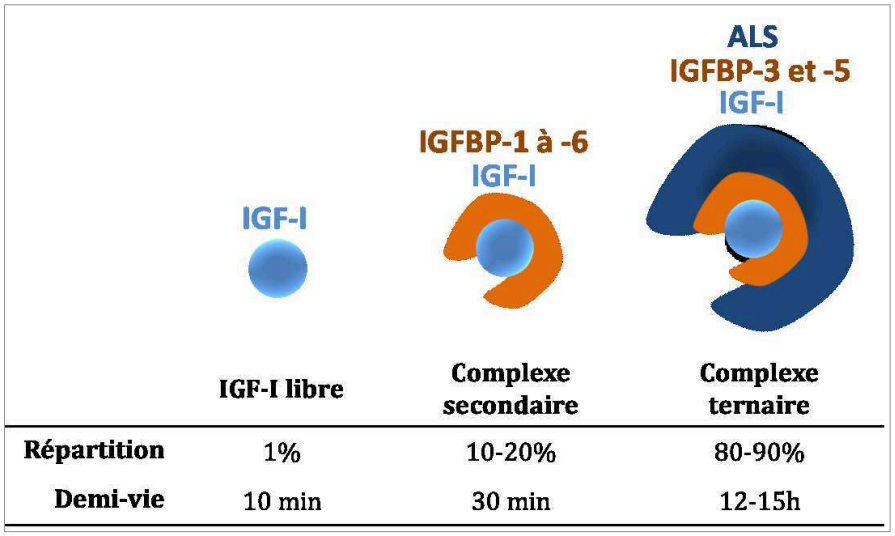


Figure 1. Structure protéique des IGF-I et IGF-II ainsi que représentation tridimensionnelle permettant la visualisation du site de liaison au récepteur IGF1R (hélice bleue). Les résidus en gras et en gras soulignés sont ceux présentant une affinité plus faible au récepteur IGF1R (Denley *et al.* 2005).

L'IGF-I sécrété a une action autocrine, paracrine et endocrine, en circulant soit sous forme libre, soit sous forme de complexe secondaire (avec les *IGF binding protein,* IGFBP) d'environ 40 kDa, soit sous forme de complexe ternaire (avec les IGFBP-3 ou -5 et l'*acid-labile subunit* (ALS)) d'environ 140 kDa (figure 2) (Le Roith 2003). Ces complexes confèrent une stabilité à l'IGF-I lié et ainsi une augmentation de la durée de sa demi-vie plasmatique. Cependant, ainsi lié, l'IGF-I ne peut se fixer à son récepteur, le récepteur de type 1 des IGF (IGF1R). IGF-I agit *via* son récepteur tyrosine kinase IGF1R au niveau de très nombreux tissus de l'organisme (voir 1.1.3.) (Nissley *et al.* 1985). De plus, IGF1R est exprimé au niveau de l'hypothalamus et de l'hypophyse, et son activation induit une régulation négative des sécrétions de GHRH et de GH respectivement. Il existe une influence importante de l'état nutritionnel et des processus inflammatoires sur les taux circulants d'IGF-I (Thissen *et al.* 1994; Bergad *et al.* 2000). Ainsi, en cas de carence nutritionnelle ou d'inflammation active, les taux d'IGF-I sont diminués. Les mécanismes en jeu sont ceux d'une résistance à la GH, que ce soit par diminution du nombre de récepteurs hépatiques de la GH ou par diminution de l'activité post-récepteur de la voie JAK2/STAT5b (carence protidique, cytokines pro-inflammatoires) (Woelfle & Rotwein 2004). Lors de l'interprétation du dosage d'IGF-I sérique, il est donc essentiel de prendre en compte l'état nutritionnel et inflammatoire du sujet.



	IGF-I libre	Complexe secondaire	Complexe ternaire
Répartition	1%	10-20%	80-90%
Demi-vie	10 min	30 min	12-15h

Figure 2. Les différentes formes circulantes d'IGF-I. L'IGF-I sérique est principalement sous forme complexée faisant intervenir les IGFBPs et l'ALS qui augmentent significativement sa demi-vie.

Les concentrations sériques d'IGF-I augmentent progressivement dans l'enfance, avec un pic important lors de la période pubertaire sous l'influence des stéroïdes sexuels pour diminuer progressivement à l'âge adulte et sont différentes selon le sexe de l'individu (figure 3) (Brabant *et al.* 2003).

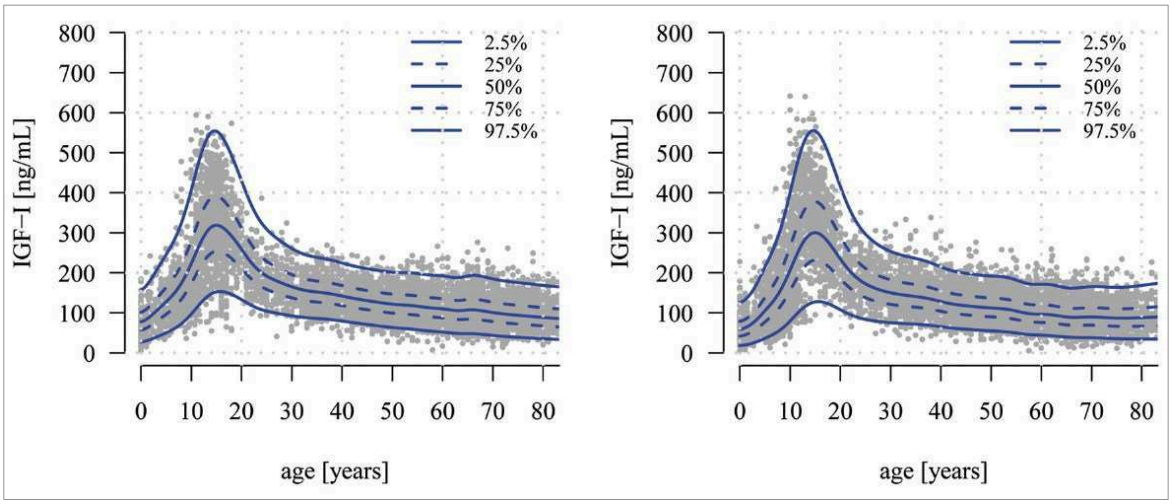


Figure 3. Normes des concentrations d'IGF-I (ng/mL) chez l'être humain selon l'âge et le sexe (masculin à gauche et féminin à droite) (Bidlingmaier *et al.* 2014).

1.1.1.2 IGF-II

La sécrétion d'IGF-II n'est pas sous la dépendance de l'hormone de croissance. Elle est relativement stable dans la vie et selon l'environnement de l'organisme (Yu *et al.* 1999). Son action biologique principale se fait par activation d'IGF1R, mais il peut également se lier au récepteur de type 2 des IGFs (IGF2R) ainsi qu'au récepteur de l'insuline (IR).

IGF2 est situé dans la région 11p15 chez l'humain et est composé de neuf exons (Rotwein 2018). Il s'agit d'un gène soumis à empreinte parentale qui s'exprime uniquement à partir de l'allèle paternel pendant la vie fœtale et qui reste soumis à empreinte parentale dans certains tissus en période postnatale (voir section 1.2.1) (DeChiara *et al.* 1990, 1991). Chez l'humain, l'expression d'*IGF2* est dépendante de cinq promoteurs (P0-P4) dont la répartition tissulaire et temporelle varie. Dans le cerveau, *IGF2* n'est pas soumis à empreinte parentale en dehors de quelques régions (hypothalamus, protubérance, globus pallidus et noyau raphé) chez l'adulte (Pham *et al.* 1998). Dans la majorité des autres tissus de l'organisme, *IGF2* est exprimé à partir des promoteurs 3 et 4 et à partir de l'allèle paternel (Rotwein 2018). Le promoteur P0 est majoritairement actif dans le placenta et de nombreux tissus fœtaux (Monk *et al.* 2006b). Le promoteur P1 n'est pas soumis à empreinte parentale (expression biallélique d'*IGF2*) et est présent uniquement dans le foie (Vu & Hoffman 1994). Le gène *IGF2R* est situé sur le chromosome 6q25.3 chez l'humain, son expression est très majoritairement biallélique, à l'exception de rares zones placentaires (Monk *et al.* 2006a). Chez la souris *Igf2r* est soumis à empreinte paternelle dans la majorité des tissus (Moore *et al.* 2015). Les KO murins de ces deux gènes ont été développés (figure 4) (Baker *et al.* 1993). Les souris KO pour *Igf2* (sur l'allèle paternel) avaient un poids de naissance diminué d'environ 60% par rapport aux contrôles, tandis que les souris KO pour *Igf2r* sur l'allèle maternel avaient une croissance fœtale excessive (130% des contrôles). Les double KO de ces deux gènes entraînaient une restriction de croissance fœtale d'environ 75%. Chez la souris, l'expression d'*Igf2* est extrêmement faible en période postnatale (et les taux circulants d'IGF-II sont indosables au bout de quelques jours) et le promoteur équivalent au P1 hépatique humain est absent (Vu & Hoffman 1994; Shmela & Gicquel 2013). IGF-II est ainsi considéré comme le facteur de croissance principal au cours de la vie fœtale, comme le suggèrent les concentrations d'IGF-II dans le sang de cordon humain qui sont environ 6 fois supérieures aux concentrations retrouvées en postnatal (Verhaeghe *et al.* 1993; Constância *et al.* 2002).

De même, *IGF2* est exprimé dans la grande majorité des tissus du fœtus humain (exception faite de l'hypothalamus et d'une grande partie du cortex), et son expression est bien supérieure à celle d'*IGF1* (Han *et al.* 1987). De plus, son rôle dans le placenta est probablement essentiel à cette action. En effet, il intervient de façon centrale dans l'allocation des ressources maternelles vers le fœtus au niveau placentaire (Sferruzzi-Perri 2018). Ainsi, des modifications environnementales chez la mère, telles que l'hypoxie sévère, la malnutrition ou l'hypovascularisation induisent une baisse d'expression d'*Igf2* dans le placenta murin et ainsi participent au retard de croissance (Coan *et al.* 2010; Habli *et al.* 2013; Cuffe *et al.* 2014). Les travaux dans les modèles murins ont montré également que c'était sous l'effet de l'IGF-II que le placenta s'adaptait (en termes de structure et de fonction) pour équilibrer les échanges de

nutriments entre le fœtus et sa mère. Bien que la structure placentaire humaine diffère de celle des modèles murins (présence de villosités placentaires et absence de zone labyrinthique), le rôle de cette surface d'échange est central de par ses interactions avec le métabolisme maternel, l'environnement et le fœtus (Sferruzzi-Perri *et al.* 2017).

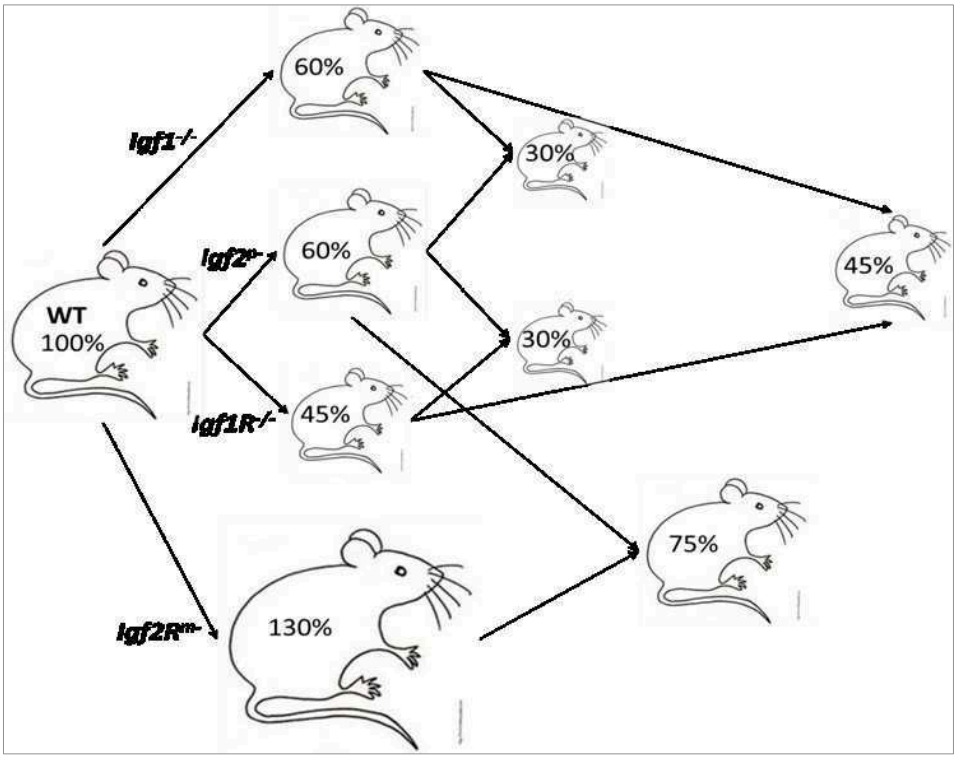


Figure 4. Poids à la naissance (en % par rapport aux contrôles (WT)) selon les différents gènes du système des IGFs invalidés dans les modèles murins (Baker *et al.* 1993; Liu *et al.* 1993; Gicquel & Le Bouc 2006).

IGF-II se lie à l'IGF1R mais peut aussi se lier au récepteur du mannose 6-phosphate cation dépendant (M6PCI ou IGF2R) ainsi qu'à l'isotype A du récepteur de l'insuline (IR) (Clemmons 1989; Frasca *et al.* 1999). L'affinité d'IGF1R pour IGF-II est moindre que pour IGF-I (Henderson *et al.* 2015). L'activation d'IGF2R après fixation d'IGF-II entraîne une clairance d'IGF-II, bien que les mécanismes exacts sous-tendant ce rôle soient imparfaitement connus (Clemmons 1989). L'affinité d'IGF2R est très largement supérieure (K_i = 0,2 nM) pour IGF-II devant celle, négligeable, pour IGF-I (K_i = 400 nM) et nulle pour l'insuline (Tong *et al.* 1988). IGF-II se compose de 67 acides aminés et possède une structure en 4 domaines B-C-A et D (dans le sens N à C-terminal) (de Pagter-Holthuizen *et al.* 1987).

1.1.1.3 IGF1R

L'IGF1R qui lie les IGFs est un récepteur tyrosine kinase composé de deux sous-unités : alpha, extra-cellulaire avec deux sites de liaison au ligand, et bêta, trans-membranaire (Steele-Perkins *et al.* 1988; Werner *et al.* 1989). Le récepteur est organisé en hétéro-tétramère (deux sous-unités alpha et deux sous-unités bêta), *via* des ponts disulfures (figure 5) (Abbott *et al.* 1992). Il est présent sur toutes les cellules de l'organisme, à l'exception des hépatocytes. Ce récepteur peut lier à la fois IGF-I, IGF-II et l'insuline. L'affinité pour IGF-I (K_i = 1,5 nM) est supérieure à celle d'IGF-II (K_i = 3 nM) et plus de 50 fois plus élevée que celle de l'insuline (K_i > 30 nM) (Steele-Perkins *et al.* 1988; Slaaby *et al.* 2006; Tian *et al.* 2016). Le gène *IGF1R* chez l'homme se situe sur le chromosome 15 en position q26.6, il est composé de 21 exons (figure 5) (LeRoith *et al.* 1995).

Les données expérimentales issues des KO sélectifs de souris illustrent parfaitement l'action propre et combinée de ces différents facteurs (figure 4) (DeChiara *et al.* 1990, 1991; Baker *et al.* 1993; Liu *et al.* 1993). On en déduit une action majeure d'Igf-I et Igf-II sur la croissance fœtale par le biais de leur récepteur commun Igf1r. Les expériences de double KO *Igf2/Igf1r* et *Igf1/Igf1r* mettaient en évidence que les souris KO *Igf2/Igf1r* étaient plus petites qu'en cas d'invalidation d'*Igf1/Igf1r* ou *Igf1r* seul (Baker *et al.* 1993; Liu *et al.* 1993). Ainsi, Igf-II semble agir *via* une autre voie, indépendante d'Igf1r, bien qu'aucune n'ait été caractérisée jusqu'à présent (Frasca *et al.* 1999).

La fixation du ligand au niveau du site de liaison de la sous-unité alpha induit des changements conformationnels de la sous-unité bêta qui permettent l'autophosphorylation du récepteur au niveau de ses domaines tyrosine kinase intra-cellulaires (Hanks *et al.* 1988). Lorsque les résidus tyrosine du domaine juxta-membranaire sont phosphorylés (tyr950), ils permettent à certaines protéines d'ancrage qui reconnaissent ces résidus tyrosine phosphorylés, comme les IRSs (*insulin receptor substrate*), d'être phosphorylées à leur tour par le récepteur (figure 6). Par la suite, d'autres protéines reconnaissent les domaines phosphorylés des IRSs – par leur domaine SH2 (*SRC-homology-2*) – et sont à l'origine des cascades de phosphorylations dans différentes voies de signalisation (Craparo *et al.* 1995; Tartare-Deckert *et al.* 1995; Dey *et al.* 1996; Saltiel & Kahn 2001).

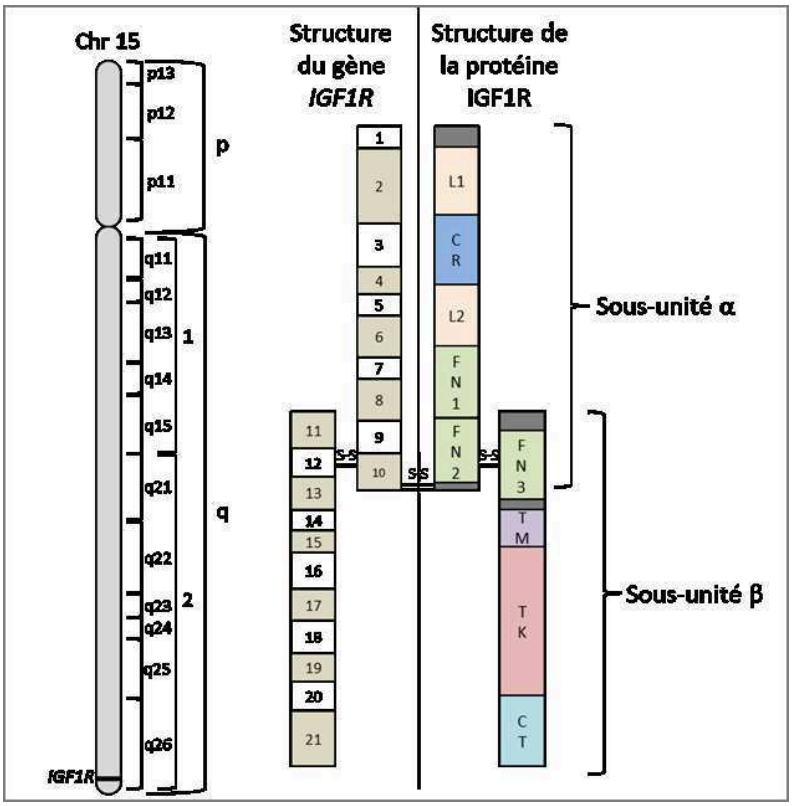


Figure 5. Localisation d'*IGF1R* en position 15q26.6, structure exonique (21 exons) et domaines protéiques correspondants. IGF-1R se compose de différents domaines : deux domaines riches en leucines (L1 et L2) séparés par un domaine riche en cystéine (CR), trois domaines type fibronectine III (FN1-3), un domaine transmembranaire (TM), un domaine tyrosine kinase (TK) et enfin une extrémité C-terminale (CT) (Adams *et al.* 2000).

Ainsi, selon la position des résidus tyrosine du récepteur qui sont phosphorylés, des protéines, reconnaissant spécifiquement ces résidus, vont pouvoir être phosphorylées et activer des voies de signalisation responsables de divers mécanismes biologiques (migration, internalisation ou dégradation du récepteur par exemple) (Li *et al.* 1994; Hernández-Sánchez *et al.* 1995; Miura *et al.* 1995). La phosphorylation d'IRS1 ou 2 par IGF1R va ainsi permettre l'activation des voies de signalisation *mitogen-activated protein kinase* (MAPK) et phosphatidylinositol 3-kinase (PI3K), responsables *in fine* de la prolifération, de la différenciation et de la survie cellulaire (figure 6). Au sein de chacune de ces voies de signalisation, différents acteurs entrent en jeu, et des anomalies génétiques impliquant ces facteurs peuvent induire des anomalies de croissance, de métabolisme et de susceptibilité aux tumeurs. Dans les modèles murins d'invalidation des IRS, des phénotypes différents ont été décrits selon les IRS et les tissus, mais l'insulinoresistance était constante, suggérant une action principalement métabolique de ces protéines. Ainsi, les KO murins d'IRS1 exprimaient un phénotype sévère de restriction de croissance et d'insulinorésistance, tandis que le KO d'IRS2 présentait également une insulinorésistance,

associée à un effet sur la croissance limité à certains tissus (rétine, système nerveux central, pancréas) (Withers *et al.* 1998; Kido *et al.* 2000).

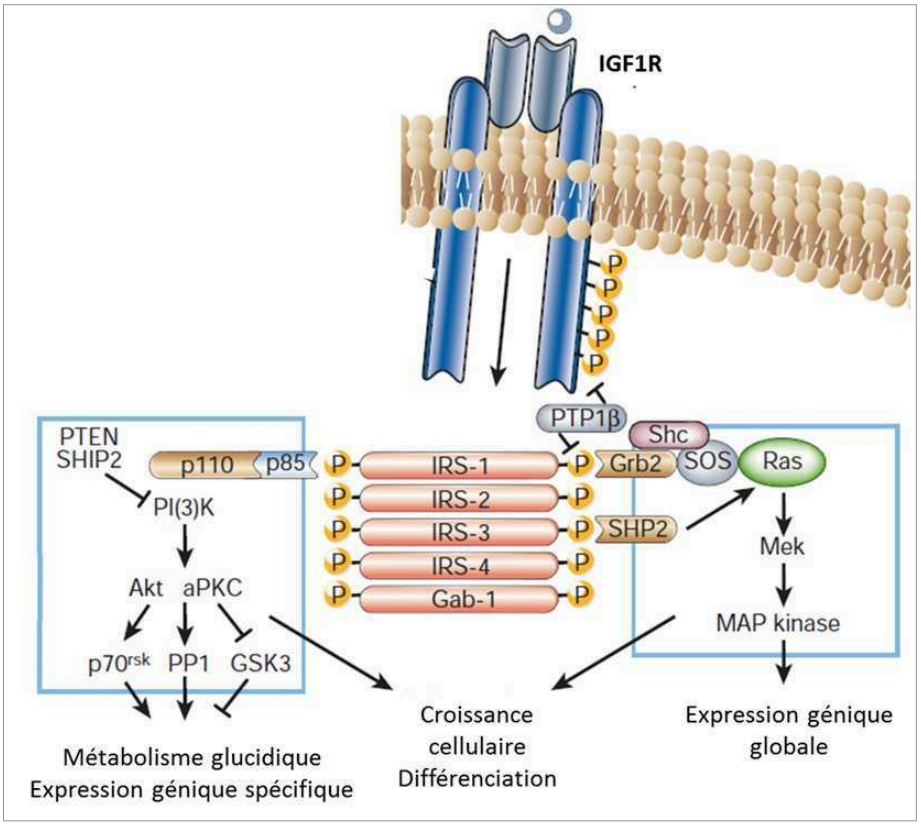


Figure 6. Signalisation d'IGF1R. Après fixation du ligand (IGF-I, IGF-II ou insuline), la sous-unité β s'auto-phosphoryle et permet le recrutement de protéines d'ancrage (IRSs et Gab-1) qui vont, une fois phosphorylées à leur tour, permettre l'activation des voies de signalisation des Ras-MAP kinases et de la PI3 kinase (Saltiel & Kahn 2001).

Dans la suite des cascades de phosphorylation, on note qu'AKT (ou PHB, protéine kinase B) est un acteur central de la voie PI3K puisque sa phosphorylation va permettre la régulation de multiples processus biologiques (différenciation cellulaire, métabolisme glucidique). Ainsi, des facteurs inhibiteurs de PI3K et AKT, tels que PTEN (*phosphatase and tensin homolog*), ont été impliqués en pathologie humaine, principalement dans des syndromes de prédisposition tumorale (Jelsig *et al.* 2014). Concernant la voie des MAPK, la phosphorylation en cascade des protéines MEK et ERK (MAP/ER kinase et *extracellular signal-regulated kinase*)induit principalement la prolifération cellulaire (Copps & White 2012). Par ailleurs, l'activation de ces voies de signalisation peut être modulée par certains facteurs comme les phospho-tyrosine phosphatases (PTP) qui vont induire une déphosphorylation rapide des protéines sur ces voies (Elchebly *et al.* 1999).

Enfin, IGF1R a la particularité de partager plus de 50% d'homologie en résidus d'acides aminés avec le récepteur de l'insuline (IR) (Ullrich *et al.* 1986). Les deux type d'IR, A et B, ont une affinité supérieure pour l'insuline (IC_{50} = 0,2-0,9 nM et 0,5-1,6 nM respectivement) que pour les IGFs, bien que l'affinité d'IRA pour IGF-II soit non négligeable (IC_{50} = 2,5 nM). En revanche, les affinités d'IGF-I pour les deux types d'IR sont très faibles (IC_{50} > 30 nM), tout comme celle d'IRB pour IGF-II (IC_{50} > 10 nM) (Rechler *et al.* 1980; Bayne *et al.* 1988). Dans certaines conditions, il peut se former des hétérodimères IGF1R/IR qui ont une affinité supérieure pour les IGFs que pour l'insuline (Moxham *et al.* 1989; LeRoith *et al.* 1995; Belfiore *et al.* 2009; Tian *et al.* 2016). De nombreux travaux se sont intéressés à caractériser les actions spécifiques de ces récepteurs, notamment par l'étude de récepteurs chimériques IR/IGF1R (Faria *et al.* 1994; Tartare *et al.* 1994). Les IGFs ont un rôle prépondérant dans l'induction d'effets cellulaires à long terme (prolifération, différenciation), tandis que l'insuline a un effet métabolique plus immédiat. Certains travaux mettent en évidence un impact de la durée de liaison du ligand au récepteur qui activerait préférentiellement les voies métaboliques si la liaison est courte, et des voies de prolifération si la liaison perdure (De Meyts 1994; De Meyts *et al.* 1995). Cela serait concordant avec les différences de taux circulants, stables pour les IGFs, et très variables, selon la glycémie, pour l'insuline. Par ailleurs, les voies de signalisation d'IGF1R, d'IR ou des hétérotétramères peuvent interagir avec d'autres systèmes moléculaires. Des travaux ont ainsi montré qu'IGF1R pouvait former des complexes avec la sous-unité α des protéines G, mais aussi que les sous-unités βγ de ces protéines G pouvaient activer la voie MAPK induite par IGF-I (Luttrell *et al.* 1995; Dalle *et al.* 2001). Cette signalisation est également influencée par de nombreux facteurs locaux (tels que les IGFBPs), d'autres facteurs de croissance (PDGF, EGF, FGF), ou des hormones (oestrogènes, glucocorticoïdes) (Ricort & Binoux 2001; Lassarre *et al.* 2013; Hakuno & Takahashi 2018). La régulation des différentes voies de signalisation d'IGF1R ou IR est donc non seulement spatiale, car dépendante du type et de l'environnement cellulaire (répartition du nombre et du type de récepteurs à sa surface, autres voies de régulation), mais également temporelle (modification entre la vie fœtale et postnatale des caractéristiques spatiales) (LeRoith *et al.* 1995).

1.1.1.4 IGFBPs

Les IGFBPs sont au nombre de six (tant chez l'humain que chez la souris) (Allard & Duan 2018). Elles constituent les protéines de liaison de l'IGF-I et de l'IGF-II qui ont pour leurs IGFBPs une affinité en moyenne dix fois supérieure que pour leur récepteur commun IGF1R (K_i entre 0,01 et 0,22 nM, voir tableau 1) (Loddick *et al.* 1998; Firth & Baxter 2002). Elles ne lient pas l'insuline. Les IGFBPs ont une affinité un peu supérieure pour IGF-II que pour IGF-I (hormis IGFBP-1 et IGFBP-4 pour lesquelles les IGF ont une affinité comparable (tableau 1) (Oh *et al.*

1993; Jones & Clemmons 1995). La structure de ces protéines est proche (elles ont des domaines C et N terminaux ayant un degré d'homologie important) et seuls leur domaines de liaison ainsi que de fines variations (glycosylation ou phosphorylation sur des sites spécifiques) les distinguent, avec des masses moléculaires variant entre 22 et 32 kDa (tableau 1 et figure 7A)(Hwa *et al.* 1999; Firth & Baxter 2002). Les IGFBP-3 et -5 peuvent lier l'ALS pour former le complexe ternaire. L'IGFBP-3 est la plus présente dans la circulation sanguine, 90% étant sous forme de complexe ternaire, contre 55% pour l'IGFBP-5 (Bach 2018). Ces IGFBPs sont protéolysées par différentes enzymes, dont les *pregnancy associated plasma protein-a (*PAPP-A et PAPP-A2) et libèrent ainsi les IGFs (Conover *et al.* 2004; Fujimoto *et al.* 2018). En dehors de leur rôle dans la biodisponibilité des IGFs, les IGFBPs sont impliqués dans d'autres processus qui peuvent être indépendants de leur liaison aux IGFs (Firth & Baxter 2002; Ricort 2004). En effet, de nombreux travaux mettent en évidence une action propre de ces IGFBPs ou de leurs formes protéolysées dans certains processus métaboliques, que ce soit par des récepteurs propres (IGFBP-1, -2, -3 et -5), par interaction avec des protéines de la matrice extracellulaire (IGFBP-5) ou des protéoglycanes de la surface membranaire (IGFBP-1 et -2) (Jones 1993; Russo *et al.* 1997; Andress 1998; Ricort 2004; Allard & Duan 2018; Clemmons 2018).

À titre d'exemple, il existe une action anti-mitogénique de la partie N-terminale de la forme protéolysée de l'IGFBP-3, indépendante de la liaison à l'IGF-I (Bach 2018; Clemmons 2018). Les mécanismes sous-jacents sont imparfaitement élucidés et il pourrait exister un récepteur propre à certaines IGFBPs qui induirait des effets intracellulaires. Cela a été suggéré principalement pour IGFBP-3 qui induit de manière indépendante à IGF-I une augmentation rapide et transitoire du calcium intracellulaire, en se fixant sur un récepteur membranaire possiblement lié à une protéine G (Ricort et al. 2002). De même, IGFBP-3 est capable de co-activer le récepteur TGFβ de type V pour inhiber la prolifération cellulaire (Leal et al. 1997; Oklü & Hesketh 2000; Gui & Murphy 2003). De plus, certaines équipes ont mis en évidence une modulation de certaines de ces IGFBPs sur l'activité des IGFs via IGF1R (Firth & Baxter 2002). Ainsi, IGFBP-5 se lie à des protéines de la membrane cytoplasmique pour faciliter et prolonger l'action d'IGF-I sur son récepteur en maintenant un ancrage plus prolongé sur le site de liaison (figure 7B) (Parker et al. 1998). Différentes interactions avec des facteurs intracellulaires du réticulum endoplasmique ou de la membrane nucléaire ont également été décrits (IGFBP-3 et -5) (Ricort 2004; Martin & Baxter 2011).

Tableau 1. Principales caractéristiques des différentes IGFBPs. Les actions en vert sont celles qui sont favorisées, en rouge celles qui sont inhibées et en orange celles dont l'effet est variable. (Salih *et al.* 2004; Ranke *et al.* 2005; Beattie *et al.* 2006; Ning *et al.* 2008; Bach *et al.* 2013; Gupta 2015; Russo *et al.* 2015; Bach 2018)

	Localisation Chromosomique	Affinité pour les IGFs		Localisations principales	Autres actions	Spécificités	Modèles murins
IGFBP-1	7p12.3	IGF-I = IGF-II	Phosphorylation ↗ affinité pour IGF	Foie++ Endomètre décidual Foie fœtal	Migration cellulaire Résorption osseuse	Régulation par l'insuline (↘)	KO: phénotype normal
IGFBP-2	2q35	IGF-II > IGF-I		Foie Adipocytes Système nerveux central	Migration cellulaire Différenciation des ostéoblastes Tumorogénèse	Régulation par l'insuline (↘) et la leptine (↗)	KO: hépatomégalie, croissance normale
IGFBP-3	7p12.3	IGF-II > IGF-I	Phosphorylation ↘ affinité pour IGF	Sérum	Sénescence Prolifération et survie cellulaires Angiogénèse	Régulation par la GH (↗)	KO: phénotype normal Surexpression: restriction de croissance (pré et post-natale), insulinoresistance
IGFBP-4	17q21.2	IGF-I = IGF-II		Os Très faible dans le sérum	Tumorogénèse Remodelage osseux Angiogénèse	Protéolyse par PAPP-A Action propre de la forme protéolysée conjointement avec IGF-II	KO: restriction de croissance fœtale
IGFBP-5	2q35	IGF-II > IGF-I	Liaison aux glycosaminoglycanes ↘ affinité pour IGF	Os Sérum	Survie cellulaire Fibrose tissulaire		KO: phénotype normal Surexpression: mortalité néonatale, restriction de croissance pré et post-natale, hépatomégalie et macrocéphalie, ostéopénie
IGFBP-6	12q13.13	IGF-II >> IGF-I			Prolifération cellulaire Apoptose Migration cellulaire Réponse auto-immune		KO: phénotype normal Surexpression: restriction de croissance postnatale immédiate, développement cérébral anormal

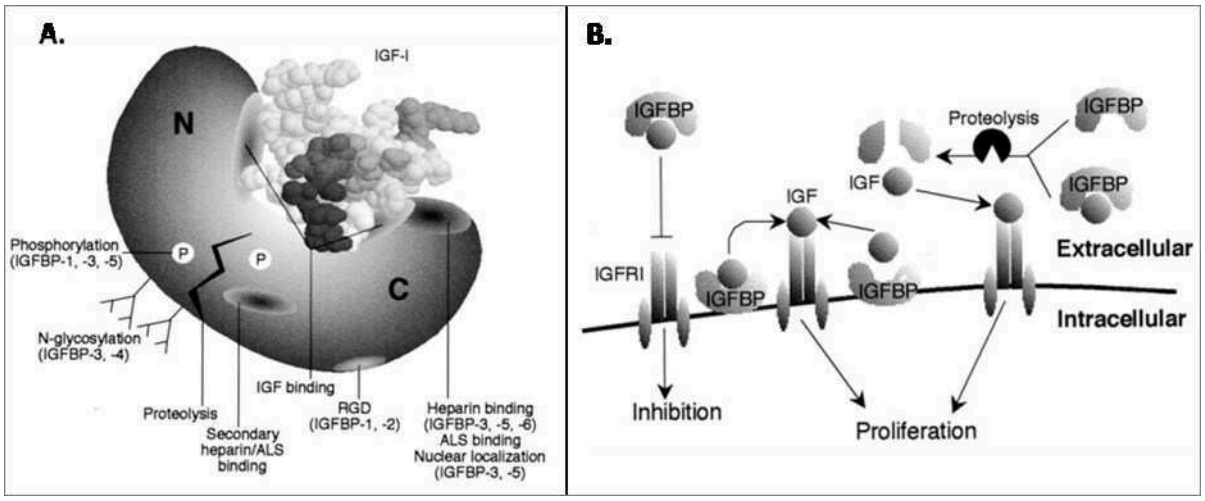


Figure 7. A. Structure commune des IGFBPs (en "*donut*") et des sites fonctionnels de chacune des IGFBPs (site de liason des IGFs dans la partie concave entre les domaines N et C terminaux, sites de glycosylation, sites de phosphorylation, domaine de liaison à l'héparine ou à l'ALS). B. Régulation des effets des IGFs par les IGFBPs au niveau cellulaire : inhibition de la signalisation IGF1R par liaison aux IGFs, activation d'IGF1R après protéolyse des IGFBPs qui libèrent les IGFs et prolongation d'activation d'IGF1R en stabilisant la liaison des IGFs à l'IGF1R (Firth & Baxter 2002).

Les modèles murins ont permis d'évaluer les rôles de ces différentes IGFBPs en physiologie, puisque les précédentes données étaient restreintes à la biologie cellulaire ; en effet, les mécanismes complexes identifiés *in vitro,* s'ils permettent une meilleure compréhension des mécanismes en jeu et l'identification de possibles cibles thérapeutiques, ne sont pas toujours transposables dans leur globalité *in vivo* (Bach 2018). Ainsi, les KO sélectifs d'*Igfbp3* ou d'*Igfbp5* ne causaient pas de phénotype particulier sur la croissance fœtale, probablement du fait d'une redondance de fonction entre elles (Ning *et al.* 2006, 2007). En revanche, dans les expériences de triple KO *Igfbp3, 4* et *5,* les souris avaient une restriction de croissance postnatale, associée à de faible taux circulant d'IGF-I et une baisse de sa bioactivité (Ning *et al.* 2006). De même, les expériences de surexpression de ces gènes (*Igfbp3* et *Igfbp5*) chez les souris mettaient en évidence des phénotypes de restriction de croissance fœtale et postnatale (Modric *et al.* 2001; Salih *et al.* 2004). La surexpression d'*Igfbp3* était également responsable d'une insulinorésistance et d'une diminution de la formation osseuse (Silha *et al.* 2001, 2002), tandis que la surexpression d'*Igfbp5* induisait un excès de mortalité néonatale, une hypertrophie cérébrale et hépatique, ainsi qu'une diminution de la densité osseuse (Salih *et al.* 2004, 2005; Bach 2018).

Il n'y a pas, à ce jour, d'anomalie génétique constitutionnelle décrite pour ces IGFBPs chez l'être humain. Une hypothèse serait celle de la létalité précoce de telles anomalies chez l'être humain, aux vues de l'implication majeure de ces IGFBPs dans les multiples mécanismes biologiques précédemment décrits et de la mortalité excessive rapportée dans certains KO murins (*Igfbp-5*).

On peut, au contraire, préjuger qu'il existe, comme chez la souris, une redondance entre les différentes IGFBPs qui n'induit pas de phénotype chez l'être humain. Les données de séquençage whole exome en population générale pourraient renseigner confirmer ce dernier point si des variants pathogènes sur le plan fonctionnel étaient retrouvés à l'état homozygote chez des sujets sains. L'étude de ces facteurs chez l'humain repose donc principalement sur l'étude des taux circulants des différentes IGFBPs (Marzullo *et al.* 2001). Les taux circulants d'IGFBP-3 (la plus représentée des IGFBPs dans le sérum) sont corrélés positivement à la survenue d'un syndrome métabolique et à l'intolérance glucidique chez l'humain et des taux élevés d'IGFBP-3 sont associés à une augmentation du risque cardio-vasculaire chez l'adulte (Yeap *et al.* 2010; Eggert *et al.* 2014; Clemmons 2018). Bien que les mécanismes d'action exacts de ces IGFBPs soient imparfaitement élucidés, il est maintenant admis qu'un équilibre entre les concentrations d'IGF-I et d'IGFBP-3 est nécessaire pour assurer une balance croissance suffisante-croissance excessive (cancers) adéquate, comme il a été rapporté dès 2004 (figure 8) (Park & Cohen 2004; Eggert *et al.* 2014).

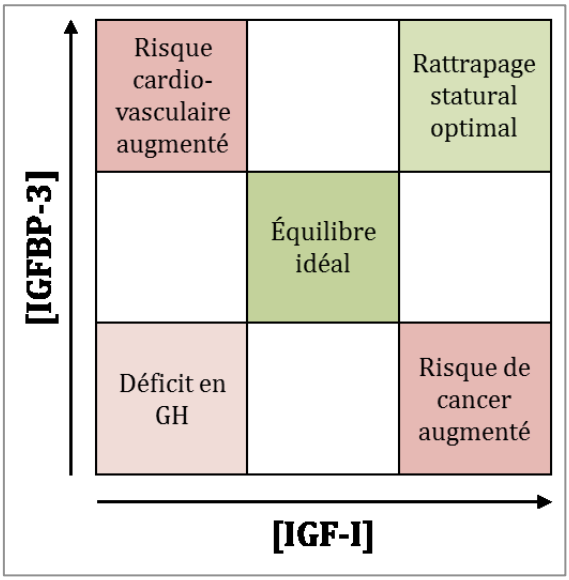


Figure 8. Effets et morbidité générale à long terme de l'équilibre entre concentrations d'IGF-I et d'IGFBP-3 lors d'un traitement par hormone de croissance (GH) recombinante (Park & Cohen 2004).

En contexte de pathologie humaine, cela a conduit à l'évaluation du ratio molaire IGF-I/IGFBP-3 comme reflet de cet équilibre, principalement au cours d'un traitement par hormone de croissance recombinante (rGH) (Juul *et al.* 1995; Cabrol *et al.* 2011). Récemment, ce ratio IGF-I/IGFBP-3 a été évalué en cours de traitement par hormone de croissance recombinante (rGH) chez des patients avec syndrome de Prader-Willi (SPW, voir section 1.2.2.3) en comparaison avec une mesure de l'IGF-I « biodisponible » (par une méthode ELISA) (Chen *et al.* 2003; Bakker

et al. 2015). Les auteurs ont identifié une corrélation significative entre le ratio IGF-I/IGFBP-3 et l'IGF-I biodisponible uniquement chez les patients les plus jeunes (ρ = 0,663, p = 0,001). Il est donc possible que ce ratio soit un bon reflet de la biodisponibilité d'IGF-I uniquement en période prépubertaire, du fait de l'intervention de facteurs spécifiques pendant la période pubertaire (protéases, modification de la signalisation IGF1R par les stéroïdes sexuels, modification de l'état nutritionnel ou durée d'exposition à la rGH) (Juul *et al.* 1995; Devi *et al.* 2000; Renes *et al.* 2014).Des travaux plus anciens avaient également montré une augmentation relative des complexes secondaires et surtout ternaires après un an de traitement par rGH, avec une corrélation positive avec la vitesse de croissance staturale (ρ = 0,72, p = 0,01) (Mandel *et al.* 1997; Bakker *et al.* 2015). Dans une étude récente, Y. Le Bouc a comparé les ratios IGF-I/IGFBP-3 lors du traitement par rGH chez des patients nés petits pour l'âge gestationnel (PAG), des patients avec un déficit en hormone de croissance et des patients avec un SPW (Gaddas *et al.* 2019). Dans les trois populations de patients, le ratio IGF-I/IGFBP-3 était corrélé positivement à l'insulinémie et à la dose de rGH. De plus, certains patients (nés PAG) avaient des taux d'IGF-I élevés (supérieurs à 2 DS) mais des ratios IGF-I/IGFBP-3 normaux, qui incitent donc à considérer que la biodisponibilité d'IGF-I était normale chez ces patients. Des résultats similaires avaient été publiés en 2017, montrant une corrélation positive entre le ratio IGF-I/IGFBP-3 et la bonne réponse au traitement en termes de gain statural (Ballerini *et al.* 2017). L'évaluation non seulement des concentrations d'IGFBP-3 conjointement à celles d'IGF-I mais également du ratio IGF-I/IGFBP-3 lors de la surveillance d'un traitement par rGH serait donc un outil important pour aider le clinicien dans l'adaptation des doses de rGH chez les patients (Ranke *et al.* 2005; Ballerini *et al.* 2017; Gaddas *et al.* 2019). Néanmoins, ce marqueur, théoriquement très intéressant, reste à valider du fait des résultats pour l'instant contradictoires obtenus sur de petites cohortes. Enfin, il est essentiel d'interpréter ces valeurs à la lumière des paramètres cliniques des sujets du fait de l'impact de la composition corporelle, de l'âge, de la puberté et d'un traitement par rGH sur ce paramètre.

1.1.1.5 ALS

L'ALS est une protéine de 85kDa qui est synthétisée par les hépatocytes sous la dépendance de la GH, et dont la fonction principale est la liaison des IGFBP-3 et IGFBP-5 liant IGF-I (mais l'ALS ne lie pas directement IGF-I) (Baxter 1988). Elle permet ainsi la formation des complexes ternaires stables dans la circulation sanguine (figure 2) (Bach 2018). Le gène codant l'ALS, *IGFALS,* est situé sur le chromosome 16 (p13.3) et est constitué de deux exons (Dai & Baxter 1992). Les modèles de KO murins ont une réduction de croissance postnatale modérée de 10-20% et une sensibilité accrue à l'insuline. Les invalidations hétérozygotes d'*IGFALS* induisaient un phénotype de croissance peu éloigné des souris contrôles. Les expériences de surexpression

d'ALS chez la souris ont montré également un phénotype de réduction modérée de la croissance postnatale (9-17% à trois semaines de vie), probablement secondaire à la baisse de biodisponibilité d'IGF-I (Silha *et al.* 2001; Domené *et al.* 2011).

Le fonctionnement global du système des IGFs est schématisé dans la figure 9 pour clore cette partie descriptive de la physiologie de ce système.

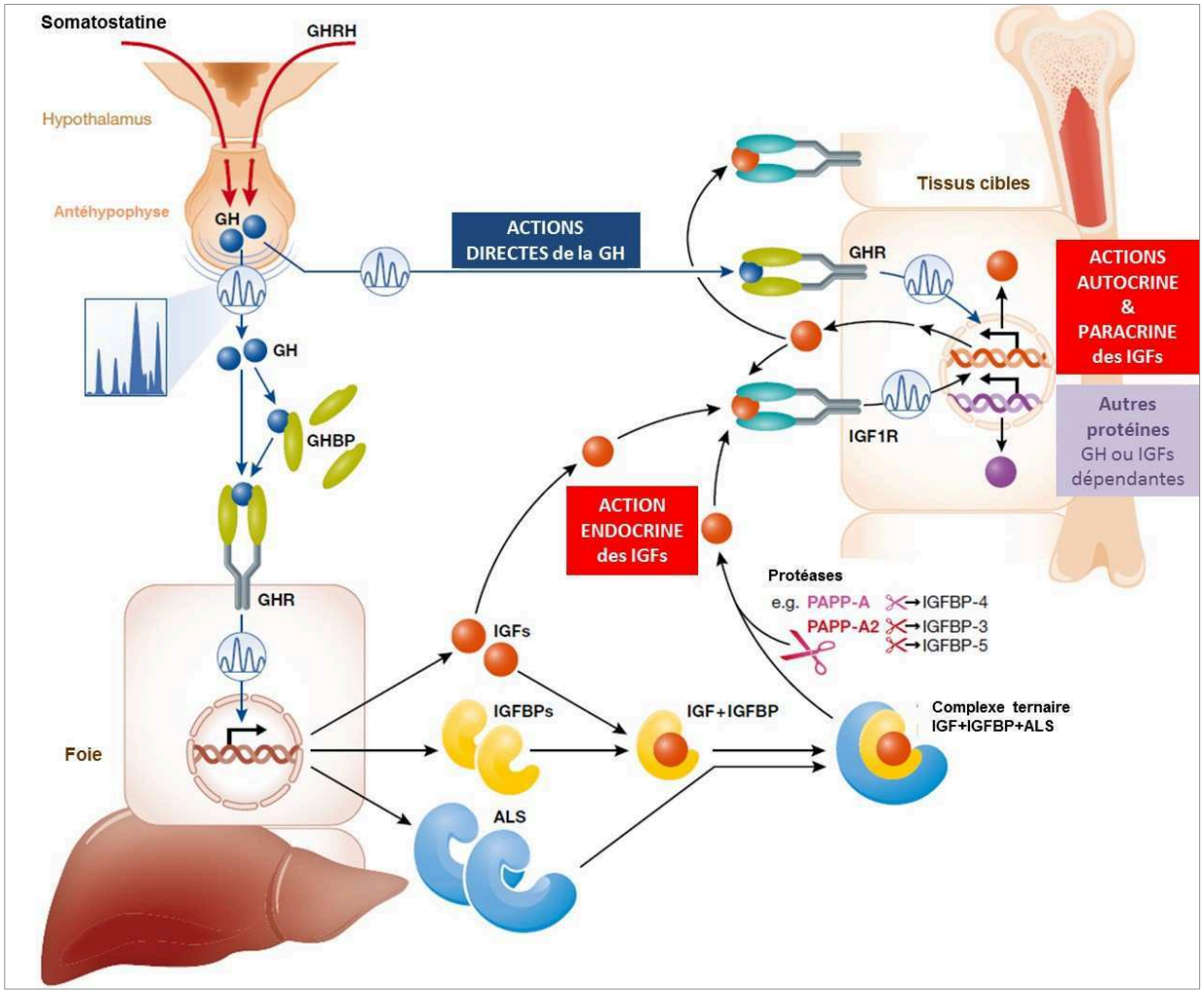


Figure 9. Régulation du système GH-IGFs. La sécretion pulsatile de la GH est sous la dépendance de la GHRH et de la somatostatine hypothalamique. La GH circule sous forme liée à la GHBP (GH *binding protein*) et exerce soit une action directe sur la prolifération et la maturation du cartilage de croissance (par l'induction de l'expression locale des IGFs), soit stimule la sécrétion hépatique des IGFs, des IGFBPs et de l'ALS, *via* la fixation à son récepteur (GHR). Les IGFs ont une action auto ou paracrine via le recepteur IGF1R, ainsi qu'une action endocrine en circulant sous forme de complexe secondaire (avec les IGFBP) ou ternaire (avec IGFBP-3 ou -5 et l'ALS). La protéolyse des IGBP par PAPP-A et PAPP-A2 libère les IGFs de leurs IGFBPs permettant l'activation d'IGF1R sur les cellules cibles (Argente *et al.* 2017).

1.1.2 Pathologies impliquant le système des IGFs

1.1.2.1 Anomalies d'IGF-I

Woods et son équipe ont rapporté en 1996 la première anomalie d'*IGF1* (délétion homozygote) chez l'humain (Woods *et al.* 1996). Le phénotype associait une restriction de croissance fœtale, un retard de croissance postnatal sévère, une microcéphalie, une surdité, une déficience intellectuelle et des taux indétectables d'IGF-I. Depuis, quelques patients avec anomalies d'*IGF1* ont été décrits avec des phénotypes variables. Ainsi la surdité est un symptôme inconstant, et le retard mental peut être modéré ou absent. La sévérité de la restriction de croissance fœtale et postnatale est également variable. Selon les anticorps utilisés (et la préservation des épitopes reconnus sur la protéine mutée) pour le dosage des concentrations d'IGF-I de ces patients, les valeurs retrouvées étaient effondrées, normales ou même élevées (David *et al.* 2011). Quand elles ont été réalisées (n = 2), les études fonctionnelles ont montré une baisse d'activité de l'IGF-I mutée par baisse de son affinité à l'IGF1R (Walenkamp *et al.* 2005; Netchine *et al.* 2009). Les patients porteurs de mutations homozygotes d'*IGF1* restent extrêmement rares, et bien que quelques patients porteurs hétérozygotes de mutations aient été rapportés, l'implication de ces mutations dans le phénotype n'a pas toujours pu être confirmée (Bonapace *et al.* 2003; Coutinho *et al.* 2007).

1.1.2.2 Anomalies d'IGF-II

1.1.2.2.1 Syndrome de Silver-Russell

Le syndrome de Silver-Russell (SRS) est une maladie soumise à empreinte parentale dont l'anomalie moléculaire la plus fréquente entraîne une baisse d'expression d'*IGF2*. Il a été décrit dans les années 50 chez des patients nés PAG sans rattrapage en postnatal, une asymétrie corporelle et une dysmorphie faciale (grand front et visage triangulaire) (Silver *et al.* 1953; Russell 1954; Black 1961). Depuis, des causes moléculaires sous-tendant ce syndrome ont été caractérisées et un score clinique (Netchine-Harbison *clinical scoring system*, NH-CSS) permet son diagnostic (Azzi *et al.* 2015a). La disomie uniparentale maternelle du chromosome 7 (mupd7) a été la première cause moléculaire identifiée de SRS en 1995 et, dix ans plus tard, la perte de méthylation au niveau du centre d'empreinte *H19/IGF2* sur le chromosome 11 (p15.5) a été décrite dans notre équipe (voir section 1.2.1) (Kotzot *et al.* 1995; Gicquel *et al.* 2005). Ces deux mécanismes représentent actuellement respectivement 10% et 50% des causes moléculaires identifiées du SRS, laissant encore 40% des patients sans diagnostic moléculaire. En 2016, nous avons publié une revue de la littérature dans laquelle sont décrits le phénotype, les anomalies moléculaires et la prise en charge de ces patients (Publication n°1, (Giabicani *et al.*

2016)). Par la suite, le premier consensus international portant sur le diagnostic clinique et moléculaire et le traitement de ces patients a été publié (Wakeling *et al.* 2016). Les discussions ont abouti à l'élaboration de 76 recommandations qui permettent une homogénéisation de la prise en charge de ses patients (article dans son intégralité en Annexe).

La description récente de mutations d'*IGF2* est venue confirmer l'implication majeure de la baisse d'expression d'*IGF2* dans le syndrome de Silver-Russell, étant donné que les patients mutés présentaient un phénotype identique à celui des patients avec SRS par anomalie de la région 11p15 (Begemann *et al.* 2015).

De plus, récemment notre équipe a identifié une voie de régulation impliquant les oncogènes *PLAG1* et *HMGA2* dans l'expression d'*IGF2* (Abi Habib *et al.* 2018). PLAG1 est un facteur de transcription agissant au niveau du promoteur P3 pour induire l'expression d'*IGF2*, les autres mécanismes sous-tendant cette voie de régulation ne sont pas élucidés (Voz *et al.* 2000). Chez des patients présentant un retard de croissance intra-utérin et postnatal chez qui une mutation dans *PLAG1* ou *HMGA2* (déjà décrit chez des patients nés PAG) a été identifiée, il existe une baisse d'expression d'*IGF2*. Cette baisse d'expression *d'IGF2* explique probablement le phénotype proche de celui des patients atteints d'un SRS. Par des méthodes d'invalidation sélective des différents gènes, W. Abi Habib *et al.* ont montré l'influence de *PLAG1* et d'*HMGA2* sur l'expression d'*IGF2* (figure 10) (Abi Habib *et al.* 2018).

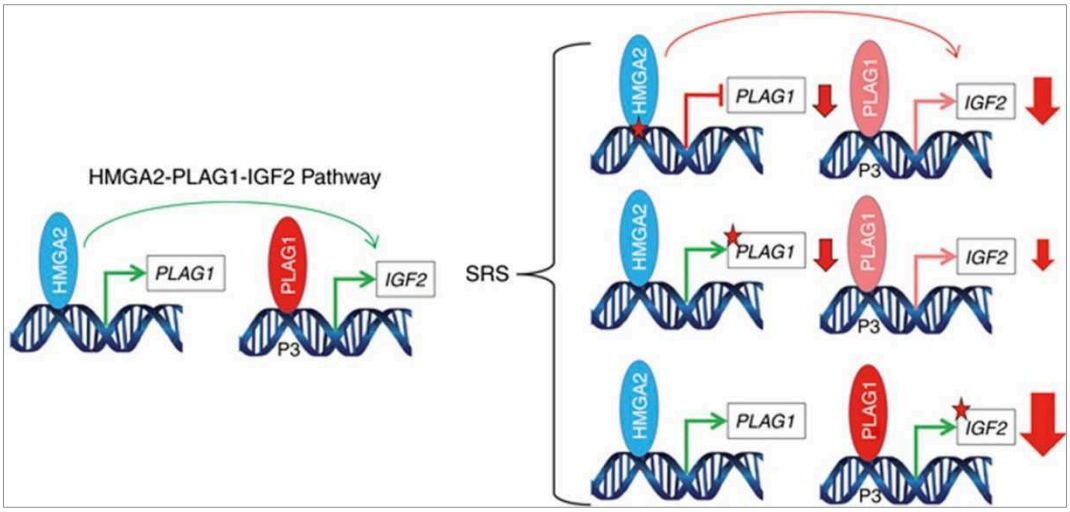


Figure 10. Régulation de l'expression d'*HMGA2*, *PLAG1* et *IGF2* (flèches rouges) chez un individu sain (à gauche) et en cas de syndrome de Silver-Russell (SRS). Les étoiles représentent les mutations identifiées dans les gènes, les flèches vertes l'activation de l'expression des gènes et les lignes fermées l'inhibition d'expression des gènes (Abi Habib *et al.* 2018).

1.1.2.2.2 Syndrome de Beckwith-Wiedemann

Le syndrome de Beckwith-Wiedemann (SBW) est considéré comme le miroir clinique et moléculaire du SRS. En effet, les patients avec SBW présentent classiquement un excès de croissance fœtale (macrosomie à la naissance), une organomégalie, une asymétrie corporelle, un hyperinsulinisme, des anomalies de la paroi abdominale (omphalocèle), une macroglossie et ont une prédisposition à développer des tumeurs d'origine embryonnaire dans la petite enfance (néphroblastome, hépatoblastome). Le consensus récent sur le diagnostic clinique et moléculaire ainsi que la prise en charge du SBW a permis une homogénéisation de la caractérisation et du suivi de ces patients (Brioude *et al.* 2018). Le diagnostic clinique repose sur un score clinique associant des critères majeurs et mineurs et permet de situer le phénotype du patient dans le spectre du SBW qui va guider le diagnostic moléculaire et la surveillance. En effet, SRS et SBW sont majoritairement dus à des anomalies intéressant la même région du chromosome 11. Là où les patients avec SRS auront une perte d'expression d'*IGF2*, les patients avec SBW peuvent avoir une surexpression d'*IGF2*. Il est essentiel de préciser le mécanisme sous-tendant le SBW car le risque de développer des tumeurs varie selon l'anomalie moléculaire en cause. Ainsi, les patients pour lesquels une surexpression d'*IGF2* est responsable du SBW (disomie uniparentale paternelle ou gain de méthylation du centre d'empreinte *H19/IGF2* sur l'allèle maternel) ont un risque important de survenue de cancer. Les SBW secondaires à une anomalie du second centre d'empreinte parentale de la région 11p15 qui ne fait pas intervenir *IGF2* mais un gène exprimé à partir de l'allèle maternel, *CDKN1C* (impliqué dans la régulation du cycle cellulaire), auront un risque plus faible. La surveillance carcinologique de ces enfants sera donc différente selon le type d'anomalie moléculaire.

1.1.2.2.3 Syndrome 3-M

Parmi les pathologies génétiques connues pour induire un retard de croissance à début prénatal, le syndrome 3-M a été décrit pour la première fois en 1975 (Miller *et al.* 1975). Le phénotype est assez pauvre en dehors du trouble de croissance et d'anomalies osseuses rachidiennes. Les principales anomalies génétiques retrouvées se situent sur les gènes *CUL7, OBSL1* ou *CCDC8* et se transmettent sur un mode autosomique récessif (Huber *et al.* 2005; Hanson *et al.* 2009, 2011). Ces gènes codent pour des protéines responsables de la régulation du protéasome. En 2013, l'implication d'*IGF2* dans le phénotype de ces patients a été démontrée. En effet, les patients atteints du syndrome 3-M ont une expression moindre d'*IGF2* ainsi qu'une production moindre d'IGF-II (dans les fibroblastes) (Murray *et al.* 2013). Bien que cette baisse d'expression puisse expliquer le phénotype, les mécanismes par lesquels elle a lieu chez ces patients ne sont pas élucidés.

1.1.2.3 Anomalies d'IGF1R

Pour mon travail de recherche, nous nous sommes intéressés aux anomalies génétiques impliquant *IGF1R*. Chez l'humain, les premières anomalies décrites ont été des délétions terminales du chromosome 15 (avec remaniement en anneau ou non) incluant *IGF1R* à l'état hétérozygote, chez des patients avec restriction de croissance fœtale, microcéphalie et retard psychomoteur de degré variable (Pasquali *et al.* 1973; Butler *et al.* 1988). En 2003 ont été décrits les premiers patients avec des variants pathogènes d'*IGF1R* à l'état hétérozygote et hétérozygote composite (Abuzzahab *et al.* 2003). Depuis, de nombreux cas ont été publiés avec majoritairement des mutations faux-sens à l'état hétérozygote et un phénotype clinique associant de manière constante une restriction de croissance fœtale et une microcéphalie (Yang *et al.* 2018). La majorité des patients présentait des taux spontanément élevés d'IGF-I dans le sérum (moyenne à 1,2 DS) (Walenkamp *et al.* 2019). Le retard de croissance postnatal est très variable, avec un éventail de tailles adultes allant de très petites tailles à des tailles normales. De même, la déficience intellectuelle est inconstante et hétérogène. Il n'existe à ce jour que deux patients porteurs de mutations à l'état homozygote rapportés (Gannage-Yared *et al.* 2012; Prontera *et al.* 2015).

Sur le plan fonctionnel, certaines équipes ont montré une baisse de phosphorylation du récepteur IGF1R et d'AKT (voie de la PI3 kinase) en western blot en réponse à la stimulation par IGF-I, soit sur des cellules de patients (fibroblastes), soit dans des lignées cellulaires après expression de récepteurs mutés par transfection (Ester *et al.* 2009; Fang *et al.* 2009; Kruis *et al.* 2010; Labarta *et al.* 2013; Solomon-Zemler *et al.* 2017).

Sur le plan thérapeutique, le traitement par hormone de croissance a un effet inconstant mais les modalités d'administration étant très variables, l'évaluation objective de son efficacité reste impossible (Yang *et al.* 2018; Walenkamp *et al.* 2019). Il ne semble cependant pas y avoir de différence en termes d'efficacité entre les patients avec une délétion d'*IGF1R* ou un variant pathogène (Walenkamp *et al.* 2019).

La prévalence des anomalies *d'IGF1R* est inconnue, une étude récente a cependant retrouvé une anomalie d'*IGF1R* dans deux cas au sein d'une population de 64 patients nés PAG sans rattrapage à quatre ans. Les auteurs suggéraient donc un dépistage plus systématique de ces anomalies dans cette population de patients nés avec PAG (Janchevska *et al.* 2018).

L'étude d'une cohorte de patients porteurs d'anomalies d'*IGF1R* a été l'objet d'un article soumis, dans le cadre de mon travail expérimental (section 2.1.1.1).

1.1.2.4 Anomalies d'ALS

Des anomalies homozygotes ou hétérozygotes composites d'*IGFALS* (MIM #601488) ont été décrites depuis 2003, se manifestant par des paramètres de naissance dans la moyenne basse (-1 DS en moyenne) et un retard postnatal modéré (entre -3 et -2 DS) avec retard pubertaire dans la moitié des cas décrits (Domené *et al.* 2004, 2011). Sur le plan fonctionnel, les taux bas d'ALS diminuent la stabilité d'IGF-I dans le plasma en raison de l'absence de formation du complexe ternaire, et il en résulte une baisse d'IGF-I dans la circulation sanguine. Les actions autocrines et paracrines sous la dépendance seule de la GH sont théoriquement peu affectées et permettent probablement de préserver la croissance fœtale, mais elles paraissent insuffisantes à assurer une croissance postnatale satisfaisante. Les patients présentaient également de manière inconstante une résistance modérée à l'insuline.

1.1.2.5 Anomalies de PAPP-A2

Très récemment, des patients porteurs de mutations du gène *PAPPA2* à l'état homozygote codant pour une des protéases du complexe ternaire clivant IGFBP-3 et IGFBP-5 ont été décrits (Dauber *et al.* 2016). Il s'agit d'un long gène de 21 exons situé sur le chromosome 1q25.2. Les patients atteints présentaient un retard de croissance postnatal modéré à sévère, une microcéphalie d'apparition progressive (inconstante) et des taux élevés d'IGF-I, d'IGFBP-3, d'IGFBP-5, d'ALS et de GH et des taux modérément élevés d'insuline. Malgré des taux sériques d'IGF-I élevés, l'IGF biodisponible et l'IGF-I bioactif étaient très abaissés. Les modèles murins KO avaient le même phénotype clinique et biologique. L'absence de protéolyse des complexes ternaires par défaut de PAPP-A2 entraîne une séquestration d'IGF-I par ses protéines de liaison au sein de ces complexes, l'IGF-I sera donc non mobilisable sous sa forme libre qui permet d'activer IGF1R. Les effets auto et paracrines d'IGF-I (sous la dépendance de la GH) sont préservés, rendant probablement compte du retard de croissance modéré chez les patients atteints. Ces travaux ont donc permis d'affiner les connaissances sur la régulation de la biodisponibilité d'IGF-I en période postnatale (figure 9) (Argente *et al.* 2017).

Les questionnements de notre travail expérimental portent essentiellement sur le système des IGFs et son implication dans la croissance. La part respective d'IGF-I, d'IGF-II et d'IGF1R dans la croissance fœtale et postnatale a été discutée dans une publication, à l'occasion de la mise en évidence, chez une enfant de sept ans présentant un retard de croissance à début postnatal, de réarrangements chromosomiques complexes impliquant une duplication de la région 11p15 incluant *IGF2* et une délétion hétérozygote d'*IGF1R* en mosaïque (Publication n°2, (Giabicani *et*

al. 2019). On y met en évidence le rôle majeur d'IGF-II en période prénatale avec une action possible par une voie indépendante d'IGF1R (comme suggéré par les modèles murins de double invalidation *Igf2*/*Igf1r*) ainsi que le rôle prépondérant de la signalisation IGF-I-IGF1R en période postnatale.

1.2 Empreinte parentale

1.2.1 Mécanismes de l'empreinte parentale

L'épigénétique se définit comme l'ensemble des modifications chromatiniennes aboutissant à une modulation de l'expression des gènes sans altérer la séquence de l'ADN. Ainsi, à un génome (séquence d'ADN) peut correspondre plusieurs épigénomes (profils d'expression des gènes) qui peuvent varier dans le temps et selon les tissus, et sont transmissibles par la cellule mère aux cellules filles. Les différents mécanismes épigénétiques décrits impliquent des modifications des histones (acétylation ou méthylation), la méthylation de l'ADN au niveau des îlots CpG (en particulier au niveau des promoteurs des gènes) ou l'expression d'ARN non codants (séquences d'ADN transcrites non traduites qui peuvent interagir avec d'autres régions du génome ou au niveau d'autres ARN ou de protéines) (Kalish *et al.* 2014). L'empreinte parentale est un processus permettant de moduler l'expression d'un gène selon son origine parentale et secondaire à la mise en place de marques épigénétiques spécifiques sur chacun des deux allèles (Kelsey & Feil 2013; Hanna & Kelsey 2014). Ainsi, il existe dans le génome humain une centaine de gènes dont l'expression va dépendre de leur origine paternelle ou maternelle (http://www.geneimprint.com). Enfin, ces régions soumises à empreinte parentale sont organisées en *clusters* au niveau de certains chromosomes (Wan & Bartolomei 2008). Un gène sera dit soumis à empreinte paternelle s'il est exprimé uniquement à partir de l'allèle maternel et réciproquement (Ferguson-Smith 2011).

1.2.1.1 Méthylation des îlots CpG

Des marques épigénétiques différentielles entre les deux allèles selon leur origine parentale vont induire une expression monoallélique. La régulation de cette expression monoallélique est actuellement imparfaitement connue, et, parmi les différents mécanismes épigénétiques décrits, la méthylation de centres d'empreinte parentale est la mieux étudiée (Holliday & Pugh 1975). La méthylation de l'ADN s'effectue sur les cytosines des dinucléotides CpG, préférentiellement au sein de régions riches en CpG appelées îlots CpG. La présence de cette méthylation va induire le recrutement de facteurs dont les MBD (*methyl CpG binding domain)* et MeCP (*methyl CpG binding protein*) qui possèdent un domaine de liaison à l'ADN et

répriment la transcription en induisant un état condensé de la chromatine (Fujita *et al.* 2000; Buck-Koehntop & Defossez 2013). D'autre part, la méthylation d'un dinucléotide CpG au niveau du promoteur d'un gène peut empêcher l'action de facteurs de transcription de l'ADN et ainsi entraîner une baisse d'expression du gène en question.

Les « *imprinting center regions* » (ICR) sont des « *differentially methylated regions* » (DMR, régions riches en îlots CpG, méthylées sur un seul des allèles) régulant l'expression de toute une région de l'ADN. Les ICR sont des DMR dites primaires, car ils acquièrent leur profil de méthylation (présence ou absence de méthylation) dans les cellules germinales et le maintiennent après la fécondation et au cours de la vie de l'individu (Feil & Khosla 1999). À l'inverse, les DMR secondaires peuvent être sujettes à des modifications de leur méthylation selon différents évènements environnementaux et selon les tissus. Chez l'humain, les ICR sont très majoritairement méthylés dans les cellules germinales féminines à l'exception des ICR situés en 11p15.5 (*H19/IGF2*:IG-DMR) et en 14q32.2 (*MEG3/DLK1*:IG-DMR) qui sont méthylés dans les gamètes mâles (Feil *et al.* 1994; Takada *et al.* 2002).

1.2.1.2 Mise en place de la méthylation

La mise en place et le maintien des marques épigénétiques régulant l'empreinte parentale sont encore imparfaitement compris. La méthylation se met en place dans les cellules germinales au cours de la gamétogénèse (Lucifero *et al.* 2002). Il existe à cette étape un dimorphisme sexuel puisque la méthylation a lieu à un stade très précoce dans les cellules germinales mâles (en période anténatale), tandis qu'elle se fait au cours des méïoses dans les cellules germinales féminines (Reik *et al.* 2001; Smallwood & Kelsey 2012). Elle est sous la dépendance des DNA methyltransférases (DNMT), enzymes permettant le transfert des groupements méthyls sur les cytosines au niveau des îlots CpG (Bestor 2000). Les DNMT3A et B sont essentielles à la mise en place de la méthylation, comme il a pu être montré tant dans des modèles murins qu'en pathologie humaine. En effet, les souris KO pour *Dnmt3b* mourraient à un stade embryonnaire précoce et expriment une hypométhylation de l'ensemble de leur génome (Okano *et al.* 1999). De même, les expériences d'invalidation de *Dnmt3a* dans les ovocytes de souris ont mis en évidence une perte de méthylation au niveau des ICRs normalement méthylés sur l'allèle maternel (et un profil de méthylation normal au niveau des ICRs méthylés sur l'allèle paternel) chez les embryons, qui décédaient en période anténatale. Dans la lignée germinale mâle, cette inactivation conditionnelle de *Dnmt3a* entraînait une azoospermie et une perte de méthylation au niveau des ICRs normalement méthylés sur l'allèle paternel (Kaneda *et al.* 2004). Chez l'humain, le syndrome ICF secondaire à des mutations de *DNMT3B* à l'état hétérozygote se manifeste par un déficit immunitaire, des anomalies morphologiques faciales ainsi que de

multiples anomalies cytogénétiques dans les régions péricentriques de certains chromosomes qui sont anormalement hypométhylées (Jeanpierre *et al.* 1993; Xu *et al.* 1999). De même, *Dnmt3l* est exprimé dans les ovocytes immatures et les prospermatogonies, au moment où les marques épigénétiques sont apposées *de novo* dans les régions soumises à empreinte. Chez la souris, l'invalidation de *Dnmt3l* chez les femelles était responsable d'une perte de méthylation des DMR normalement méthylées dans les ovocytes, qui persiste dans les tissus embryonnaires ; en revanche, la méthylation des DMR normalement méthylées sur l'allèle paternel était conservée (Bourc'his *et al.* 2001). Dnmt3l est donc nécessaire à l'apposition de l'empreinte dans la lignée germinale femelle en agissant comme cofacteur de Dnmt3a et Dnmt3b (Hata *et al.* 2002). Néanmoins ces mécanismes décrits dans des modèles murins sont probablement en partie différents chez l'humain, des travaux ayant montré l'absence d'expression de DNMT3L dans les ovocytes chez la femme (Petrussa *et al.* 2014).

Un autre acteur principal identifié est CTCF (*CCTC-binding factor*), qui est une protéine à doigts de zinc ubiquitaire qui maintien non méthylés certains ICR (Singh *et al.* 2012). En effet, des sites de liaison de CTCF (*CTCF binding sites* (CBS)) ont été identifiés dans certains ICR (dont IGF2/H19:IG-DMR) et des expériences d'invalidation de ces CBS chez la souris ont montré des gains de méthylation anormaux chez les souriceaux, après le stade de blastocyste (Schoenherr *et al.* 2003; Shmela & Gicquel 2013). Au contraire, la protéine ZFP57 (*zinc finger protein* 57) est elle impliquée dans le maintien de la méthylation, comme cela a été montré pour *KCNQ1OT1*:TSS-DMR (Li *et al.* 2008 p. 57; Monk *et al.* 2019).

Enfin, certaines équipes ont montré l'asynchronisme de mise en place de la méthylation au niveau des différents DMR dans les lignées germinales mâle ou femelle, et permettent de supposer des fenêtres de vulnérabilité qui sont différentes selon les loci (Obata *et al.* 2002; Weaver & Bartolomei 2014).

1.2.1.3 Cycle de l'empreinte parentale

Après la fécondation, l'ensemble du génome du nouvel individu va subir une phase de déméthylation puis une reméthylation (figure 11)(Santos *et al.* 2002). La particularité des ICRs va être le maintien de leur profil de méthylation (propre à leur origine parentale) au cours de ces phases (Olek & Walter 1997; Tremblay *et al.* 1997). La déméthylation des pronucléi est sous la dépendance de l'action oxydative de la protéine TET3 (*10-11 translocation protein*) (Wossidlo *et al.* 2011). Cependant, dans le pronucleus ovocytaire, il existe des facteurs de protection contre ce mécanisme de déméthylation, dont DPPA3 (*developmental pluripotency-associated protein 3*). Au cours de cette phase, différents acteurs interviennent dont DNMT1 et ZFP57 formant un

complexe avec TRIM28 (*tripartite motif-containing 28*) et SETDB1 (*histone-lysine N-methyltransferase*) (figure 12) (Messerschmidt 2012; Monk 2015).

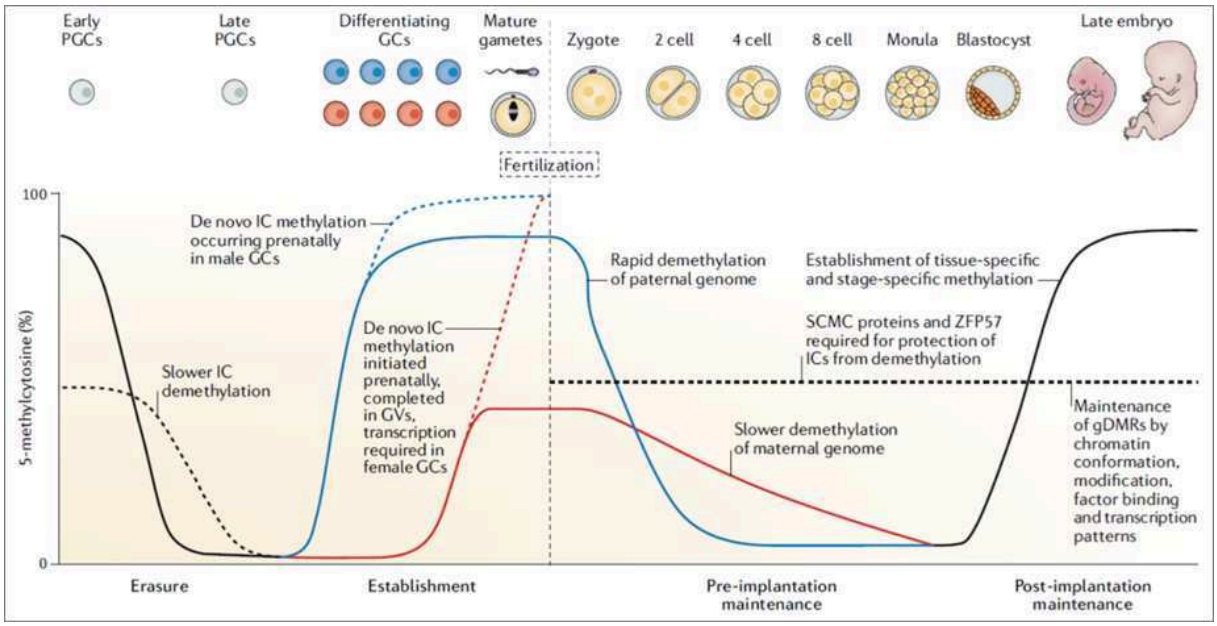


Figure 11. Modifications de la méthylation au cours du développement, au niveau des DMR (gDMR, lignes pleines) et des ICR (IC, lignes pointillées). La méthylation maternelle/féminine est figurée en rouge et la paternelle/masculine en bleu. Après la fécondation le zygote subit une vague de déméthylation des DMRs jusqu'au stade de blastocyste (avec une cinétique différente selon l'origine parentale de la méthylation), puis une phase de reméthylation pendant la vie embryonnaire. Les ICRs sont protégés par les facteurs du complexe maternel sous-cortical (SCMC, dont les NLRPs) et les protéines de maintien (comme ZFP57). Dans les cellules germinales (GC ou PGC pour cellules germinales primordiales) du nouvel individu une nouvelle vague de déméthylation touche les DMRs en incluant les ICRs qui sont ensuite, dans les gamètes, le siège de la mise en place d'un profil de méthylation correspondant au sexe de l'individu (Monk *et al.* 2019).

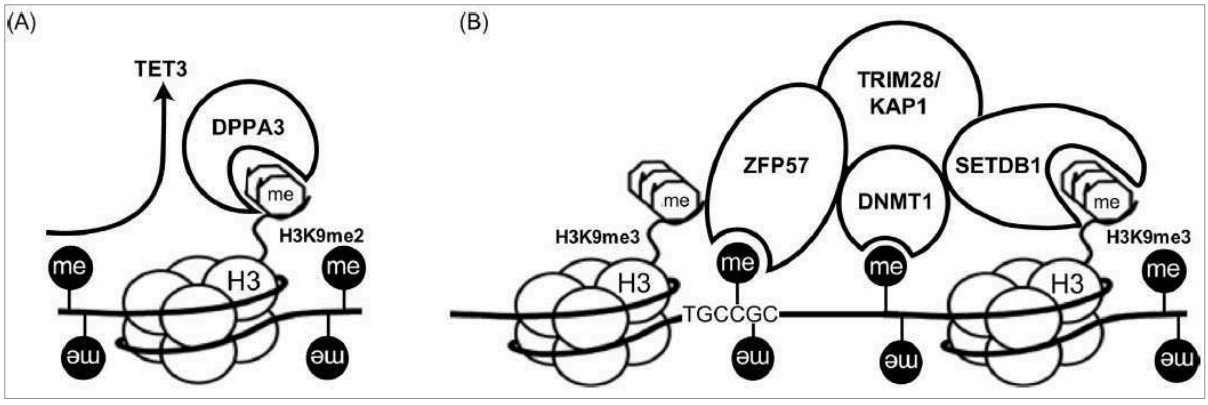


Figure 12. A. Protection par DPPA3 de la déméthylation des ICRs aux stades précoces de développement du zygote par TET3. B. Protection de la methylation des ICRs et des histones H3K9 respectivement par le complexe ZFP57-TRIM28 et SETDB1, DNMT1 (Monk 2015).

D'autres facteurs ovocytaires semblent jouer un rôle primordial dans le maintien de la méthylation aux stades précoces du développement et forment le complexe maternel sous-cortical (Monk *et al.* 2017). La famille des NLRPs (*nucleotide-binding oligomerisation domain, leucine-rich repeat and pyrin domain containing protein*), qui intervient dans des mécanismes régulant l'imminuté innée et l'apoptose, est très présente dans les ovocytes et est nécessaire au développement au-delà du stade deux cellules après fécondation (Tian *et al.* 2009). Des mutations de NLRP7 sont ainsi responsables de môles hydatiformes dites biparentales mais avec une perte de méthylation des ICR normalement méthylés sur l'allèle maternel (Murdoch *et al.* 2006). Les mécanismes d'actions ne sont pas élucidés, bien qu'il semble qu'une interaction directe avec l'ADN soit possible (Mahadevan *et al.* 2014). Enfin, leur rôle pourrait être plus global, comme le suggère la description d'un patient avec des anomalies génétiques non restreintes aux régions soumises à empreinte parentale, dont la mère était porteuse d'une mutation de NLRP7 (Soellner *et al.* 2017). La mise en évidence de ces NLRPs et d'une machinerie ovocytaire qui est nécessaire au maintien des marques épigénétiques de l'empreinte parentale ouvre un champ de recherche majeur pour la compréhension des maladies soumises à l'empreinte parentale.

Les mécanismes connus en jeu dans le cycle de l'empreinte sont donc complexes et ne sont pas strictement identiques entre les modèles murins et l'être humain, ce qui suggère une grande prudence dans l'extrapolation des nombreuses données issues de l'expérimentation animale devant les beaucoup plus rares travaux chez l'humain (Sanchez-Delgado *et al.* 2016a; Monk *et al.* 2019).

1.2.1.4 Empreinte parentale et évolution

Une dizaine de pathologies humaines impliquant des anomalies d'expression de ces gènes soumis à empreinte parentale ont été décrites. La majorité d'entre elles a un retentissement sur la croissance fœtale et/ou postnatale, sur le métabolisme et le développement cérébral. De manière générale, les gènes exprimés à partir de l'allèle paternel ont un impact positif sur la croissance, tandis que les gènes exprimés à partir de l'allèle maternel ont tendance à la restreindre (voir section 1.2.2) (Patten *et al.* 2016). La théorie évolutionniste dite du « conflit parental » (*kinship theory*) semble prévaloir pour expliquer l'existence de l'empreinte parentale (Haig 2000). Le rôle de modulation de l'expression de gènes que tient l'empreinte parentale viendrait d'un « désaccord » sur le niveau d'expression optimal de certains gènes impliqués dans le développement. Les gènes exprimés à partir de l'allèle maternel aurait un rôle d'épargne énergétique lors de la vie fœtale afin de préserver les ressources maternelles (et il en résulte un effet négatif sur la croissance fœtale) ; tandis que les gènes à expression

paternelle ont un rôle d'attribution de l'énergie tournée principalement vers le développement fœtal.

1.2.2 Pathologies liées à l'empreinte parentale

Dans la base de données Mendelian Inheritance in Man (MIM), on recense un peu plus de 9000 pathologies génétiques responsables de restriction de la croissance fœtale. Parmi elles, on retrouve les anomalies touchant les gènes codant pour les différents acteurs du système des IGFs, mais également de nombreux gènes impliqués dans le développement osseux.

Au sein de cette liste, on retrouve également les gènes responsables de pathologies dites liées à l'empreinte parentale (tableau 2) (Eggermann *et al.* 2015b). Parmi elles, le syndrome de Silver-Russell, le syndrome de Temple, le syndrome de Prader-Willi, le diabète néonatal transitoire ou les pseudohypoparathyroïdies (iPPSD) peuvent induire un retard de croissance prénatal et/ou postnatal. D'autre part, les syndromes de Beckwith-Wiedemann ou Kagami-Ogata induisent une croissance excessive et une prédisposition tumorale (tableau 2).

La survenue de pathologies liées à l'empreinte parentale peut être secondaire à des anomalies génétiques ou épigénétiques qui vont modifier l'expression des gènes de la région (Eggermann *et al.* 2015a). Les duplications ou les délétions vont entraîner une modification de l'expression des gènes si elles intéressent l'allèle parental à partir duquel sont normalement exprimés ces gènes (Demars *et al.* 2011; Heide *et al.* 2018). Il peut s'agir également de mutations survenant au sein d'un gène exprimé uniquement à partir d'un des allèles parentaux (avec un effet de perte ou de gain de fonction). Dans ce cas, le patient n'exprimera le phénotype que si la mutation est portée par l'allèle qui exprime normalement ce gène. Les disomies uniparentales résultent de la présence de deux copies issues d'un même parent pour un chromosome (ou une région chromosomique) et donc de l'absence de copie issue de l'autre parent. Ces disomies uniparentales peuvent concerner le même chromosome (isodisomie) ou deux chromosomes homologues différents mais issus du même parent (hétérodisomie). Dans ce cas, certains gènes seront exprimés de manière biallélique et d'autres ne seront pas exprimés. Enfin, il peut survenir une perte ou un gain de méthylation sur un ICR, entraînant, comme dans la disomie uniparentale, une expression biallélique de certains gènes alors que d'autres ne s'exprimeront pas. Ces anomalies sont illustrées en prenant comme exemple la région 11p15.5 sur la figure 13.

Syndrome OMIM	Région chromosomique	Gènes impliqués	Phénotype
Silver-Russell #180860	11p15.5 7p12.1/q32.2	*IGF2, CDKN1c,* *GRB10, MEST PLAG1, HMGA2*	RCIU, retard de croissance, relative macrocéphalie, asymétrie corporelle, difficultés alimentaires, front bombant (Azzi *et al.* 2015a) (Wakeling *et al.* 2016; Abi Habib *et al.* 2018)
Beckwith-Wiedeman #130650	11p15.5	*IGF2, CDKN1c*	Macrosomie, asymétrie corporelle, défaut de la paroi abdominale, risque tumoral, hyperinsulinisme (Brioude *et al.* 2018)
Temple #616222	14q32.2	*DLK1, RTL1 MEG3, MEG8*	RCIU, hypotonie, retard de croissance, puberté précoce, obésité, retard de développement (Ioannides *et al.* 2014; Geoffron *et al.* 2018)
Kagami-Ogata #608149	14q32.2	*DLK1, RTL1 MEG3, MEG8*	Macrosomie, dysmorphie faciale, insuffisance respiratoire restrictive (thorax en cloche), anomalies de la paroi abdominale, retard de développement, risque d'hépatoblastome (Kagami *et al.* 2008)
Prader-Willi #176270	15q11.2	*SNRPN, SNORD116*	Hypotonie, retard de croissance, dysmorphie, troubles du comportement alimentaire, obésité, retard pubertaire, déficits endocriniens centraux, retard de développement (Goldstone *et al.* 2008)
Angelman #105830	15q11.2	*UBE3A, ATP10A*	Trouble envahissant du développement avec rires immotivés, épilepsie, déficience intellectuelle (Tan *et al.* 2011)
Puberté précoce #615346	15q11.2	*MKRN3*	Puberté centrale précoce (Abreu *et al.* 2013)
iPPSD #10380 #603233 #612462	20q13.22	*GNAS*	Ostéodystrophie, résistances hormonales, retard de développement, obésité, calcifications sous-cutanées (Mantovani *et al.* 2018)
Diabète néonatal transitoire #601410	6q24.2	*PLAGL1*	RCIU, hyperglycémie (Docherty *et al.* 2013)

Tableau 2. Principales pathologies liées à une anomalie de l'empreinte parentale, les régions et gènes impliqués, les principales caractéristiques cliniques et les références dans le diagnostic et la prise en charge de ces patients.

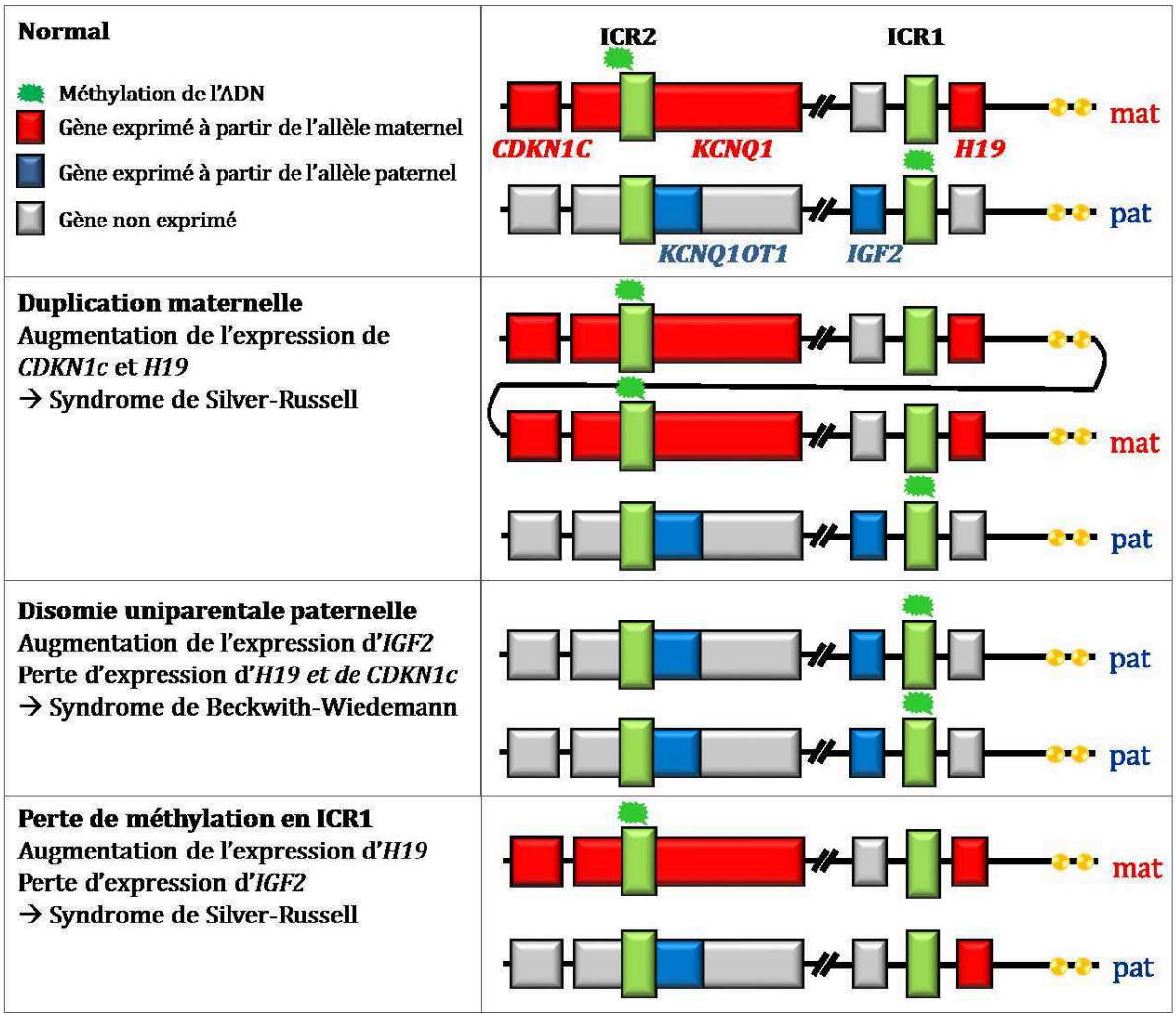


Figure 13. Représentation schématique de la région 11p15.5 pour illustrer les principales anomalies génétiques et épigénétiques responsables de pathologies liées à l'empreinte parentale. ICR1 : *H19/IGF2*:IG-DMR et ICR2 : *KCNQ1OT1*:TSS-DMR.

Nous détaillons ci-après les syndromes qui peuvent être associés à une restriction de croissance fœtale.

1.2.2.1 Syndrome de Silver-Russell

Voir section 1.1.2.2.1 et Publication n°1 (Giabicani *et al.* 2016).

1.2.2.2 Syndrome de Temple

Le syndrome de Temple (TS) a été décrit au début des années 90 par le Pr K. Temple et son équipe à l'université de Southampton (Temple *et al.* 1991). Le phénotype des patients atteints associe une restriction de croissance fœtale, une hypotonie néonatale, une obésité précoce, une puberté centrale précoce, un possible retard de développement et des particularités morphologiques (acromicrie, dysmorphie faciale). Certains travaux ont rapporté

le chevauchement clinique entre TS et SRS ou SPW, identifiant des anomalies de la région 14q32 chez des patients avec des suspicions cliniques de SRS (enfants nés PAG avec difficultés alimentaires et périmètre crânien conservé à la naissance) ou de SPW (hypotonie néonatale, difficultés alimentaires puis obésité et retard cognitif) (Poole *et al.* 2013; Azzi *et al.* 2015a; Kagami *et al.* 2015, 2017). L'anomalie moléculaire la plus souvent identifiée était une disomie uniparentale maternelle du chromosome 14 puis des pertes de méthylation au niveau du centre d'empreinte *MEG3/DLK1:*IG-DMR, et enfin des délétions paternelles de la région 14q32.2 (Ioannides *et al.* 2014). Physiologiquement, la méthylation du centre d'empreinte *MEG3/DLK:*IG-DMR sur l'allèle paternel entraîne l'expression de gènes codant pour les facteurs *DLK1*, *DIO3* et *RTL1* à partir de l'allèle paternel, et d'ARNs non codants (*MEG3*, *MEG8*) de microARNs et de small nucleolar (sno)ARNs à partir de l'allèle maternel (figure 14). L'expression phénotypique en cas d'anomalie de cette région n'est pas clairement expliquée, du fait des fonctions encore imparfaitement connues des gènes et ARNs non codants qui la composent. La publication n°4 rapporte les données cliniques et moléculaires d'une cohorte de 28 patients (voir partie Travail expérimental section 2.2.1 (Geoffron *et al.* 2018)).

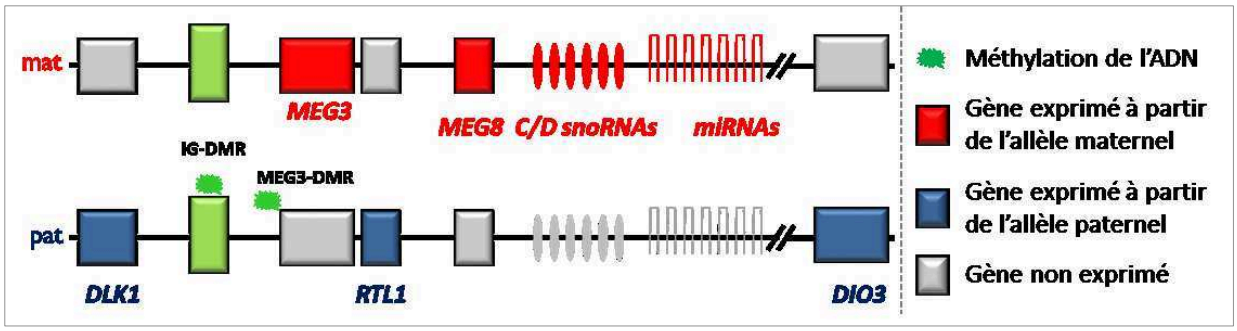


Figure 14. Région 14q32.2. L'allèle maternel (mat) et l'allèle paternel (pat) ont des profils de méthylation et d'expression qui diffèrent.

1.2.2.3 Syndrome de Prader-Willi

Les nouveaux-nés atteints du syndrome de Prader-Willi (SPW) présentent une hypotonie importante et caractéristique à la naissance, et des difficultés alimentaires, généralement dans les premiers mois de vie. Par la suite, ces patients présentent une hyperphagie avec des troubles du comportement handicapants et un retard cognitif dont le degré est variable. Ils présentent classiquement un hypogonadisme hypogonadotrope qui peut être associé à d'autres insuffisances centrales (corticotrope et thyréotrope principalement). De plus, ils ont des traits dysmorphiques communs (microstomie, bouche en chapeau de gendarmes, élargissement des

fentes palpébrales) (Gunay-Aygun *et al.* 2001; Goldstone *et al.* 2008). Le SPW est secondaire à une anomalie moléculaire de la région 15q11-q13 qui contient de nombreux gènes à expression paternelle (*MKRN3, MAGEL2, NDN, NPAP1* et *SNURF-SNRPN*) ainsi que de nombreux snoARN (*SNORD64, 107, 108, 109A, 109B, 115, 116)*, tandis que le gène *UBE3A* est à expression maternelle dans certaines régions du cerveau. Le mécanisme moléculaire principal est la délétion paternelle (75-80%) puis la disomie uniparentale maternelle (20-25%) et les anomalies de méthylation ne concernent que moins d'un pourcent des patients. La région critique responsable du phénotype complet (hypotonie néonatale, difficultés alimentaires puis hyperphagie, obésité infantile, hypogonadisme et retard de développement) semble être celle intéressant les snoARN, principalement *SNORD116* (Cassidy *et al.* 2012).

1.2.2.4 Diabète néonatal transitoire

Le diabète néonatal transitoire (TNDM) se manifeste classiquement par des hyperglycémies dans les premières semaines de vie chez un nouveau-né qui était né PAG. Ce diabète est résolutif habituellement dans la petite enfance, bien que certaines équipes aient décrit la survenue ultérieure de rechutes nécessitant parfois une insulinothérapie (Metz *et al.* 2002). Peuvent également s'associer des signes inconstants appartenant au spectre du syndrome de Beckwith-Wiedemann tels qu'une macroglossie ou des anomalies de fermeture de la paroi abdominale (Docherty *et al.* 2013; Brioude *et al.* 2018). La région soumise à empreinte parentale impliquée est située sur le chromosome 6q24.2 et régule l'expression paternelle des gènes *PLAGL1* et *HYMA1* bien que les mécanismes physiopathologiques sous-tendant l'expression phénotypique ne soient pas élucidés. Les anomalies identifiées chez les patients sont des disomies uniparentales paternelles (40%), des duplications paternelles (33%) ou des pertes de méthylation de l'ICR sur l'allèle maternel (26%).

1.2.2.5 Anomalies inactivatrices de la signalisation PTH/PTHrp (iPPSD)

Le terme pseudohypoparathyroïdie (PHP) a été remplacé par iPPSD lors du premier consensus international sur le diagnostic clinique et moléculaire et la prise en charge de ces syndromes (Thiele *et al.* 2016). Une cause moléculaire est identifiée chez environ 80% des patients et touche la voie de signalisation des récepteurs couplés aux protéines G. Le diagnostic est envisagé devant la présence de critères majeurs : résistance à la parathormone (PTH), ossifications sous-cutanées, obésité précoce (avant l'âge de 2 ans) associée à une autre résistance hormonale (TSH, GH), des antécédents familiaux et/ou une ostéodystrophie héréditaire d'Albright (qui associe une brachydactylie et une petite taille adulte à d'autres anomalies inconstantes telles que des ossifications ectopiques, un faciès lunaire ou un aspect

trapu) (Mantovani *et al.* 2018). Il existe un phénotype variable qui peut inclure des manifestations multisystémiques, neurologiques (retard cognitif, malformation de Chiari, etc), endocriniennes (hypogonadisme, déficit en GH, etc), des anomalies de minéralisation, et certains patients naissent PAG. La restriction de croissance fœtale s'observe principalement chez les patients avec mutation de *GNAS* sur l'allèle paternel (pseudoPHP ou hétéroplasie osseuse progressive) ou une acrodysostose (par mutation dans *PRKAR1A* ou *PDE4D* de transmission autosomique dominante). La majorité des patients (80% en cas de PHP1A, 50-70% des pseudoPHP et tous ceux atteints d'acrodysostose) a une petite taille à l'âge adulte. Le traitement par hormone de croissance est recommandé en cas de déficit ou de résistance à la GH ainsi que chez les patients nés PAG, avec une attention particulière sur les patients présentant des calcifications ectopiques devant l'absence de données sur leur évolution sous traitement.

Dans une revue récente, nous avons repris les principales anomalies génétiques et épigénétiques responsables de restriction de la croissance fœtale (principalement *via* leur action sur le système des IGF), ainsi que les conséquences et les enjeux à long terme chez les patients affectés (Publication n°3 (Giabicani *et al.* 2018)). Ce travail permet de mettre en avant l'importance de la programmation fœtale principalement sur le métabolisme (glucidique et lipidique) mais également les régulations hormonales (adrénarche et puberté) et le développement cérébral. Nous y proposons également une prise en charge adaptée aux enfants nés PAG pour limiter ces conséquences à long terme (renutrition précoce progressive, surveillance de l'adrénarche, dépistage et traitement des facteurs de risque cardio-vasculaires).

1.2.3 Défauts de méthylation multiloci

Des anomalies de méthylation au niveau de plusieurs ICR ont été mises en évidence chez des patients présentant une maladie liée à l'empreinte parentale (Mackay *et al.* 2006a; b). La proportion de ces défauts multiples de méthylation (*multiloci imprinting defects,* MLID) est variable selon les syndromes identifiés. Ainsi, dans une revue récente, Mackay *et al.* ont rapporté une prévalence des MLID (évaluée sur de l'ADN leucocytaire) de 50% chez les patients atteints de diabète néonatal transitoire, 25% chez les patients avec syndrome de Beckwith-Wiedemann (SBW, avec perte de méthylation en *KCNQ1OT1*:TSS-DMR) et, dans une moindre mesure, autour de 10% chez les patients atteints de pseudohypoparathyroïdie (PHP) ou de syndrome de Silver-Russell (SRS, par perte de méthylation d'*H19/IGF2*:IG-DMR) (Mackay *et al.* 2015). Cette proportion de MLID est indicative mais varie en fonction des tissus analysés et du nombre de loci étudiés (Azzi *et al.* 2015b). Une revue récente a recensé et quantifié les proportions de MLID selon les syndromes présentés par les patients et selon les loci les plus souvent atteints, montrant ainsi le caractère fréquent et étendu (nombreux loci concernés) de ces atteintes

(figure 15) (Sanchez-Delgado *et al.* 2016b). Par la suite, des anomalies génétiques concernant des facteurs impliqués dans la mise en place (NLRP7) ou le maintien (ZFP57) de la méthylation sur les ICR ont été identifiées chez les patients ou leur mère (Begemann *et al.* 2018). Ainsi, les mutations homozygotes ou hétérozygotes composites de *ZFP57* sont identifiées chez la moitié des patients avec diabète néonatal transitoire et MLID (Mackay *et al.* 2008). Les défauts génétiques identifiés sur les gènes *NLRP5, NLRP2* ou *NLRP7* chez les mères de patients avec MLID sont eux responsables d'anomalies de la mise en place ou du maintien de la méthylation dans les cellules germinales maternelles ou en tout début de fécondation (Meyer *et al.* 2009; Docherty *et al.* 2015; Soellner *et al.* 2017). Il est donc suggéré de rechercher des MLID chez les patients présentant des maladies liées à l'empreinte parentale atypiques, en cas de formes familiales, ou encore de difficultés de conception (fausses couches répétées ou môle hydatiforme, qui correspond à une perte plus ou moins complète des marques épigénétiques d'origine maternelle) (Begemann *et al.* 2018). La mise en évidence et l'étude de ces anomalies *multiloci* permettent d'améliorer la connaissance des mécanismes physiopathologiques impliqués dans la mise en place et le maintien de la méthylation dans ces régions soumises à empreinte parentale. Le développement de techniques robustes et de haut débit permettant d'une part d'étudier rapidement la méthylation de tous les ICR, et d'autre part les anomalies génétiques des facteurs agissant en *trans,* devrait apporter des données cruciales dans la compréhension de ces MLID (Court *et al.* 2013; Soellner *et al.* 2019). Bien que, l'utilisation de ces données pour le conseil génétique reste encore très complexe, c'est également une perspective majeure de ce champs de recherche.

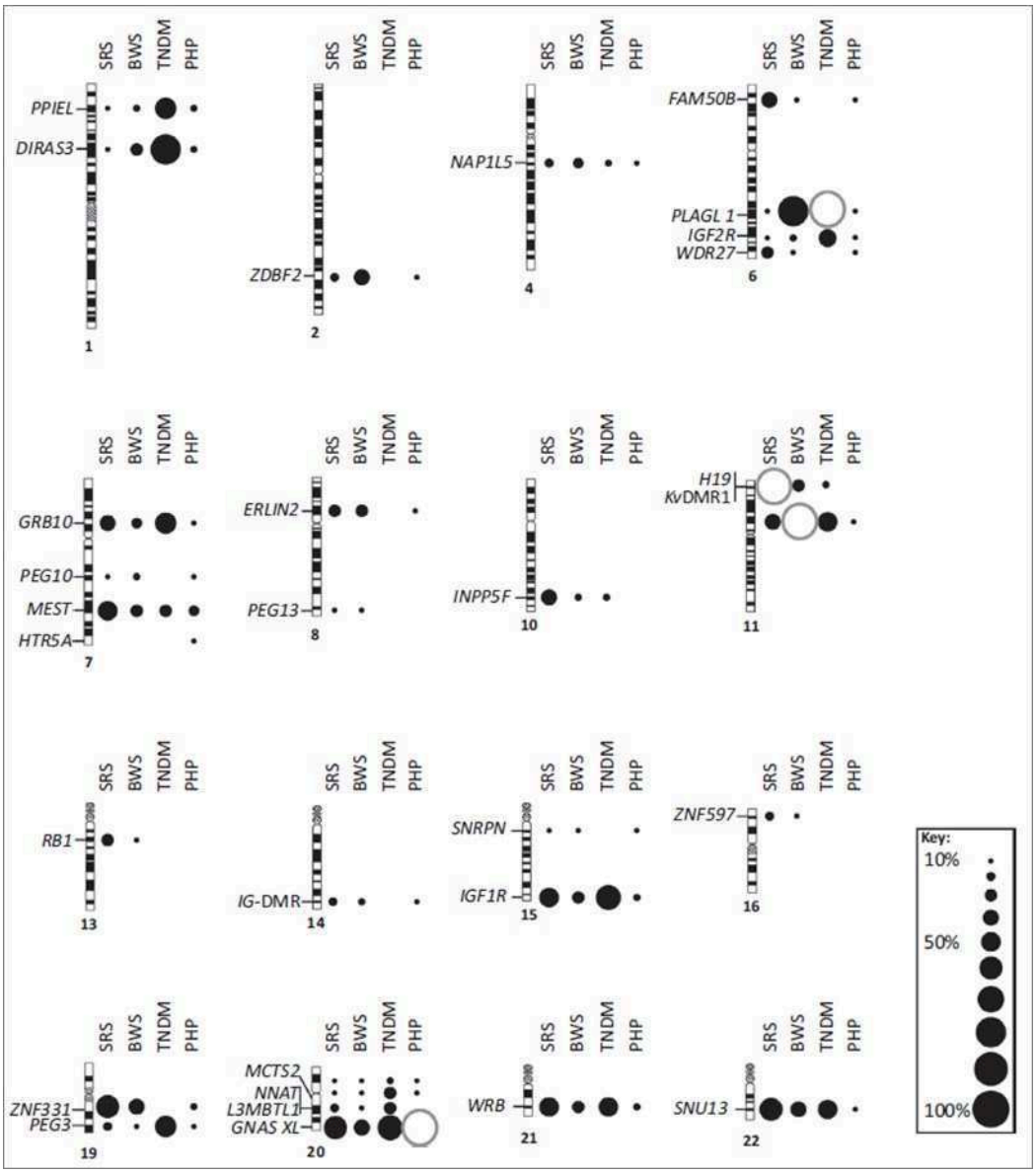


Figure 15. Représentation schématique de la proportion d'anomalies de la méthylation aux différents ICR chez les patients atteints de pathologies liées à l'empreinte parentale. Les cercles blancs correspondent à l'anomalie responsable du syndrome. (Sanchez-Delgado *et al.* 2016b).

1.2.4 Réseau de gènes soumis à empreinte parentale

Du fait du chevauchement clinique observé entre les différents syndromes liés à l'empreinte parentale, des co-régulations entre ces différents gènes ont été étudiées (Eggermann *et al.* 2015b). En 2006, à l'aide d'un modèle murin avec KO paternel de *Zac1 (*équivalent de *PLAGL1* chez l'humain, impliqué dans le TNDM), une équipe française a montré l'existence d'un réseau de gènes soumis à empreinte parentale (figure 16) (Varrault *et al.* 2006). En effet, l'absence de *Zac1* induit des modifications de l'expression de certains gènes soumis à empreinte parentale

ayant un rôle clé dans la croissance fœtale (incluant notamment *Igf2*, *Dlk1*, *Igf2r*, *Cdkn1c*, *Grb10*). En 2009, une équipe montre dans un modèle de souris avec une délétion maternelle d'*H19 (*un long ARN non codant dont l'expression à partir de l'allèle maternel est contrôlé par la méthylation de l'*H19/IGF2*:IG-DMR sur l'allèle paternel, dans la région 11p15 (figure 13)) la surexpression d'autres gènes soumis à empreinte parentale exprimés à partir de l'allèle paternel (*Igf2*, *Dlk1*, *Rtl1*, *Gnas*) ou maternel (*Cdkn1c*, *Igf2r*) dont certains sont localisés en dehors de la région 11p15 (Gabory *et al.* 2009). Les mécanismes en jeu dans ce cas étaient soit médiés par *H19* directement soit par un micro ARN (*miR675*) co-exprimé avec *H19*. Par la suite, c'est dans des cellules pluripotentes induites à partir de cellules de patients avec syndrome de Prader-Willi que Stelzer *et al.* ont mis en évidence que la perte d'expression d'*IPW* (un long ARN non codant situé en 15q11, exprimé à partir de l'allèle paternel) induisait l'augmentation d'expression de gènes exprimés à partir de l'allèle maternel et la baisse d'expression des gènes exprimés à partir de l'allèle paternel dans la région 14q32.2 (région du syndrome de Temple) (Stelzer *et al.* 2014). Les mécanismes en jeu dans cette régulation faisaient intervenir des modifications de la méthylation des histones de la région 14q32.2. Enfin, toujours chez la souris, des travaux ont montré que des doubles KO d'*Igf1r* et du récepteur de l'insuline entraînaient une dérégulation de l'expression des gènes soumis à empreinte dans ces cellules (Boucher *et al.* 2014). Les travaux mentionnés précédemment (section 1.1.2.2.1) montrant une régulation de l'expression d'*IGF2* par *PLAG1* ou *HMGA2* confirment donc chez l'humain que le réseau des gènes soumis à empreinte parentale interagit également avec des gènes non soumis à empreinte parentale, impliqués dans les mécanismes de croissance et de métabolisme (Abi Habib *et al.* 2018).

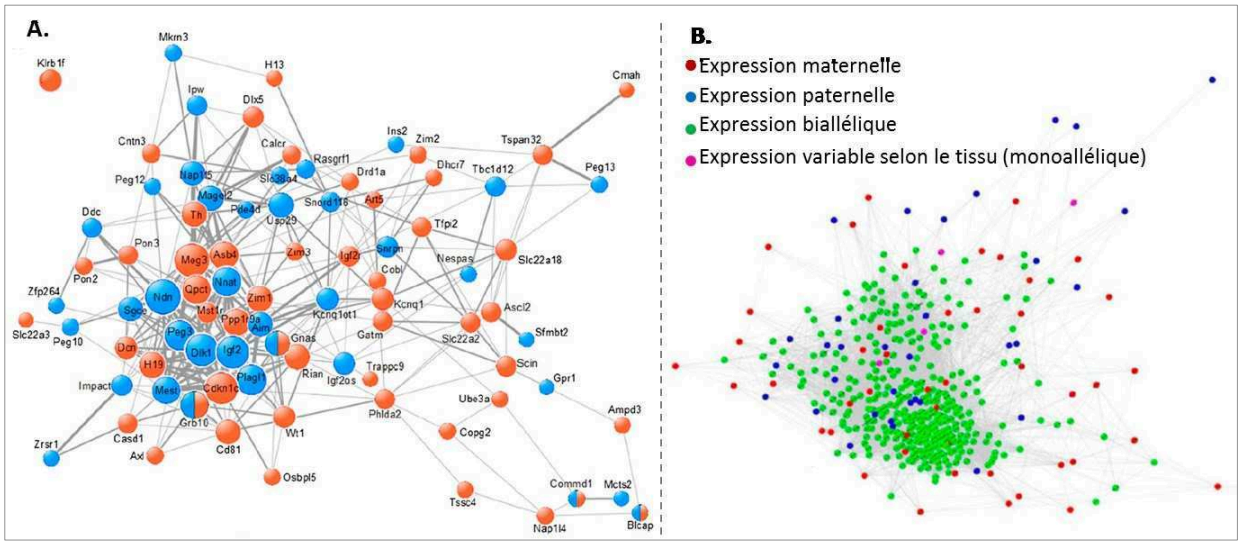


Figure 16. Réseau des gènes soumis à empreinte parentale. A. Représentation des régulations décrites dans l'expression des différents gènes soumis à empreinte chez la souris. B. Représentation incluant la régulation des gènes à expression biallélique (Al Adhami *et al.* 2015).

La compréhension de ces interrelations complexes entre gènes soumis à empreinte ou à expression biallélique et l'étude de cet IGN dans les différents tissus sont essentielles dans l'interprétation des anomalies moléculaires dépistées chez les patients. Contrairement aux cas de MLID, la méthylation d'un ICR peut être normale mais l'expression des gènes soumis à empreinte parentale qu'elle régule normalement, être modifiée par d'autres gènes soumis à empreinte (Al Adhami *et al.* 2015). Là encore, les techniques d'analyse haut-débit des profils d'expression des gènes (RNAseq) sont d'un grand intérêt pour élucider la physiopathologie de ce réseau dans la croissance, le métabolisme et le développement, mais nécessitent une interprétation bioinformatique ardue et sont rendues d'autant plus complexes car ces profils d'expression peuvent varier selon l'âge, le sexe et les tissus. L'étude de ces profils d'expression dans les tissus fœtaux et humains apportera en effet des informations cruciales sur les mécanismes sous-tendant les phénotypes des pathologies soumises à empreinte parentale. Actuellement, la difficulté d'accès à ces tissus d'interêt pour la pathologie étudiée (comme le cartilage de conjugaison pour les pathologies retentissant sur la croissance) représente un obstacle majeur, ce pourquoi les travaux dans les modèles animaux sont cruciaux.

2 TRAVAIL EXPERIMENTAL

Les objectifs de notre travail expérimental étaient de répondre à deux questions principales.

La première était d'élucider les mécanismes qui sous-tendent la résistance à l'IGF-I observée cliniquement chez les patients ayant un syndrome de Silver-Russell par anomalie de la région 11p15. Notre première hypothèse était celle d'une altération de l'activité d'IGF1R, ce qui nous a conduits à étudier précisément les patients porteurs d'anomalies d'*IGF1R* et à élaborer un test fonctionnel permettant d'évaluer l'activité d'IGF1R chez ces patients, puis chez les patients avec SRS. La seconde hypothèse était celle d'une baisse de la biodisponibilité d'IGF-I chez les patients avec SRS. Nous avons ainsi analysé les principaux acteurs du système des IGFs dans les sérums de patients avec SRS.

La seconde était de caractériser le chevauchement clinique et moléculaire entre deux pathologies liées à l'empreinte parentale : le syndrome de Silver-Russell (lié à des anomalies de la région 11p15.5) et le syndrome de Temple (lié à des anomalies de la région 14q32.2). Ce travail a fait l'objet de la publication n°4 (Geoffron *et al.* 2018). L'approfondissement sur le plan moléculaire des constatations apportées par cette publication a permis de mettre en évidence l'implication commune d'*IGF2* dans ces deux syndromes (Abi Habib *et al.* 2019).

2.1 Résistance à l'IGF-I et restriction de croissance fœtale

2.1.1 Étude d'IGF1R

Chez les patients atteints d'un SRS, il a été décrit des taux circulants d'IGF-I élevés, d'autant que leur statut nutritionnel suggèrerait plutôt des taux d'IGF-I bas du fait de leur dénutrition chronique, du moins dans les premières années de vie (Binder *et al.* 2006). Malgré ces taux spontanément élevés d'IGF-I, ils présentent une restriction de croissance pendant la vie intra-utérine qui se poursuit dans l'enfance et aboutit à une petite taille à l'âge adulte (Binder *et al.* 2008, 2013). La compréhension de ces taux sériques paradoxalement élevés chez ces patients est importante d'un point de vue physiopathologique, mais également d'un point de vue thérapeutique. En effet, le consensus international concernant le traitement par rGH en cas de restriction de croissance fœtale sans rattrappage statural recommande de maintenir les taux d'IGF-I sérique dans les normes pour l'âge et le sexe (Clayton *et al.* 2007). Cependant s'il existe un mécanisme de résistance à l'IGF-I constitutionnel chez ces patients, leurs taux d'IGF-I circulant ne sont pas le reflet de son activité et cette surveillance n'est pas opportune (Johannsson *et al.* 2018). Certaines équipes ont déjà mis en évidence des taux d'IGF-I élevés chez les patients nés PAG, cependant il est compliqué d'interpréter ces données d'un point de vue physiopathologique tant ces groupes sont hétérogènes de part les étiologies multiples aboutissant à une restriction de croissance fœtale (Finken *et al.* 2018). Nous avons donc

concentré notre étude aux pathologies de restriction de croissance fœtale impliquant le système des IGFs pour avancer dans les connaissances de cette programmation anténatale. En effet, ce profil biologique est retrouvé chez les patients porteurs d'anomalies d'*IGF1R*. Dans un premier temps, nous avons donc étudié le phénotype des patients porteurs d'anomalies d'*IGF1R* ainsi que l'activité *in vitro* de leur IGF1R. Secondairement, nous avons évalué le fonctionnement d'IGF1R chez les patients avec SRS.

2.1.1.1 Patients avec anomalies d'IGF1R

Nous avons étudié les données cliniques et moléculaires des patients chez qui une anomalie d'*IGF1R* avait été identifiée au laboratoire. Il s'agit d'une cohorte nationale de 35 individus (20 cas index et 15 apparentés) dont les caractéristiques cliniques (retard de croissance intra-utérin et postnatal et taux normaux ou élevés d'IGF-I) avaient conduit les cliniciens à demander la recherche d'anomalie d'*IGF1R* à notre laboratoire de diagnostic. Très récemment, un score clinique a été proposé pour identifier les patients devant être dépistés pour une anomalie d'*IGF1R* (Walenkamp *et al.* 2019). Nous avons donc évalué la sensibilité de ce score au sein de notre cohorte de patients et confirmé son intérêt (sensibilité de 95,2%). Ainsi, nous avons rapporté chez 20 patients, 21 anomalies génétiques. Ces anomalies étaient identifiées principalement à l'état hétérozygote, à l'exception de deux patients porteurs de variants à l'état homozygote et hétérozygote composite. Parmi les 13 variants identifiés, huit n'avaient jamais été décrits. Après une analyse *in silico* en faveur du caractère pathogène pour la majorité de ces variants, nous avons mis au point un test fonctionnel permettant d'apprécier le degré d'activité d'IGF1R chez sept patients. Les variants faux-sens étudiés induisaient une baisse de phosphorylation d'AKT dans les fibroblastes de ces patients après stimulation par IGF-I, ce qui nous a permis d'affirmer *in vitro* le caractère pathogène de deux variants dont la pathogénicité était incertaine *in silico*. Ce travail est l'objet de d'un article récemment soumis, en cours de revue dans *Journal of Medical Genetics.* À ce jour, il n'existe pas de description d'une telle cohorte de patients sur le plan clinique, moléculaire et fonctionnel.

Ce travail est important dans la perspective d'améliorer le diagnostic et le conseil génétique des les patients présentant un retard de croissance intra-utérin. L'identification de variants de signification indéterminée allant croissant avec l'expansion des techniques de séquençage haut débit, il est essentiel de pouvoir disposer d'un test fonctionnel permettant d'apprécier le caractère pathogène de ces variants. En effet, cela permet de guider la prise en charge mais également de ne pas méconnaître des diagnostics différentiels en cas d'activité normale *in vitro* (Inagaki *et al.* 2007; Kansra *et al.* 2012; Wang *et al.* 2013).

Increasing knowledge in IGF1R defects in fetal growth retardation: lessons from 35 new patients.

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ABSTRACT

Background: The IGF1R is a keystone of fetal growth regulation by mediating the effects of IGF-I and IGF-II. Recently, a cohort of patients carrying an IGF1R defect was described, from which a clinical score was established for diagnosis. We assessed this score in a large cohort of patients with identified IGF1R defects, as no external validation was available. Furthermore, we aimed to develop a functional test to allow the classification of variants of unknown significance (VUS) in vitro.

Methods: DNA was tested for either deletions or single nucleotide variant (SNV) and the phosphorylation of downstream pathways studied after stimulation with IGF-I by western blotting of fibroblast of nine patients.

Results: We detected 21 IGF1R defects in 35 patients, including eight deletions and 10 heterozygous, one homozygous, and one compound-heterozygous SNVs. The main clinical characteristics of these patients were being born small for gestational age (90.9%), short stature as adults (78.3%), and microcephaly (74.1%). Feeding difficulties and varying degrees of developmental delay were highly prevalent (54.5%). There were no differences in phenotypes between patients with deletions and SNVs of IGF1R. Functional studies showed that the six missense SNVs tested were associated with decreased AKT phosphorylation.

Conclusion: We report eight new pathogenic variants of IGF1R and an original case with a homozygous SNV. We found the recently proposed clinical score to be accurate for the diagnosis of IGF1R defects with a sensitivity of 95.2%. We developed an efficient functional test to assess the pathogenicity of SNVs, which is useful, especially for VUS.

INTRODUCTION

Insulin-like growth factors IGF-I and IGF-II are major factors which stimulate fetal growth. Both bind to the type 1 IGF receptor (IGF1R). Binding of IGFs to this receptor leads to autophosphorylation of intracellular tyrosine residues, which in turn leads to activation of the phosphatidyl-inositide 3-kinase

(PI3K)/AKT and mitogen-activated protein kinase (MAPK)/ERK signaling pathways, resulting in cellular proliferation and growth [1]. IGF1R is located at chromosome 15q26, contains 21 exons, and leads to the expression of a dimeric transmembrane tyrosine-kinase receptor (Figure 1A. and 1C.) [2]. IGF1R and the insulin receptor (IR) share more than 50% homology and hybrid dimers can be generated, the function of which is still unclear [3].

The first description of the involvement of IGF1R defects in pre and postnatal growth failure was made by Pasquali et al. in the late seventies [4]. The authors described patients with intra-uterine growth retardation (IUGR), postnatal growth failure, and microcephaly due to a 15q26 terminal deletion that included IGF1R (OMIM #612626). Butler et al. then described a similar phenotype in patients with ring chromosome 15 with IGF1R deleted [5]. The phenotype-genotype correlation was unclear because of the difference in gene content of these large deletions. In 2013, Abuzzahab et al. reported a loss of function of the IGF1R in two children with either compound heterozygous pathogenic missense variants or a heterozygous pathogenic nonsense variant of IGF1R (OMIM #270450) [6]. Since then, many others have reported pathogenic variants in IGF1R, mainly in the heterozygous state and rarely in the compound heterozygous state (n = 3) [7]. Finally, in 2012, Gannagé-Yared et al. reported the first patient with a homozygous pathogenic variant [8]. Most of the reported cases were born small for gestational age (SGA) with no or poor catch-up growth, but the final heights are widely variable and can be in the normal range. Indeed variable phenotypic expression has already been reported, even in relatives carrying the same molecular defect, which makes the diagnosis of IGF1R defects difficult [9,10]. Furthermore, some authors have highlighted that the phenotype of patients with IGF1R defects overlaps with that of either Silver-Russell syndrome (SRS, OMIM #180860) or SHORT syndrome (OMIM #269880), leading to the late diagnosis of IGF1R defects [11,12]. However, the presence of microcephaly appears to be highly specific for IGF1R defects. Therefore, head circumference should be assessed in a patient born SGA with poor catch-up growth to distinguish between these etiologies [13]. From the first descriptive cohort of 25 patients with IGF1R defects, Walenkamp et al. proposed a clinical score to drive molecular investigations [14]. This score combines the following four items: birth length or weight < -1 standard deviation score (SDS), head circumference < -2 SDS at first presentation, height at first presentation < -2.5 SDS, and plasma IGF-I levels above the mean for age and gender. Molecular testing for IGF1R should be proposed if three or more items are present, with a sensitivity of 76% in their cohort [14]. Recently, Janchevska et al. identified two anomalies of IGF1R in a cohort of 64 patients born SGA with no catch-up growth, supporting the hypothesis that the prevalence of these defects is high enough to search for them in this particular group of patients [15]. Concerning the treatment of postnatal short stature, the efficiency of recombinant growth hormone (rGH) therapy in patients with IGF1R defects is still controversial and only isolated cases with variable age at onset, duration, and dose of treatment have been reported [7,14].

A few functional studies in either fibroblasts or cell lines have been reported, generally showing the inability of the mutated receptor to activate downstream pathways, especially phosphorylation of the receptor itself and/or AKT and rarely ERK [15–18]. In 2009, Fang et al. demonstrated IGF1R haploinsufficiency due to a mRNA decay phenomenon in a nonsense variant in exon 18 [10]. Most groups have not observed any effect on the expression of the transmembrane IGF1R in patients with missense variants [6,10,20].

We report here a large cohort of 21 IGF1R defects, including eight previously unreported pathogenic variants. Furthermore, we established phenotype-genotype correlations and assessed the efficiency of rGH therapy in these patients. Finally, we developed a reproductive functional test to assess the responsibility of variants of unknown significance (VUS) in the phenotype.

MATERIALS AND METHODS

Patients

Patients were either followed in our clinic or referred by other clinical centers for molecular analysis. IGF1R molecular testing was performed in patients with IUGR with no catch up growth, usually associated with microcephaly and/or elevated serum IGF-I. A clinical file, including comprehensive clinical and biological data, growth charts, and treatment was completed for all patients. Each patient had been examined by a geneticist and/or a pediatric endocrinologist. Written informed consent for participation was received either from the patients themselves or their parents, in accordance with French national ethics rules for patients recruited in France (Assistance Publique – Hôpitaux de Paris authorization n°681).

Auxologic methods

Length, weight, and head circumference at birth are expressed as SDS according to Usher and McLean charts [21]. Postnatal growth parameters are expressed as SDS according to Sempé charts [22]. The age of puberty onset (breast development for girls and testis enlargement for boys) was considered to be normal from 8 to13 years for girls and 9 to 14 years for boys.

Molecular analysis

All molecular diagnosis of the IGF1R defects was performed in the same laboratory of molecular genetics. DNA was extracted from blood leukocytes using an in-house protocol after cell lysis by a salting out procedure, as previously described [23]. DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Invitrogen, France).

The main known molecular causes of SRS (loss of methylation at 11p15, maternal uniparental disomy of chromosome 7) or Temple syndrome (OMIM#616222) at the 14q32.2 locus were ruled out by methylation analysis, as detailed in a previous study [23].

IGF1R deletions were assessed by multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA P217 IGF1R probe mix (MRC Holland, Amsterdam, Netherlands), following the manufacturer's instructions. MLPA data were analyzed using the Novel Software Coffalyser.NET provided by MRC-Holland.

For single nucleotide polymorphism (SNP) microarray analysis, samples were processed using cytoSNP-12, or HumanOmniExpress-24 microarrays (Illumina, San Diego, CA, USA). Automated Illumina microarray experiments were performed according to the manufacturer's instructions. Images were acquired using an iScan System (Illumina). Image analysis and automated CNV calling were performed using GenomeStudio v.2011.1 and CNVPartition v.3.1.6. SNP profiles were analyzed by examination of signal intensity (Log R ratio, i.e. ln (sample copy number/reference copy number)) and allelic composition (BAF, i.e. B Allele Frequency).

For the detection of IGF1R SNV, DNA was amplified and sequenced by direct Sanger sequencing procedures, using the ABI PRISM Big Dye Terminator v3.0 Cycle Sequencing Kit and an ABI 3100 Genetic Analyzer (Life Technologies, Courtaboeuf, France). Sequences were then analyzed with SeqScape v2.6 (Life Technologies).

Variants are described in accordance with the recommendations of the Human Genome Variation Society. All the new variants were recorded in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar).

Variant interpretation was performed following the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) classification of variants [24].

Functional test

Patient fibroblasts, obtained from skin biopsies after informed consent and control fibroblasts obtained from the Coriell Institute of Medical Research (Camden, N.J.), were cultured in Dulbecco's Modified Eagle Medium enriched with glutamate, sodium pyruvate, penicillin, streptomycin, and 10% fetal-calf serum at 37°C. After 24 h of serum-free culture in six-well plates, cells were stimulated with [50 ng/ml] IGF-I (Peprotech, US) for 10 min before lysis. We found these stimulation conditions to be the most accurate to assess both AKT and ERK phosphorylation in controls. For both non-stimulated and stimulated conditions, 4.2 to 12.1 µg of protein was deposited in a NuPAGETM 4-12% Bis-Tris Gel (Thermo Fischer Scientific, US). Electrophoresis was performed on an XCell SureLock™ Mini-Cell Electrophoresis system (Thermo Fischer Scientific, US). Membranes were incubated with polyclonal antibodies against either phospho-AKT (Ser473, Cell Signaling, US, 1:2000), pan-AKT (Cell Signaling, 1:1000), phospho-ERK1/2 (Tyr204, Cliniscience, France, 1:800), ERK1/2 (Cell Signaling, 1:1000), or GAPDH for normalization (Cell Signaling, 1:2000). Then, membranes were incubated with an HRP-conjugated secondary antirabbit antibody (1:3,000), revealed with ChemiDoc™ XRS+ System (Bio-Rad, US), and analyzed with Quantity One v4.6.6 software. Immunoblot images were quantified using ImageJ 1.50 software (https://imagej.nih.gov).

mRNA quantification

Total mRNA was extracted from non-stimulated cells using NucleoSpin miRNA® (Macherey-Nagel) and cDNA obtained by reverse-transcriptase polymerase chain reaction (RT-PCR, Superscript II, Invitrogen, France). cDNA was then amplified and quantified on a QuantStudio 7 Flex Real-Time PCR system (Thermo Fischer) using primers localized in exons 7-8 (see supplemental data) by SYBR Green technology (Applied Biosystem, US).

Biological assays

IGF-I serum concentrations were determined by different techniques, as patients were followed in different centers. However, IGF-I levels were determined along with the normal values for most patients. We express IGF-I levels as SDS according to age and gender from control matched references [25].

Statistical analysis

Characteristics of the population are described as percentages for qualitative variables or as SDS and mean (range) for continuous variables. For statistical analysis, Pearson's test was used for correlations, Fisher's test for dichotomous variables, and the t-test for continuous variables.

RESULTS

Genetic results

Between 2006 and 2018, 111 samples of DNA were tested for IGF1R mutations/deletions. We identified IGF1R defect in 35 patients from 20 different families. Aside from the 20 index cases, we

identified IGF1R defects in 15 relatives, including three siblings, seven fathers, and five mothers. Among the 20 index cases, molecular analysis was prescribed for 13 patients for a clinical suspicion of an IGF1R defect and for seven for a clinical suspicion of SRS. The molecular diagnosis of the IGF1R defect was made at 9.2 years of age (0.8 to 18.1) for the index cases. Eight patients carried a heterozygous deletion (Figure 1B.). Eleven carried a single nucleotide variant (SNV): 8 missense, two nonsense, and one insertion at the boundary of intron 5-exon 6. Sequencing of the cDNA of the latter variant obtained from lymphocytes confirmed that the inserted guanine was present in the cDNA, leading to a frameshift and a premature stop codon (N417Efs*52, Supplementary figure 1). One patient carried two missense SNVs. Among the 13 SNVs identified, 10 patients had a heterozygous SNV, one patient had compound heterozygous missense SNV, and one carried a homozygous missense SNV (Figure 1). Parental DNA samples were available for 13 patients. Three inherited the anomaly from their mother, five from their father, two from both parents, and the anomaly arose de novo for three patients. Among the 13 variants, we identified eight new pathogenic or likely pathogenic variants (Table 1). Five deletions included the entire IGF1R gene, one interstitial and four terminal lengthening from 3.13 to 5.01Mb (Figure 1B., Del1 to 5), whereas three included only part of IGF1R with length from 19kb to 234kb (Figure 1B., Del6 to 8).

Clinical features

The intragenic deletions (exon 2, Del6, n = 3) and IGF1R terminal deletions (Del7, n = 2 and Del8, n = 1) did not include other disease-causing OMIM genes and were thus analyzed together with the SNVs for the clinical study. Clinical characteristics are shown in Table 2. There was no statistical difference in clinical presentation between patients with large deletions and pathogenic variants of IGF1R. We calculated the clinical score recently proposed by Walenkamp et al. for 21 patients for whom clinical data required for this scoring system were fully available (birth weight or length < -1 SDS, height at presentation < -2.5 SDS, head circumference at presentation < -2 SDS and IGF-I level > 0 SDS) [14]. Twenty patients (95.2%) met at least three of the four criteria and 11 (52.4%) fulfilled all four. Among them, all had a birth weight or length < -1 SDS, 17 (81.0%) had a height at presentation below -2.5 SDS, and 19 (90.5%) had microcephaly. All 21 patients scored positive for elevated IGF-1 levels if considered at the different endpoints (including during rGH treatment). However, five patients (23.8%) would have not met this criterion if IGF-I levels were considered only prior to the initiation of rGH treatment. One patient (carrying Del6) did not achieve a positive clinical score, with only two items [being born with a height or weight < -1 SDS and high levels of IGF-I (during rGH treatment only)].

Given the clinical overlap between SRS and IGF1R defects, the Netchine-Harbison clinical scoring system (with a positive clinical diagnosis of SRS for a score of at least 4/6) was assessable for 10 patients and only one scored 4/6 [13,26] (lacking relative macrocephaly at birth and body asymmetry items). Nevertheless, most patients scored 3 out of 6, comprising the following items: being born SGA, postnatal growth retardation, and feeding difficulties.

No deafness was reported in our cohort. One girl (carrying a missense SNV) had a slightly delayed onset of puberty (onset at 13.3 years), whereas the onset of puberty of the other 16 patients (10 boys) occurred at the normal age. Three patients were treated with GnRH analogs at the onset of puberty to preserve the duration of growth due to a low predicted final height, despite the onset of puberty at a normal age. Four patients carrying a missense SNV had attention deficit hyperactivity disorder (ADHD), which required medication. Three patients developed obesity in childhood with metabolic syndrome for one as a young adult. One patient (father of two affected children) had early type 2 diabetes and one patient had episodes of hypoglycemia in infancy. Noticeably, four patients (two with

IGF1R deletion, two with a missense SNV) had cardiac defects, including one case of transient inter-auricular communication (IAC), one of IAC and rhythmic troubles, one of patent foramen ovale, and one of severe cardiac insufficiency, which led to heart transplantation (carrying a missense SNV).

Only two cases of homozygous pathogenic variants have yet been reported [8,12]. Thus, the pedigree and growth curves of the girl with the F112L homozygous pathogenic variant are shown in Figure 2. Although the girl with the homozygous pathogenic variant (II.4) showed severe growth retardation of approximately -4 SDS, both parents (I.1 and I.2) and one older sister (II.3) with the heterozygous pathogenic variant showed impaired postnatal growth of approximately -2 SDS, with a final height in the lower range of normal curves. The unaffected younger siblings (II.1 and II.2) showed normal growth around the mean. Furthermore, the homozygous carrier (II.4) had a patent foramen ovale, severe oeso-gastric reflux, anorexia requiring enteral support for one year (naso-gastric tube), and psychomotor delay with learning disability, whereas no other member of the family presented with such clinical features.

All clinical data are available for each patient in Supplementary Table 1.

rGH therapy

Eighteen patients received rGH treatment, starting at an age of 7.5 years (1.5;15.3) under the SGA European Medicines Agency (EMA) indication, with a mean height at the start of therapy of -3.8 SDS (-5.6; -1.6). The starting dose was 46.6 µg/kg/day (35.0;85.5) and was significantly increased for only five of the 15 patients for whom data on the dose evolution was available. For most patients (60.0%), the dose of rGH was not raised because of high serum levels of IGF-I. Among the 12 patients that completed rGH treatment and reached their final height, the mean height gain was 1.0 SDS (0.2;2.5), which positively correlated with the duration of treatment ($\rho = 0.76$, $p = 0.004$) and negatively correlated with the age at the start of rGH ($\rho = -0.68$, $p = 0.01$). IGF-I serum levels were high (over 1 SDS) for 11 (47.8%) patients before any treatment, with a mean of 1.9 SDS (-2.0;7.1), which rose to 3.3 SDS (0.3;9.5) under rGH therapy.

IGF1R functional test

We performed functional analysis on fibroblasts for seven index cases, two affected parents and three controls (Figure 3). The six missense SNVs all showed a decrease in phosphorylated AKT, although the results for the S1180Y variant did not reach statistical significance ($p = 0.065$). Both G1352V and E1356K were predicted as VUS after in silico analysis and showed a significant decrease in AKT phosphorylation in vitro ($p = 0.009$ and $p = 0.002$, respectively), suggesting that these variants are likely pathogenic. There were no alterations of AKT phosphorylation for two patients carrying either a nonsense SNV or a chromosome 15q26.6 deletion that included the entire IGF1R gene. The results concerning ERK phosphorylation were highly variable and we observed no significant modifications in this pathway (Supplementary Figure 2). All but one patient showed normal IGF1R expression. This patient, who carries a 15q26.6 heterozygous deletion, including IGF1R, showed expression of 37.7% of controls (Supplementary Figure 3).

DISCUSSION

We report a large cohort of patients carrying various IGF1R defects and describe eight new pathogenic variants. Furthermore, we developed an in vitro functional test to assess the pathogenic impact of VUS.

As previously described, IGF1R defects are mainly present in the heterozygous state. Nevertheless, we found two patients with missense SNVs on both alleles, including one patient with compound heterozygous pathogenic variants and one with a homozygous one. The patient carrying the compound heterozygous SNVs did not phenotypically differ from the other patients although both variants were pathogenic. On the other hand, the homozygous pathogenic variant was associated with a more severe phenotype in terms of growth, microcephaly, and mental retardation relative to that of her relatives who carry the same variant in the heterozygous state.

With the advent of next-generation sequencing (including exome sequencing or a gene panel of growth disorders, microcephaly, or cognitive impairment, which can include IGF1R), the identification of SNVs will increase in the future. Thus, the description and registration of new SNVs with a precise phenotypic description is necessary to distinguish between those that are benign and those that are pathogenic. Furthermore, we demonstrated that functional characterization of such SNVs is sometimes necessary. In our cohort, such experiments were helpful for the classification of two SNVs reported as SNPs with a very low allele frequency and classified as VUS based on the ACMG/AMP recommendations because of incomplete penetrance (E1356K and G1352V) [24]. However, the definition of "unaffected" carrier was only based on the reported final heights of the two fathers who carried the variants, as other criteria were not available (birth parameters, head circumference and IGF-I levels).Those two variants were finally classified as likely to be pathogenic after demonstration of their functional consequences.

The in vitro studies showed impairment in the ability to activate downstream pathways for the receptors affected by missense SNVs, especially the AKT pathway. We were unable to demonstrate any significant functional consequences of deletions or nonsense SNV, unlike previous studies [10,27,28]. It is possible that discrepancies between our results on deletions and those of previous studies may be due to different IGF-I concentrations used for stimulation. Indeed, Choi et al. showed a progressive increase in AKT phosphorylation in fibroblasts from a patient with an IGF1R deletion in response to increasing IGF-I concentrations from 1 to 400 ng/mL [28]. Ester et al. reported the same pattern with lower concentrations (5 to 20 ng/mL) [18]. Thus, it is possible that the IGF-I concentration we used (50 ng/mL) did not allow proper discrimination of AKT phosphorylation between deletions and controls [29]. Nevertheless, the aim of this functional study was to assess pathogenic impact of SNVs of unknown significance, and we found IGF-I concentration of 50ng/mL to be effective. Unlike missense pathogenic variants, which may lead to a dominant-negative effect, deletions or nonsense variants may lead to haploinsufficiency. Although we could not quantify membrane IGF1R, we demonstrated that IGF1R mRNA levels were low in fibroblasts from one patient with a deletion, favoring haploinsufficiency [10,28,30].

Very recently, a scoring system has been proposed for a clinical suspicion of an IGF1R defect [14]. This clinical score showed 95.2% sensitivity for our cohort. All patients were born with weight or length < -1 SDS and microcephaly was almost always present. However, postnatal short stature (with a threshold set at -2.5 SDS) was inconstantly observed in our cohort. However, height at first evaluation was usually below -2 SDS. We suggest adapting this clinical scoring system for this item, so as not to miss patients with IGF1R defects for whom height is not severely affected. Elevated circulating IGF-I levels were absent prior to rGH therapy for 23.8% of the patients in our cohort but IGF-I levels rose markedly after initiating rGH treatment. The absence of high IGF-I levels prior to rGH therapy can be explained by the previously described feeding difficulties of some patients with IGF1R defects, which can lead to nutritional deficiency and low basal levels of IGF-I [31]. This pattern of low IGF-I levels which increase rapidly after initiating rGH therapy, should alert clinicians to the possibility of an IGF1R defect in a child born SGA, especially with the presence of

microcephaly. However, the high sensitivity of this clinical score favors its use in routine diagnosis to drive genetic tests. The specificity of this clinical score should be assessed in large cohorts of SGA patients with the help of molecular studies.

This cohort allowed us to better characterize the phenotype of patients with an IGF1R defect. As previously described, fetal and postnatal growth retardation, microcephaly, and elevated IGF-I serum levels were highly prevalent in our cohort [7,14]. As in previously reported cases, we identified several cardiac anomalies in these patients, mostly benign. However, one patient underwent heart transplantation because of severe cardiac failure. These findings are in accordance with previous observations and raise the question of whether to systematically perform cardiac ultrasound when an IGF1R defect is identified [8,12,19,32]. Another interesting feature of our cohort is the presence of ADHD in several patients, which was only been previously reported for one case [29]. Furthermore, as reported in the Dutch cohort, we found a high prevalence of feeding difficulties, sometimes requiring nutritional support, [9,11,14]. This latter feature may have misled some clinicians to consider a clinical diagnosis of SRS at first evaluation. Indeed patients with SRS or IGF1R defects share several symptoms, including being born SGA, postnatal growth retardation, and high circulating levels of IGF-I [33,34]. However patients with IGF1R defects usually present with microcephaly, which distinguishes them from SRS patients, for whom head circumference is relatively preserved at birth [13,26]. The recent international consensus on the diagnosis and management of SRS stated that IGF1R defects represents a differential diagnosis and may be considered easily after the major molecular defects of SRS are ruled out, especially for those patients with no relative macrocephaly [13].

The efficiency of rGH therapy in this cohort is difficult to ascertain, as this was a retrospective and multicentric analysis with varying management in terms of the age at onset, the initial dose, dose adaptation, and discontinuation of treatment. Since the duration and age at the start of treatment significantly correlate with height gain, rGH treatment should be considered for patients with no catch-up growth at four years of age, under the EMA SGA indication. However, both clinicians and patients (or parents) should be aware of this unpredictable response to rGH therapy and the expected high IGF-I levels encountered during treatment. Our in vitro experiments, accounting for the functional consequences of the variants, were unable to distinguish between those patients who responded well or poorly to rGH therapy. It would be of interest however to set up such a prognostic tool.

In conclusion, we provide extensive clinical data on a large cohort of patients carrying IGF1R defects. We identified eight new pathological variants, including one homozygous pathogenic variant. We validated the clinical scoring system that has been recently proposed for patients with IGF1R defects. Finally, we developed a functional test to assess IGF1R activity in vitro that is useful for sorting VUS, which is of particular importance, especially for accurate genetic counseling.

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TABLES

Table 1. Description of the identified single nucleotide variants in the cohort and predictions of the pathological consequences. ACMP/AMP: American College of Medical Genetics and Genomics and the Association for Molecular Pathology classification of variants [24]; AF: allele frequency. # Classification performed using the InterVar classification system; § First reported in Walenkamp et al. [14]. ¤ Classified as "variant of unknown significance" before the functional test results.

Table 2. Clinical features of the patients with IGF1R defects. SNV: Single nucleotide variant. WA: weeks of amenorrhea; SDS: standard deviation score; SGA: born small for gestational age; HC: head circumference; rGH: recombinant growth hormone. # Items included in the Netchine-Harbison clinical scoring system for Silver-Russell syndrome diagnosis.

Table 1.

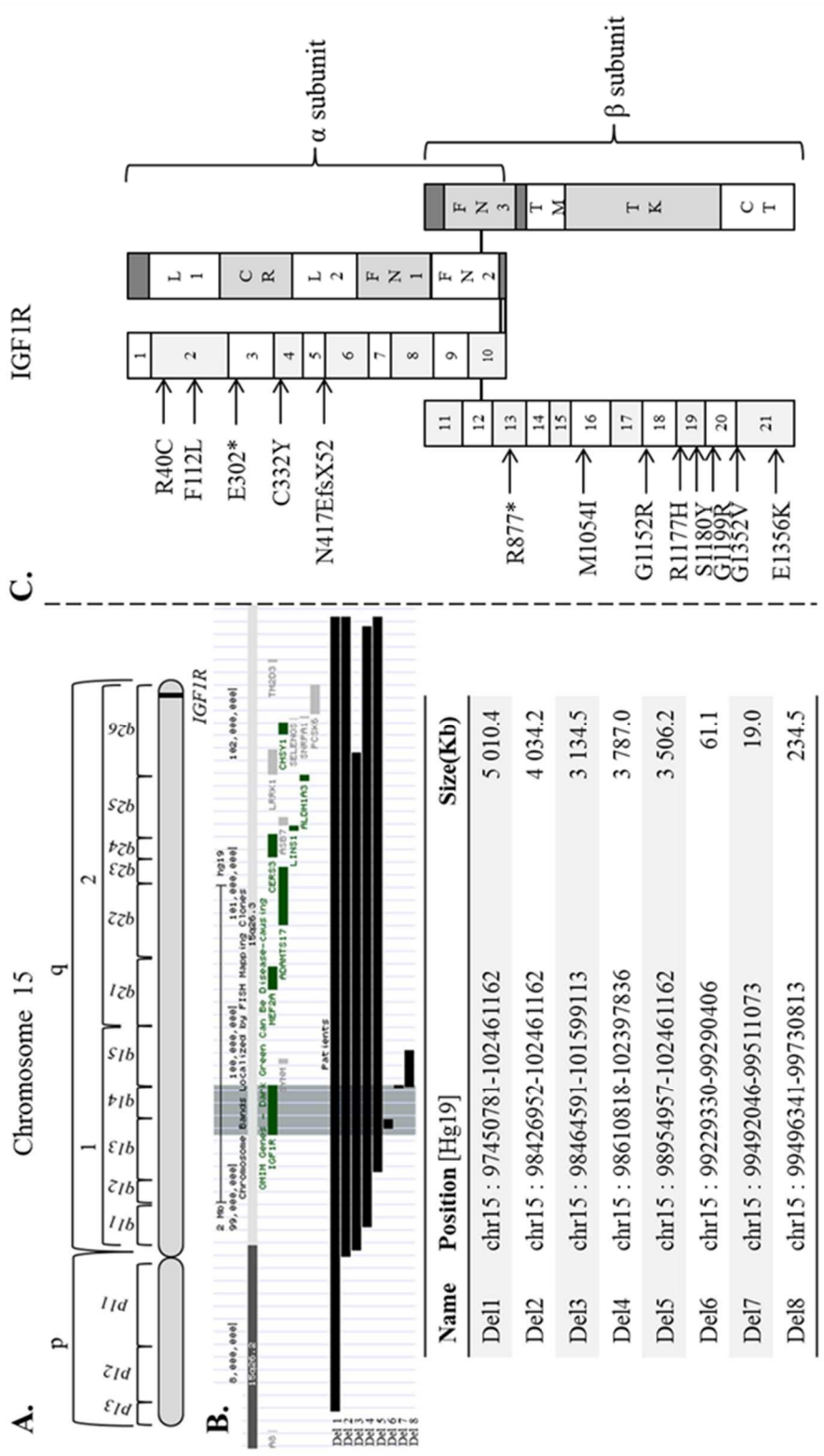
				ACMP/AMP	
cDNA nomenclature NM_000875.4	**Amino-acid substitution** NP_000866.1	**ClinVar**	**GnomAD**	**Detailed staging**	**Variant classification#**
c.118C>T	R40C	In progress	Not reported	PM1 PM2 PP2 PP3	Likely pathogenic
c.384T>C	F112L	In progress	Not reported	PS4 PM1 PM2 PP1 PP2 PP3 PS3	Pathogenic
c.904G>T	E302*	In progress	Not reported	PVS1 PM2 PP3 PP4	Pathogenic
c.995G>A	C332Y	In progress	Not reported	PM1 PM2 PP2 PP3 BS4	Likely pathogenic
c.1247+1-1247+2insG	N417Efs*52	In progress	Not reported	PS3 PM2 PM4 PP1 PP4	Pathogenic
c.3162G>A	M1054I	In progress	Not reported	PM1 PM2 PP2 PP3 PP4	Likely pathogenic
c.3454G>A	G1152R	In progress	Not reported	PS4 PM1 PM2 PP1 PP2 PP3 PP4	Pathogenic
c.3539C>A	S1180Y	In progress	Not reported	PS4 PM1 PM2 PP2 PP1 PP3	Pathogenic
c.2629C>T	R877*	rs150221450	AF: $3.977.10^{-6}$	PVS1 PM2 PP3 PP4	Pathogenic
c.3530G>A	R1177H	In progress	Not reported§	PM1 PM2 PP2 PP3	Likely pathogenic
c.3595G>A	G1199R	rs886044448	Not reported	PS4 PM1 PM2 PP1 PP2 PP3 PP4	Pathogenic
c.4055G>T	G1352V	rs759808066	AF: $1.607.10^{-5}$	PM2 PP2 PP3 PS3 BS4	Likely pathogenic¤
c.4066G>A	E1356K	rs746562843	AF: 642.10^{-5}	PM2 PP2 BS4 PS3	Likely pathogenic¤

Table 2.

	All n=35			Deletions n=7			Mutations n=28			p
	Mean	**Range**	**n (%)**	**Mean**	**Range**	**n (%)**	**Mean**	**Range**	**n (%)**	
Birth parameters										
Term (WA)	37.7	31.0;41.5	23	38.4	37.0;40.0	5	37.5	31.0;41.5	18	0.82
Preterm (<37WA)			5/23 (21.7)			0/5 (0)			5/18 (27.8)	0.55
Weight (SDS)	-2.5	-3.8;-1	23	-2.3	-3.2;-1.4	5	-2.5	-3.8;-1.0	18	0.58
SGA (weight<-2SDS)			17/23 (73.9)			3/5 (60.0)			14/18 (77.8)	1
Length (SDS)	-3.3	-5.2;-1.4	22	-3.0	-4.2;-2	5	-3.4	-5.2;-1.4	17	0.46
SGA (length<-2SDS)			20/22 (90.9)			4/5 (80.0)			16/17 (94.1)	1
SGA(weight/length<-2SDS)			20/22 (90.9)			4/5 (80.0)			16/17 (94.1)	1
Head circumference (SDS)	-2.6	-3.9;-0.3	17	-2.4	-3.0;-1.6	5	-2.7	-3.9;-0.3	12	0.63
Microcephaly (<-2SDS)			14/17 (82.4)			4/5 (80.0)			10/12 (83.3)	1
Relative macrocephaly			3/17 (17.6)			1/5 (20.0)			2/12 (16.7)	1
Clinical features										
Gender (Female/Male)			15/20			2/5			13/15	0.70
Microcephaly			20/27 (74.1)			4/4 (100)			16/23 (69.6)	0.70
Feeding difficulties			12/22 (54.5)			4/5 (80.0)			8/17 (47.1)	0.69
Anorexia/thinness			6/14 (42.9)			2/4 (50.0)			4/10 (40.0)	0.17
Enteral nutrition			5/14 (35.7)			2/4 (50.0)			3/10 (30.0)	1
Developmental delay			12/22 (54.5)			4/5 (80.0)			8/17 (47.1)	0.69
Langage			6/22 (27.3)			3/5 (60.0)			3/17 (17.6)	0.31
Motor			6/22 (27.3)			1/5 (20.0)			5/17 (29.4)	1
Cognitive			12/22 (54.5)			4/5 (80.0)			8/17 (47.1)	0.69
Assisted scholarship			14/23 (60.9)			4/5 (80.0)			10/18 (55.6)	0.70
Final height (SDS)	-2.5	-4.2;1.0	23	-2.9	-4.1;-2.1	5	-2.3	-4.2;1.0	18	0.4
Without rGH	-2.1	-4.2;1.0	11	-3.2	-4.1;-2.1	2	-1.9	-4.2;1.0	9	0.36
With rGH	-2.8	-4.2;-1.0	12	-2.8	-3.8;-2.1	3	-2.8	-4.2;-1.0	9	0.99
Short stature (<-2SDS)			18/23 (78.3)			5/5 (100)			13/18 (72.2)	0.72

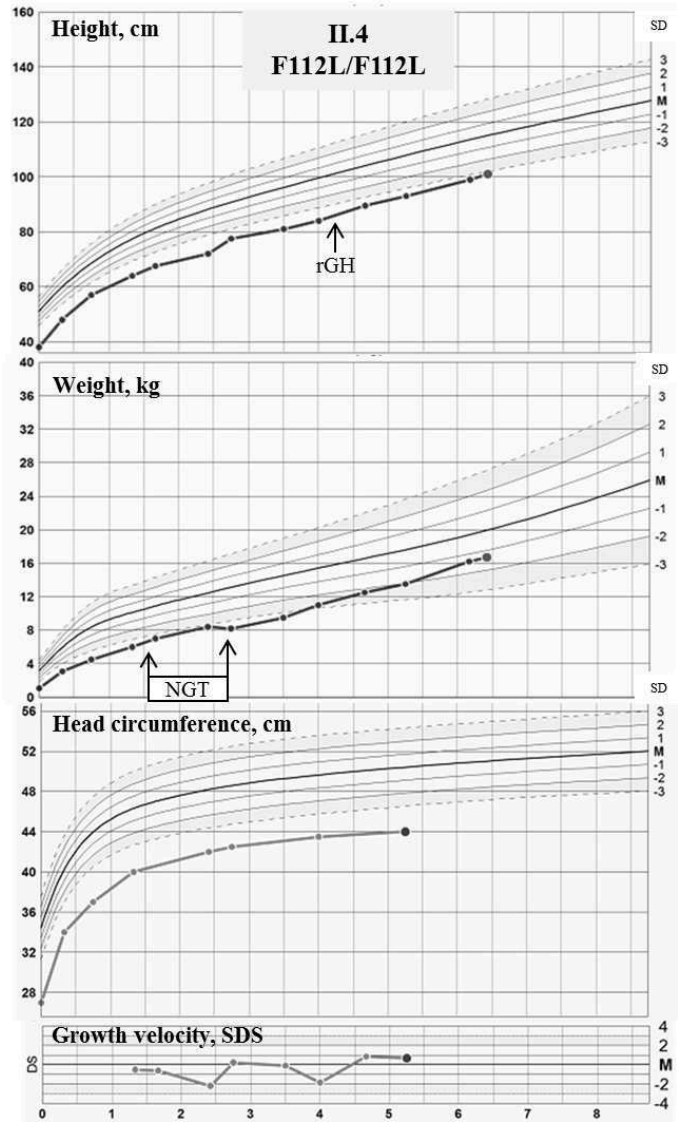
FIGURES

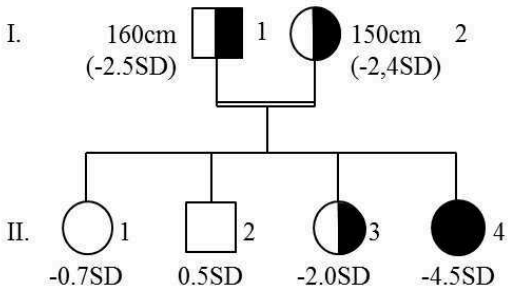
Figure 1. A. Schematic representation of the position of IGF1R on chromosome 15. B. Representation of the eight identified deletions using the UCSC (University of California Santa Cruz) software. C. Representation of the identified single nucleotide variants (SNV) from exons 1 to 21. Arrows indicate the SNVs identified in the cohort. The corresponding functional domains of the protein are shown to the right. L1 and 2: leucine-rich repeat domains; CR: cysteine-rich region; FN1 to 3: fibronectine type III domains; TM: trans-membrane region; TK: tyrosine kinase domain; CT: C-terminal segment.



Name	Position [Hg19]	Size(Kb)
Del1	chr15 : 97450781-102461162	5 010.4
Del2	chr15 : 98426952-102461162	4 034.2
Del3	chr15 : 98464591-101599113	3 134.5
Del4	chr15 : 98610818-102397836	3 787.0
Del5	chr15 : 98954957-102461162	3 506.2
Del6	chr15 : 99229330-99290406	61.1
Del7	chr15 : 99492046-99511073	19.0
Del8	chr15 : 99496341-99730813	234.5

Figure 2. Growth curves andpedigree of the family with the patient carrying the homozygous variant F112L/F112L. SDS: standard score deviation; NGT: naso-gastric tube.





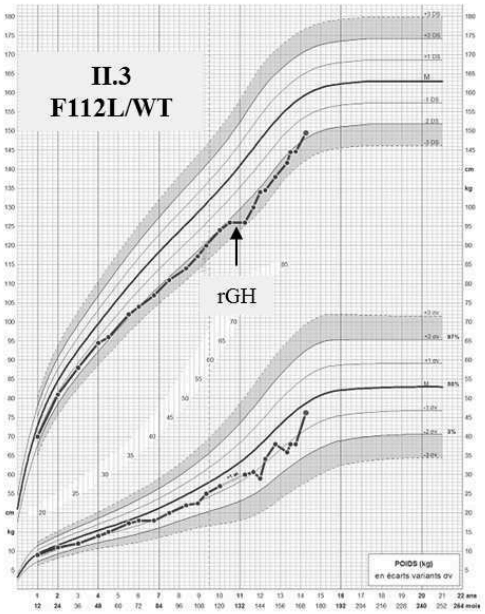
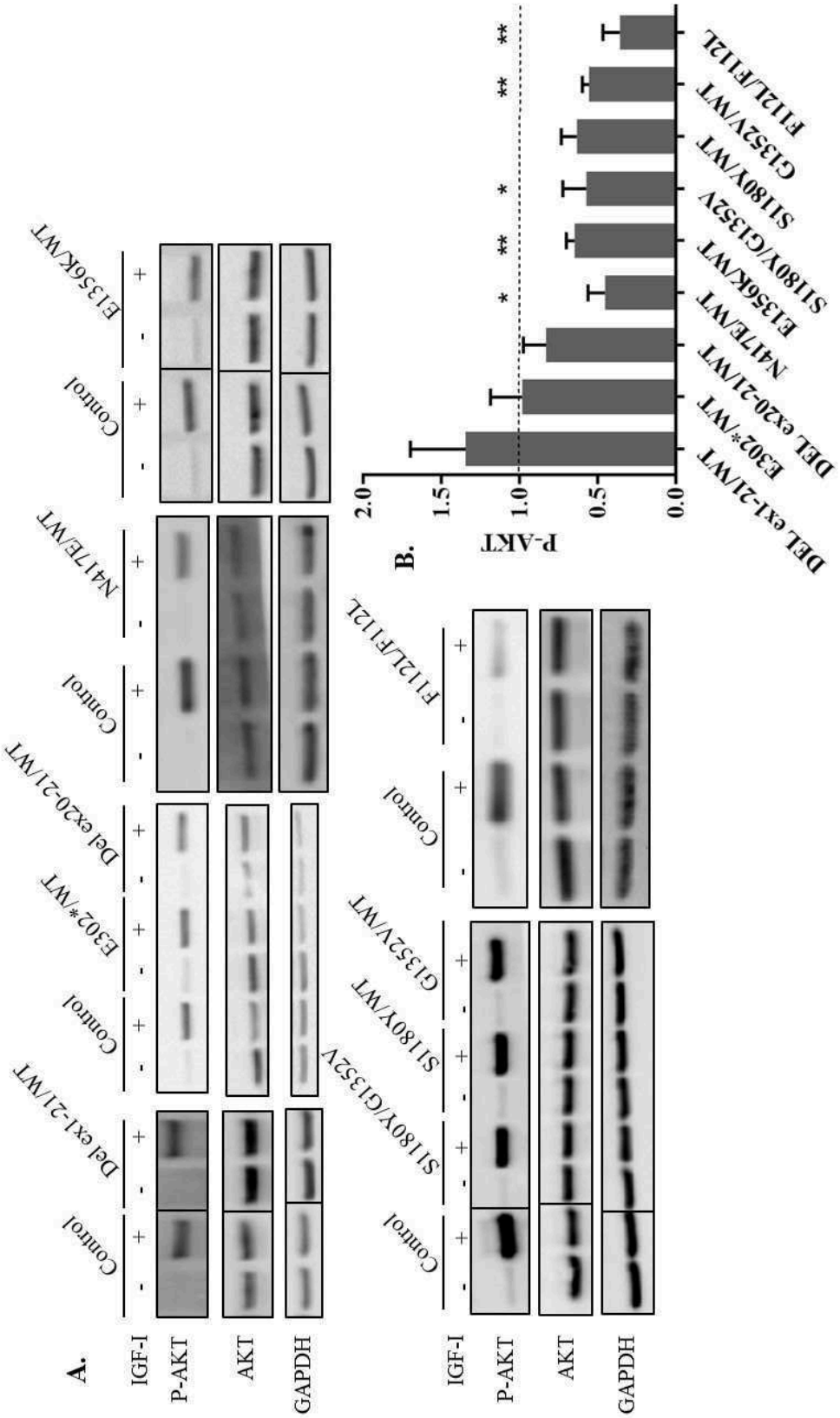


Figure 3. A. Western blot showing phosphorylated-AKT (P-AKT), total AKT, and GAPDH for patients and controls. B. Quantification of AKT phosphorylation calculated as: [(P-AKTpatient/AKTpatient)/GAPDHpatient]/ [(P-AKTcontrol/AKTcontrol)/GAPDHcontrol]. *p < 0.05; **p < 0.01; WT: wildtype allele. Experiments were repeated from 3 to 6 times for each individual. Error bars represent the standard error of the mean. Del ex1-21 corresponds to Del4 in figure 1 and Del ex20-21 to Del7.



SUPPLEMENTARY DATA

Figure 1. cDNA sequencing for the patient carrying the NM_000875.4:c.1247+1_1247+2insG variant, responsible for a one-base-pair insertion, leading to a frameshift and premature stop codon (N417EfsX52).

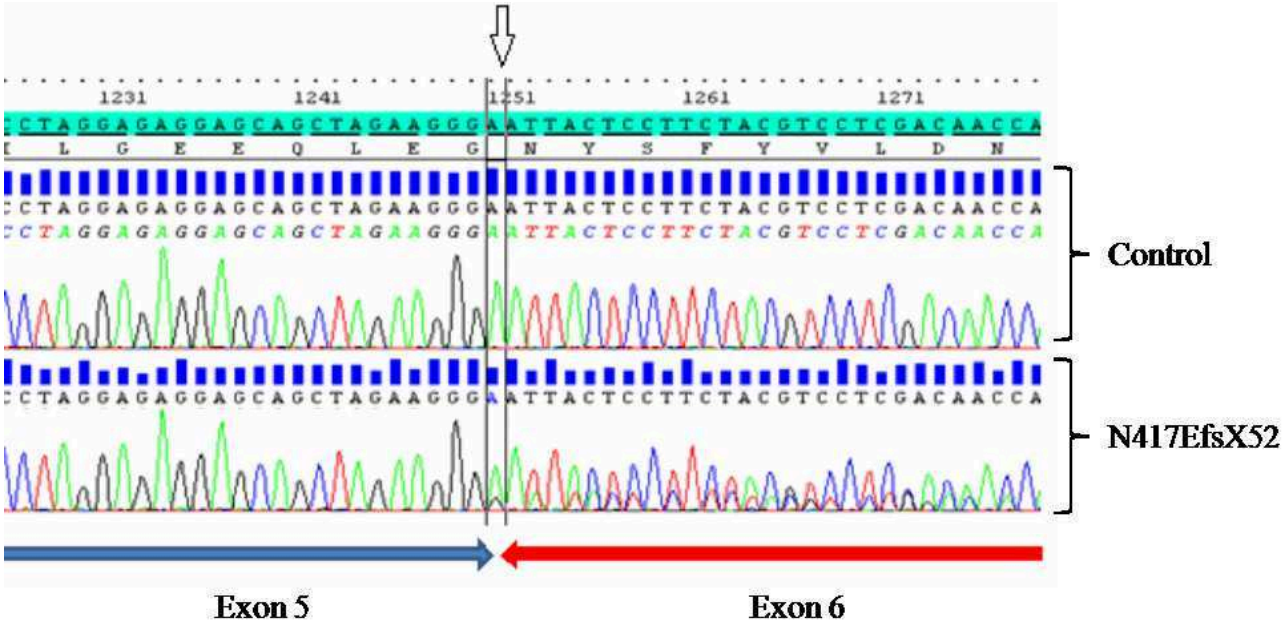


Figure 2. Quantification of ERK phosphorylation calculated as:

[(P-ERKpatient/ERKpatient)/GAPDHpatient]/ [(P-ERKcontrol/ERKcontrol)/GAPDHcontrol]. WT: wildtype allele. Experiments were repeated from 3 to 6 times for each individual. Error bars represent the standard error of the mean. Del ex1-21 corresponds to Del4 in figure 1 and Del ex20-21 to Del7.

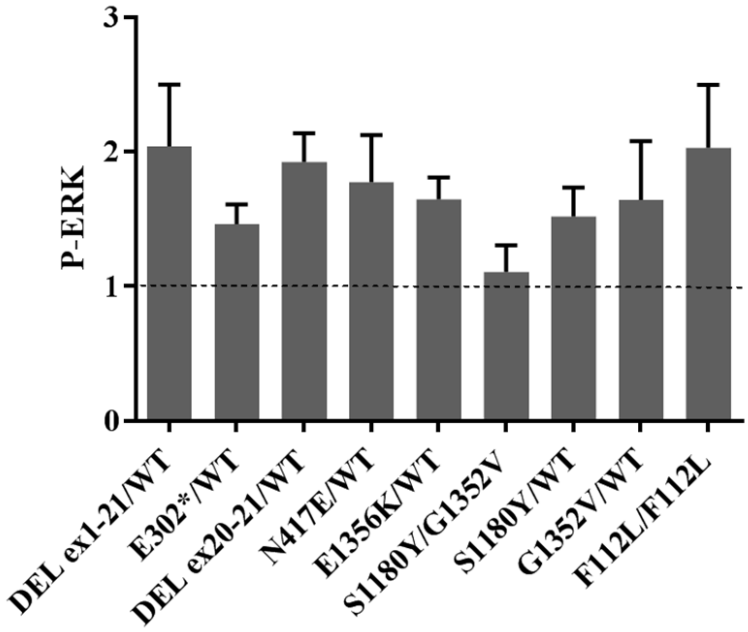
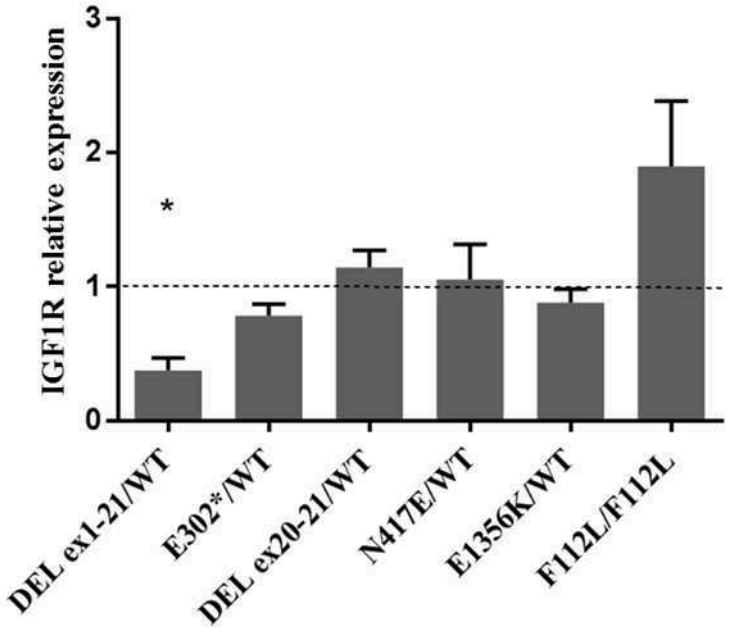


Figure 3: IGF1R cDNA expression in fibroblasts. Primers used: Igf1R-219-F: ACAGGGATCTCATCAGCTTCAC and Igf1R-219-R: TCCACCATGTTCCAGCTGTT. The amplicon length was 109 bp, spanning exons 7 and 8. Del ex1-21 corresponds to Del4 in figure 1 and Del ex20-21 to Del7.



STATEMENTS

Competing interests: The authors have nothing to disclose.

Contribution of the authors: EG and FB wrote the manuscript. EG, SCB, WAH, VS, SA, BL, NT, and SR performed the experiments. EG, FB, IN, SCB, and MW analyzed the data and helped in revising the manuscript. All other authors collected data and helped to revise the manuscript.

2.1.1.2 Patients avec syndrome de Silver-Russell

À ce jour, il existe deux publications contradictoires portant sur l'activité d'IGF1R chez les patients avec SRS (Montenegro *et al.* 2012; Iliev *et al.* 2014). La première a montré, dans les fibroblastes d'un patient avec un SRS par anomalie de la région 11p15, une diminution de la prolifération cellulaire, une phosphorylation normale d'AKT après stimulation par différentes concentrations d'IGF-I (10-20-50 ng/mL) et abaissée d'ERK par rapport à des fibroblastes de sujet contrôle (Montenegro *et al.* 2012). Cependant, cette étude ne portait que sur un cas isolé. La seconde équipe n'a pas répliqué ces résultats dans les fibroblastes de quatre patients avec une anomalie de la région 11p15 (Iliev *et al.* 2014). Les auteurs ont retrouvé une phosphorylation d'AKT après stimulation prolongée (72h) par des concentrations croissantes d'IGF-I (10-200 ng/mL) qui est identique aux contrôles. Par ailleurs, la vitesse de prolifération était également inchangée. Néanmoins, les index de méthylation en *H19/IGF2*:IG-DMR étaient peu abaissés dans les fibroblastes de ces patients et les taux d'IGF-I circulants étaient normaux (-0,7 à 0,8 DS).

Nous avons donc utilisé le test fonctionnel développé précédemment chez les patients avec anomalies d'*IGF1R* chez six patients avec SRS par atteinte de la région 11p15 et présentant des taux circulants d'IGF-I élevés (tableau 3). Tous présentaient une perte de méthylation en *H19/IGF2*:IG-DMR dans les fibroblastes, et certains avaient des anomalies de méthylation *multiloci*.

Nom	Sexe	Anomalie moléculaire	Index de méthylation (*H19/IGF2*:IG-DMR)	IGF-I
Patient 1	Féminin	Perte de méthylation *IGF2/H19*:IG-DMR *MEG3/DLK1*:IG-DMR	0/10	5DS sous traitement
Patient 2	Masculin	Perte de méthylation *IGF2/H19*:IG-DMR	24/29	2,5DS avant traitement
Patient 3	Féminin	Perte de méthylation *IGF2/H19*:IG-DMR	6/11	Élevé
Patient 4	Masculin	Perte de méthylation *IGF2/H19*:IG-DMR Délétion du centre d'empreinte	0/6	2,8DS avant traitement
Patient 5	Masculin	Perte de méthylation *IGF2/H19*:IG-DMR	4/13	2,8DS avant traitement
Patient 6	Féminin	Perte de méthylation *IGF2/H19*:IG-DMR *MEG3/DLK1*:IG-DMR	0/1	2,5DS avant traitement

Tableau 3. Description des six patients avec SRS chez qui a été effectué le test fonctionnel d'IGF1R. L'index de méthylation indiqué est celui retrouvé dans les fibroblastes (deux sites de méthylation en *H19/IGF2*:IG-DMR).

2.1.1.2.1 Résultats

Les profils de phosphorylation d'AKT ou d'ERK dans les fibroblastes de ces patients n'étaient pas différents de ceux des contrôles (figure 17A). De même, la quantification de l'expression d'*IGF1R* était comparable chez les patients avec SRS et chez les contrôles (figure 17B.).

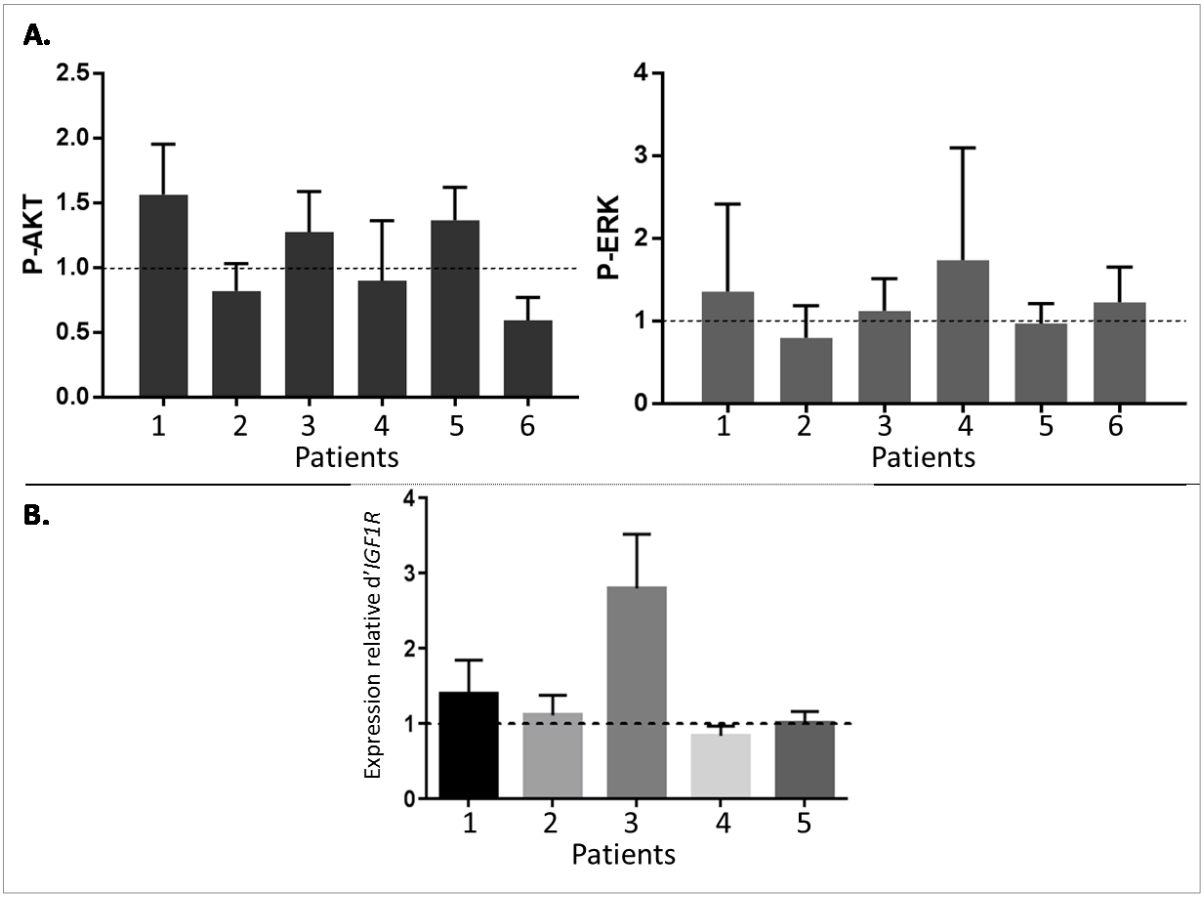


Figure 17. A. Quantification de la phosphorylation d'AKT (droite) et d'ERK (gauche) dans les fibroblastes de patients avec SRS après stimulation par IGF-I [50 ng/mL]. Les expériences ont été répétées entre 3 et 5 fois. La quantification se fait par la formule suivante (identique pour ERK)[(P-AKT patient/AKT patient)/GAPDH patient]/ [(P-AKTcontrôle/AKT contrôle)/GAPDH contrôle]. La ligne 1 correspond donc à la phosphorylation chez les contrôles B. Expression d'*IGF1R* dans les fibroblastes des patients avec SRS en PCR quantitative par rapport aux contrôles (ligne pointillée avec fold change à 1). (Voir *Méthodes* article soumis ci-dessus).

2.1.1.2.2 Discussion

L'étude de la phosphorylation d'AKT et ERK sur les deux voies de signalisation d'IGF1R n'a pas mis en évidence d'anomalie d'activité du récepteur chez les patients avec SRS par anomalie de la région 11p15. Ces résultats, conjointement à ceux publiés par Iliev *et al.* sont en faveur d'un fonctionnement normal du récepteur IGF1R chez les patient avec SRS (Iliev *et al.* 2014). Cependant, les index de méthylation dans les fibroblastes, les taux circulants d'IGF-I, les

concentrations d'IGF-I et la durée de stimulation étaient très variables entre ces trois études. Il est envisageable que le protocole expérimental utilisé (avec des concentrations d'IGF-I ou une durée de stimulation variables) modifie ces résultats. Une autre hypothèse est celle d'un dysfonctionnement en aval des protéines étudiées (AKT et ERK) chez les patients avec SRS. En conséquence, l'étude des gènes cibles de l'activation d'IGF1R pourrait être intéressante afin de préciser le fonctionnement d'IGF1R chez les patients avec un SRS par hypométhylation d'*H19/IGF2*:IG-DMR. Une autre possibilité est que dans le tissu que nous avons étudié, ce phénomène de résistance à l'IGF-I soit moindre. En effet, le tissu cible principal de l'action de l'IGF-I sur la croissance staturale étant le cartilage de croissance, la résistance à l'IGF-I est potentiellement restreinte à ce tissu. Les difficultés d'accès et d'expérimentation *in vitro* font néanmoins du cartilage de croissance un tissu extrêmement ardu à étudier chez l'humain. Néanmoins, les profils normaux de phosphorylation d'AKT et ERK chez ces patients et l'absence d'hypothèse sur les mécanismes qui sous-tendraient l'anomalie moléculaire et le défaut d'IGF1R nous ont incités à élaborer d'autres hypothèses pour expliquer cette résistance à l'IGF-I.

2.1.2 Étude de la biodisponibilité d'IGF-I chez les patients avec SRS

2.1.2.1 Contexte

Notre seconde hypothèse était non pas une baisse d'activité d'IGF1R, mais une baisse de la disponibilité sérique d'IGF-I. Nous entendons par « biodisponibilité », la quantité d'IGF-I mobilisable pour agir sur son récepteur, elle comprend donc l'IGF-I deux formes circulantes : libre (immédiatement active) ou liée (sous formes de complexes secondaires ou ternaires) qui constitue une réserve mobilisable après protéolyse des IGFBPs. Des travaux ont mis en évidence différents mécanismes d'altération de la biodisponibilité d'IGF-I en pathologie humaine, soit par anomalie d'ALS (et diminution de l'IGF-I lié sous forme de complexe ternaire) soit par anomalie de la protéolyse (anomalies de PAPP-A2, entraînant une séquestration de l'IGF-I en complexes secondaires et ternaires), responsable de restriction de croissance (Domené *et al.* 2004; Dauber *et al.* 2016). Dans le dernier cas, la concentration élevée d'IGF-I total ne rend pas compte de sa biodisponibilité qui est abaissée (Bakker *et al.* 2015). Nous avons donc exploré cet aspect chez les patients avec SRS dont les concentrations d'IGF-I sont paradoxalement élevées par rapport à leur vitesse de croissance et leur statut nutritionnel.

La difficulté de cette évaluation vient du fait que le dosage sérique d'IGF-I comprend l'IGF-I total, sans distinction entre la forme libre ou liée à ses protéines de liaison. Ainsi, des taux élevés d'IGF-I ne sont pas nécessairement équivalents à des taux élevés d'IGF-I libre. De plus, l'équilibre

physiologique entre IGF-I libre et IGF-I lié sous forme de complexes secondaire ou ternaire pour satisfaire à une croissance optimale n'est pas déterminé. Certains auteurs proposent donc l'utilisation des taux d'IGFBP-3 ou d'ALS pour évaluer de manière indirecte la fraction d'IGF-I libre par rapport à l'IGF-I total. L'IGFBP-3, contrairement à l'ALS, peut être dosée en routine, de manière concomitante à l'IGF-I. Ainsi, a été proposé l'utilisation du ratio molaire IGF-I/IGFBP-3 comme reflet de la fraction d'IGF-I libre dans la circulation. L'analyse des dosages d'IGF-I par radio-immuno-dosage chez les patients atteints de SRS par hypométhylation en *H19/IGF2*:IG-DMR (*loss of methylation*, LOM 11p15) ou disomie uniparentale maternelle du chromosome 7 (mupd7) disponibles antérieurement au laboratoire confirmait les taux spontanément élevés d'IGF-I (uniquement chez les patients avec LOM 11p15) qui se majoraient avec la mise en place du traitement par hormone de croissance (rGH) (figure 18).

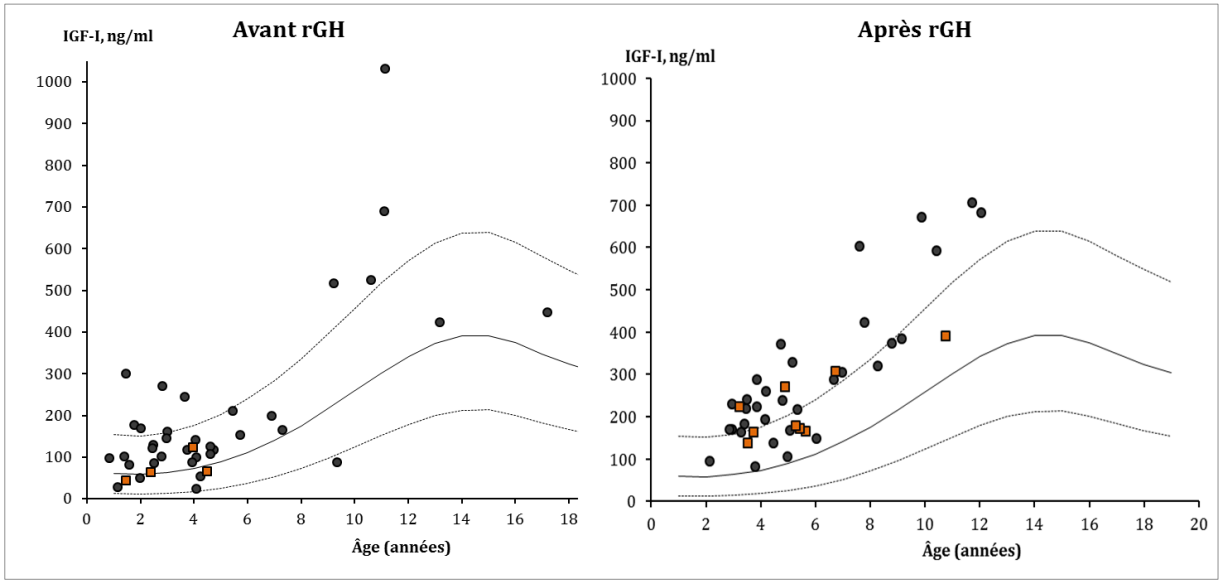


Figure 18. Répartition des taux d'IGF-I, dosés en RIA, chez les patients SRS avec LOM 11p15 (ronds gris, n=36) ou par mupd7 (carrés oranges, n=4), avant et pendant traitement par hormone de croissance (rGH), en fonction des valeurs d'une population de référence (Brabant *et al.* 2003). La médiane est au centre et les lignes supérieure et inférieure figurent respectivement les 97,5ème et 2,5ème percentiles.

2.1.2.2 Méthodes

Nous avons caractérisé le profil biologique des différents acteurs du système des IGFs dans une cohorte de patients avec SRS LOM 11p15 ou par mupd7. Les concentrations d'IGF-I et d'IGFBP-3 étaient mesurées par chemiluminescence sur un automate IDS ISYS instrument (Immunodiagnostic Systems, Paris, France). Pour IGF-I, le seuil de sensibilité était de 8,8ng/ml et les coefficients de variation intra-essai et inter-essai étaient de 1,9-2,9% et 3,9-7,2% respectivement. Pour IGFBP-3, le seuil de sensibilité était de 80ng/ml et les coefficients de variation intra-essai et inter-essai étaient de 1,4-2,6% et 5,8-7,2% respectivement. Le ratio molaire IGF-I/IGFBP-3 était calculé après conversion des deux paramètres en nmol/L, pour IGF-I

le facteur multiplicatif était de 0,1307 et pour IGFBP-3 de 0,03478, comme préconisé par les articles de références concernant les normes de ces trois paramètres (Bidlingmaier *et al.* 2014; Friedrich *et al.* 2014). Le dosage de l'ALS était réalisé manuellement avec le kit ELISA E35 (Mediagnost, Allemagne), dosage « sandwich » dont le seuil de sensibilité était de 0,53 ng/mL et les coefficients de variation intra-essai et inter-essai étaient de 6,5-6,8% et 7,0-10,0% respectivement. La zone de lecture des résultats allait de 0,11 à 6000 mU/mL soit 0,53 - 30000 ng/mL (1 mU est équivalent à 5 ng). Le dosage de l'IGF-II a été réalisé successivement par deux types d'immunoanalyses : de 2011 à fin 2017 avec le kit *radio-immuno-assay* (RIA) E30 (Mediagnost, Allemagne), et depuis 2018 avec le kit ELISA E30 (Mediagnost, Allemagne). La méthode RIA avait un seuil de sensibilité de 0,039 ng/mL et les coefficients de variation intra-essai et inter-essai étaient de 0,9-2,3% et 5,0-8,1% respectivement. La méthode ELISA avait un seuil de sensibilité de 0,02 ng/mL et les coefficients de variation intra-essai et inter-essai étaient de 3,07-6,61% et 7,06-7,22% respectivement. Les dosages d'IGF-II et d'ALS ont été réalisés en respectant la procédure proposée par le fabricant.

Les normes utilisées pour l'interprétation des dosages selon l'âge et le sexe des patients avaient soit fait l'objet de publications sur de large cohortes pour IGF-I, IGFBP-3 et le ratio IGF-I/IGFBP-3, soit avaient été établies à partir de patients contrôles au laboratoire (ALS) ou étaient fournies par le fabricant (IGF-II RIA et ELISA) (Bidlingmaier *et al.* 2014; Friedrich *et al.* 2014). Pour analyser les résultats d'IGF-I, IGFBP-3 et du ratio IGF-I/IGFBP-3 de nos patients en fonction des normes publiées, nous les avons séparés par sexe (graphes en rouge pour les filles et en bleu pour les garçons). En parallèle, nous avons regroupé les valeurs de tous les patients sur des graphiques aux normes moyennées (graphe en noir).

Concernant le dosage d'IGF-II, nous avons présenté les résultats selon la technique utilisée (RIA et ELISA). Les normes des brochures étaient identiques quelle que soit la méthode utilisée.

Seuls les prélèvements pour lesquels les concentrations contemporaines d'IGF-I et d'IGFBP-3 étaient disponibles ont été inclus. Nous avons analysé au maximum trois dosages différents par patient (avant ou après hormone de croissance).

Les tests statistiques utilisés étaient paramétriques (t-test) pour comparer les moyennes entre elles ou à une moyenne de référence, et les variables étaient appariées pour comparer des valeurs avant et après traitement par hormone de croissance recombinante (rGH) chez un même patient. En cas de petits effectifs (entre 4 et 10 patients), le test non paramétrique de Mann-Whitney était utilisé. Les comparaisons de variables qualitatives étaient réalisées par le test exact de Fischer du fait des petits effectifs. Le seuil de significativité était à 0,05 et les conventions utilisées pour figurer les différents seuils étaient : *$p<0,05$, **$p<0,01$ et *** $p<0,001$.

2.1.2.3 Résultats

Le tableau 3 récapitule les différents dosages obtenus pour ces patients selon l'anomalie moléculaire, le sexe et le moment du prélèvement en fonction de la présence ou non d'un traitement par hormone de croissance. Les résultats des différents dosages ont montré que les concentrations d'IGF-I, d'IGFBP-3 et les ratios IGF-I/IGFBP-3 étaient spontanément élevés chez les patients avec LOM 11p15 et qu'ils augmentaient significativement avec le traitement par hormone de croissance (figures 19 à 24). À l'inverse, chez les patients avec mupd7, les concentrations d'IGF-I étaient spontanément basses mais les ratios IGF-I/IGFBP-3 étaient normaux. Les taux d'ALS étaient spontanément hauts et augmentaient avec le traitement, tandis que les taux d'IGF-II étaient dans les normes et le restaient avec le traitement (figures 25 et 26). Le tableau 4 reprend la proportion de patients ayant des valeurs d'ALS ou d'IGF-II au-delà du 97,5ème percentile. Le nombre de patients ayant des taux élevés est resté stable pour les taux d'ALS (p = 0,85) et augmentait pour IGF-II (p = 0,04). De même, la proportion de patients avec LOM 11p15 ayant des concentrations d'IGF-I (p = 0,003), d'IGFBP-3 (p = 0,02) et des ratios IGF-I/IGFBP-3 (p = 0,006) supérieurs à 2DS augmentait significativement avec l'instauration du traitement (figure 27).

		IGF-I; IGFBP3; IGF-I/IGFBP3	ALS	IGF-II	
				RIA	ELISA
AVANT TRAITEMENT	Filles	22/5	57/10	20/4	37/5
	Garçons	16/3			
	Tous	38/8			
PENDANT TRAITEMENT	Filles	12/2	42/9	13/4	9/0
	Garçons	9/2			
	Tous	21/4			

Tableau 4. Nombre de prélèvements disponibles pour chaque dosage (IGF-I et IGFBP-3 en chemiluminescence automatisée, ALS en ELISA et IGF-II en RIA et en ELISA), pour chaque sexe, avant et pendant le traitement par rGH. Le premier chiffre correspond aux patients avec LOM 11p15 et le second aux patients avec mupd7.

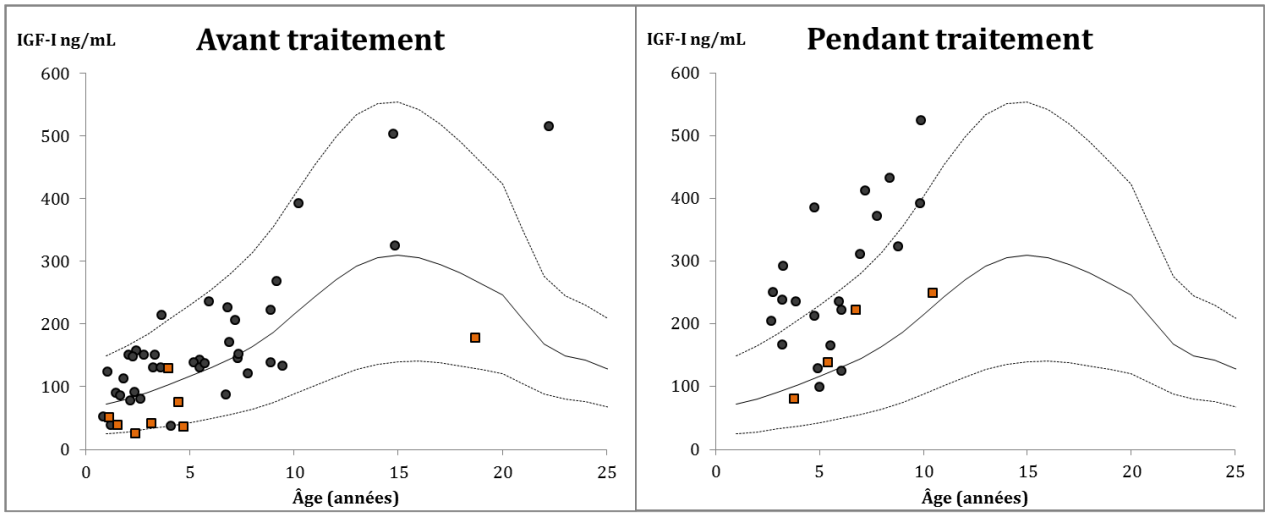


Figure 19. Répartition des taux d'IGF-I (mesurés en chemiluminescence automatisée) chez les patients SRS avec LOM 11p15 (ronds gris) ou par mupd7 (carrés oranges), avant et pendant traitement par rGH, en fonction des valeurs d'une population de référence. La médiane correspond à la ligne centrale, les lignes supérieure et inférieure figurent respectivement les 97,5ème et 2,5ème percentiles (Bidlingmaier *et al.* 2014). Les résultats sont présentés ensemble sur une courbe moyennée (courbes propres à chaque sexe en Annexe 6.1).

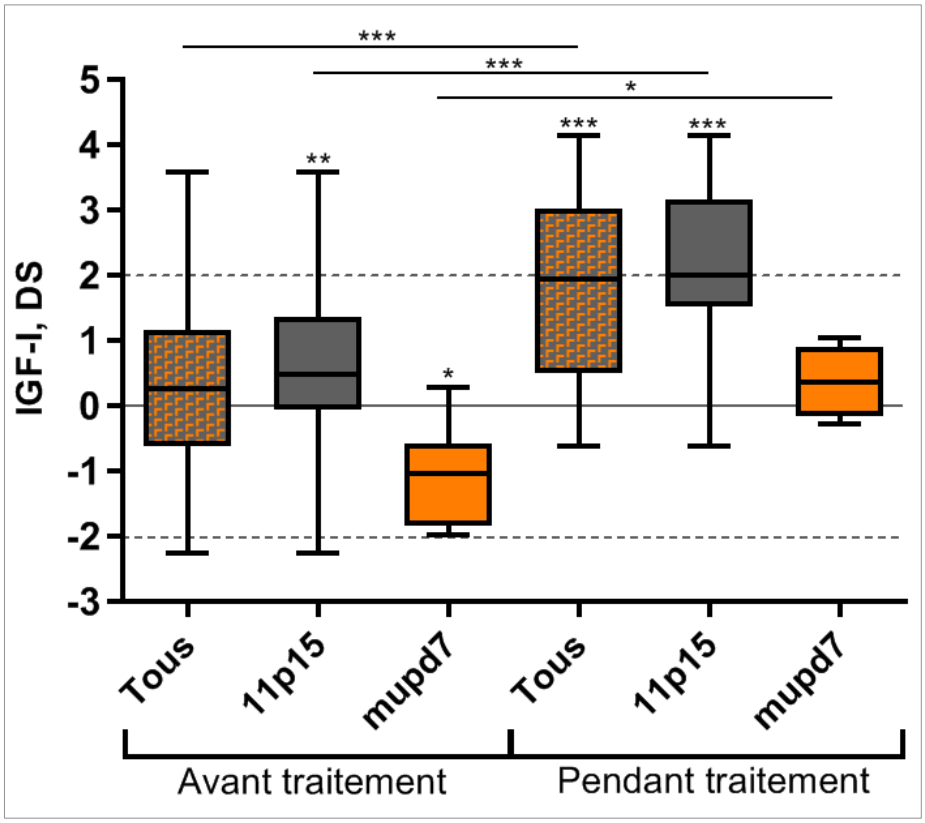


Figure 20. Comparaison des IGF-I chez les patients avec LOM 11p15 (gris) et mupd7 (orange) avant ou pendant traitement par rGH (Bidlingmaier *et al.* 2014). Comparaison statistique des distributions (présentées avec moyenne, minimum et maximum) à la répartition normale (figurée au dessus de chaque distribution) et entre elles, avant et pendant traitement (figurées au-dessus des ponts).

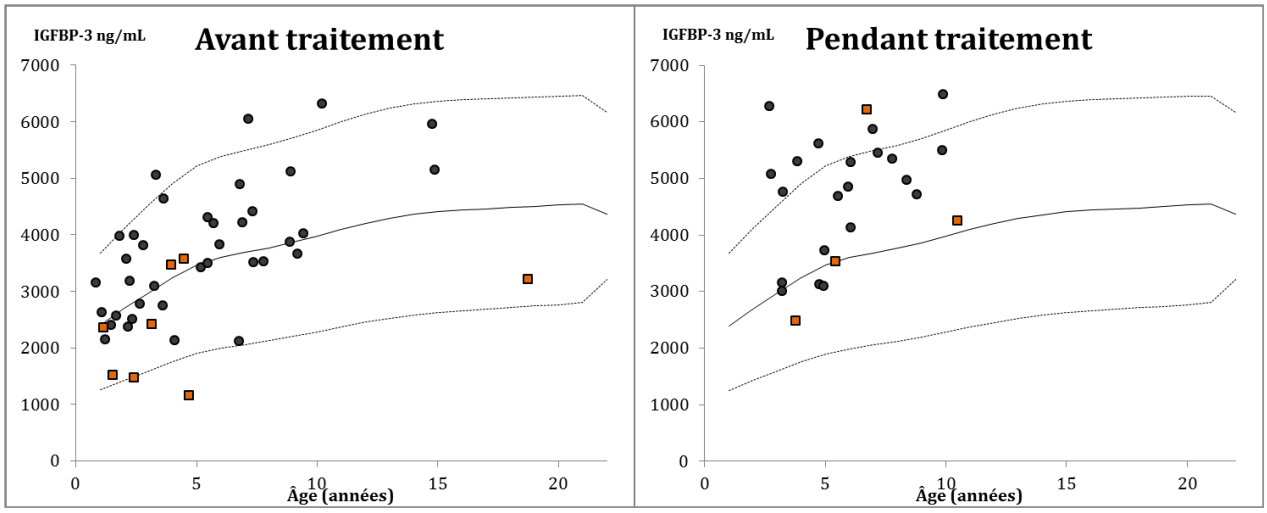


Figure 21. Répartition des taux d'IGFBP-3 (mesurés en chemiluminescence automatisée) chez les patients SRS avec LOM 11p15 (ronds gris) ou par mupd7 (carrés oranges), avant et pendant traitement par rGH, en fonction des valeurs d'une population de référence. La médiane correspond à la ligne centrale, les lignes supérieure et inférieure figurent respectivement les 97,5ème et 2,5ème percentiles (Friedrich *et al.* 2014). Les résultats sont présentés ensemble sur une courbe moyennée (courbes propres à chaque sexe en Annexe 6.1).

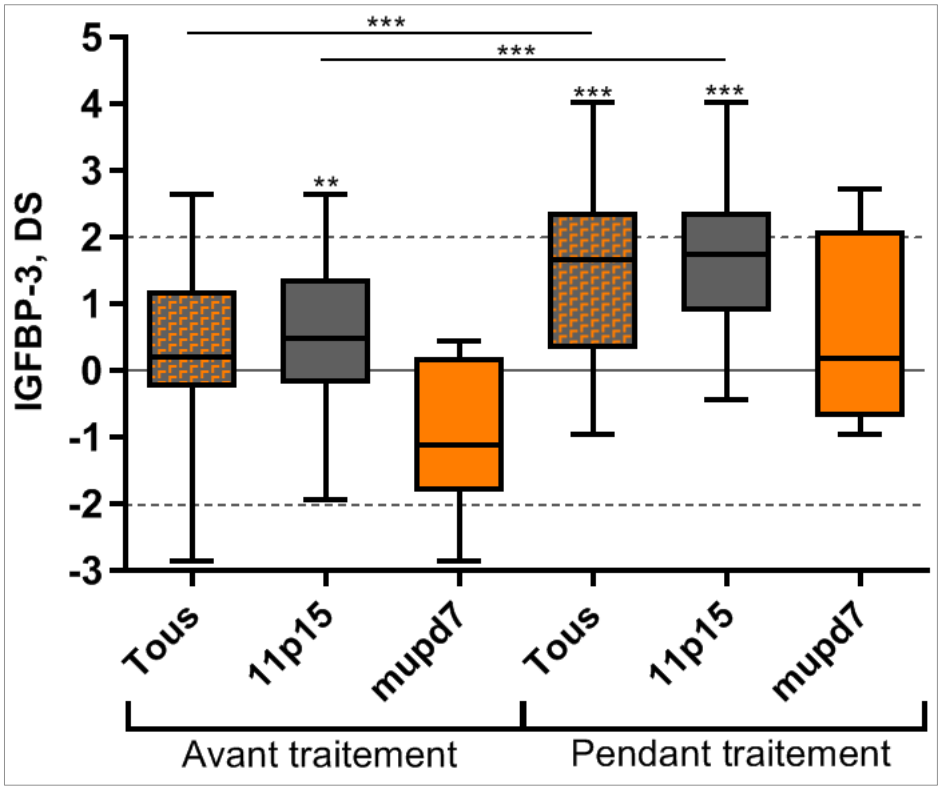


Figure 22. Comparaison des IGFBP-3 chez les patients avec LOM 11p15 (gris) et mupd7 (orange) avant ou pendant traitement par rGH (Friedrich *et al.* 2014). Comparaison statistique des distributions (présentées avec moyenne, minimum et maximum) à la répartition normale (figurée au-dessus de chaque distribution) et entre elles, avant et pendant traitement (figurées au-dessus des ponts).

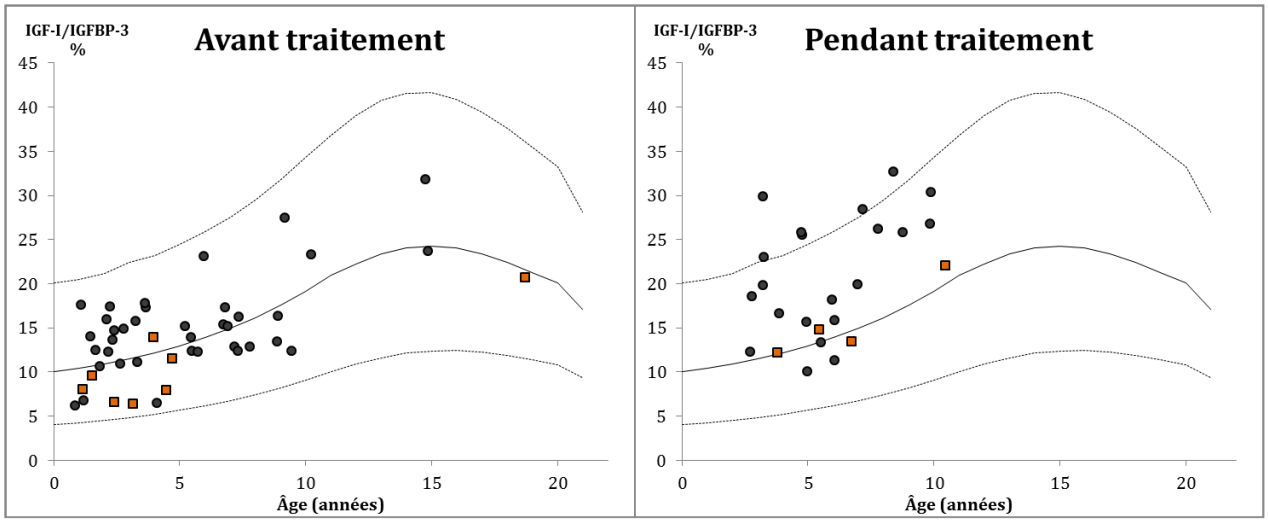


Figure 23. Répartition des ratios IGF-I/IGFBP-3 (mesurés en chemiluminescence automatisée) chez les patients SRS avec LOM 11p15 (ronds gris) ou par mupd7 (carrés oranges), avant et pendant traitement par rGH, en fonction des valeurs d'une population de référence. La médiane correspond à la ligne centrale, les lignes supérieure et inférieure figurent respectivement les 97,5ème et 2,5ème percentiles (Friedrich *et al.* 2014). Les résultats sont présentés ensemble sur une courbe moyennée (courbes propres à chaque sexe en Annexe 6.1).

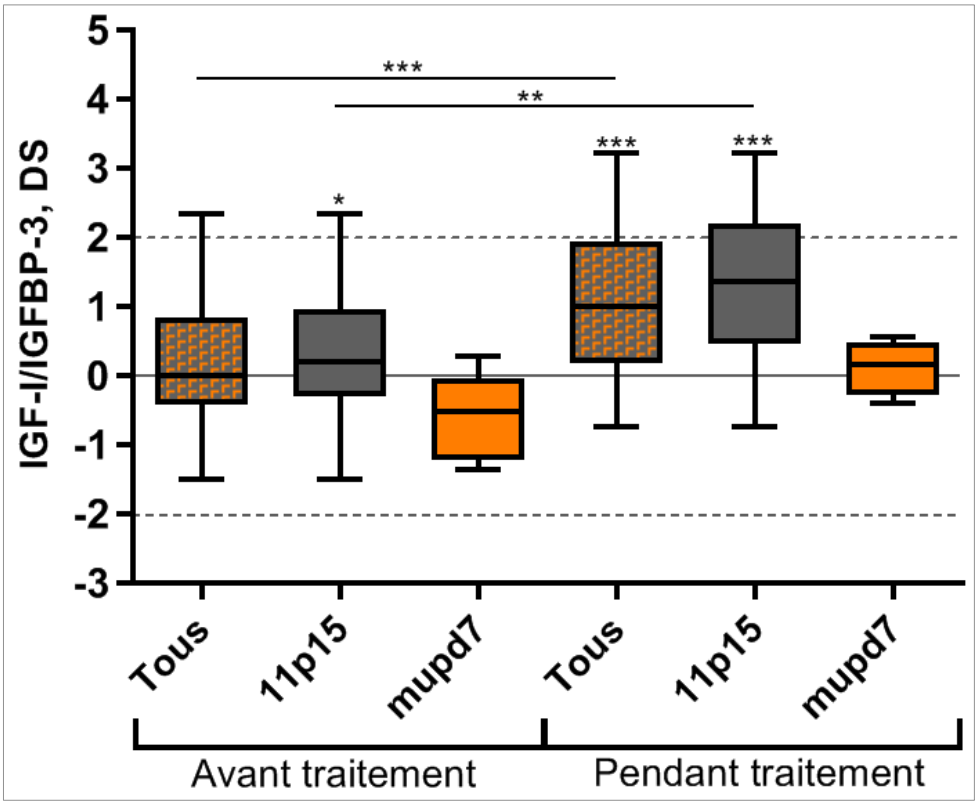


Figure 24. Comparaison des ratios IGF-I/IGFBP-3 chez les patients avec LOM 11p15 (gris) et mupd7 (orange) avant ou pendant traitement par rGH (Friedrich *et al.* 2014). Comparaison statistique des distributions (présentées avec moyenne, minimum et maximum) à la répartition normale (figurée au-dessus de chaque distribution) et entre elles, avant et pendant traitement (figurées au-dessus des ponts).

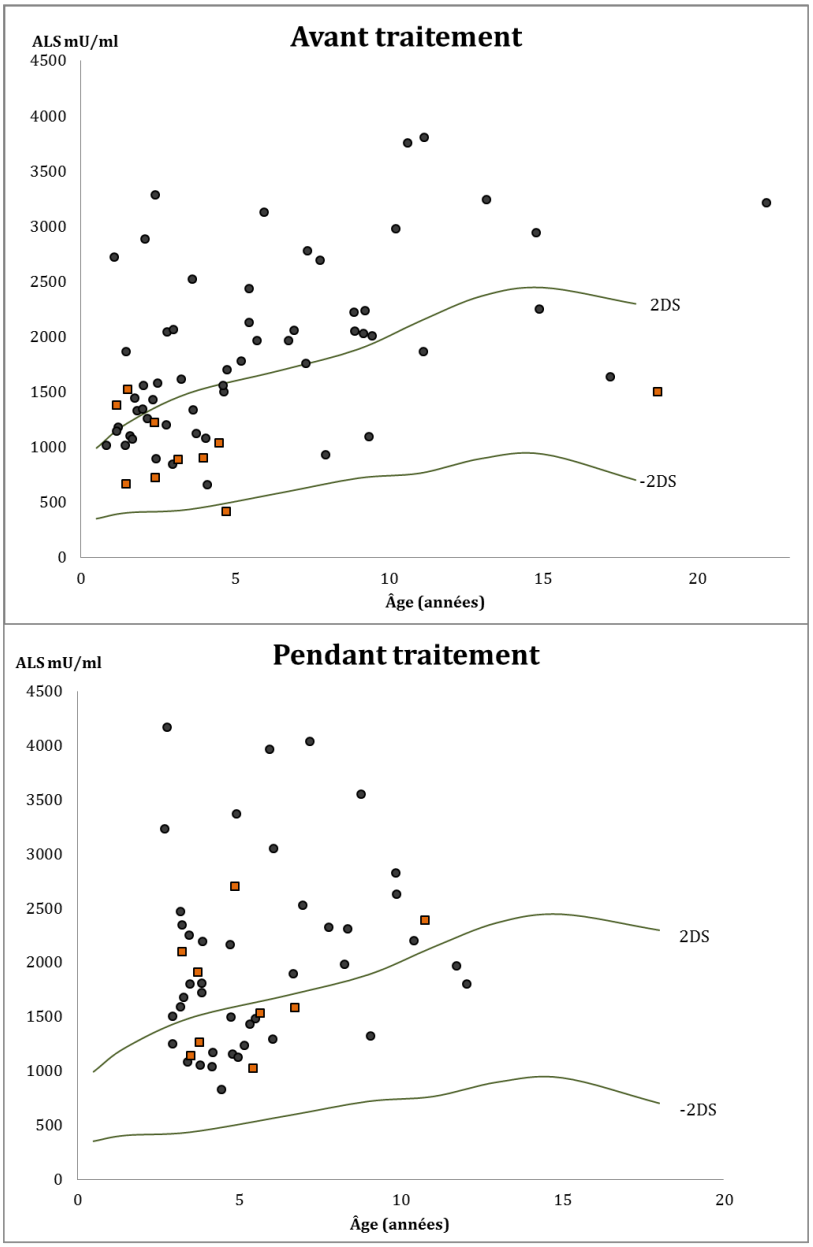


Figure 25. Répartition des taux d'ALS (kits MEDIAGNOST RIA et ELISA) chez les patients SRS par LOM 11p15 (ronds gris) ou par mupd7 (carrés oranges), avant et pendant traitement par rGH, en fonction des valeurs normales établies chez des témoins [Y. Le Bouc et L. Périn, non publié]. Les lignes supérieure et inférieure figurent respectivement les 2DS et -2DS. Une valeur très élevée a été exclue pour les besoins de la présentation (ALS pendant traitement à 5826 mU/mL à 7,6 ans)

	ALS		IGF-II	
	Avant rGH	Pendant rGH	Avant rGH	Pendant rGH
	n=67	n=51	n=67	n=26
<2,5ème p (%)	1,5	0	3,0	0
>97,5ème p (%)	58,2	61,5	4,5	19,2

Tableau 5. Évolution du nombre de patients (en %) avec SRS avec des taux d'ALS et d'IGF-II inférieurs au 2,5ème percentile (-2DS pour ALS) ou supérieurs au 97,5ème percentile (2DS pour l'ALS) avant et pendant le traitement par rGH.

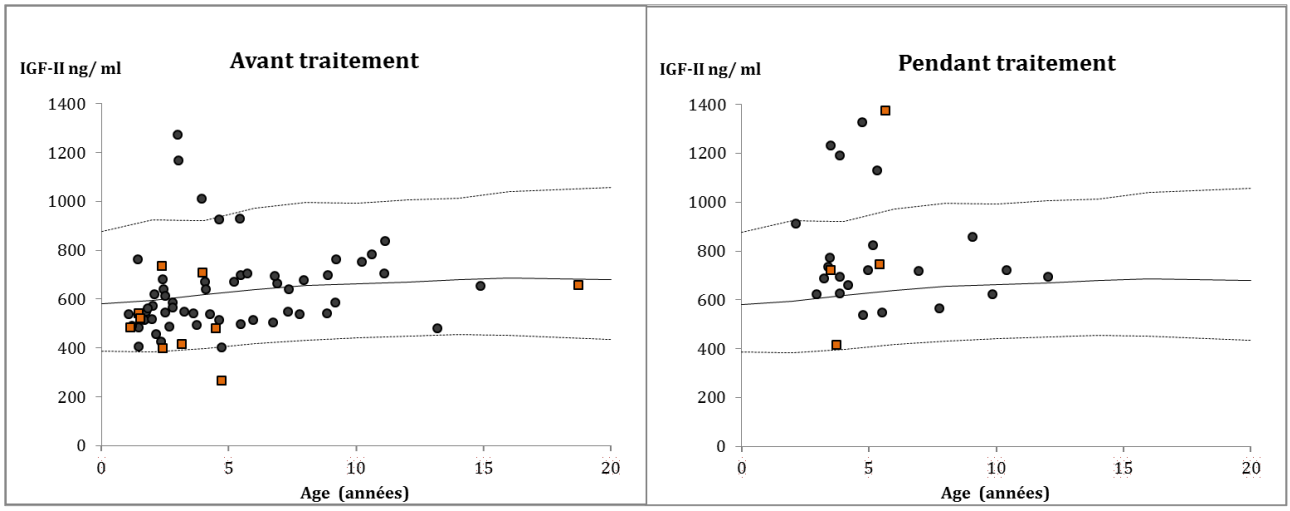
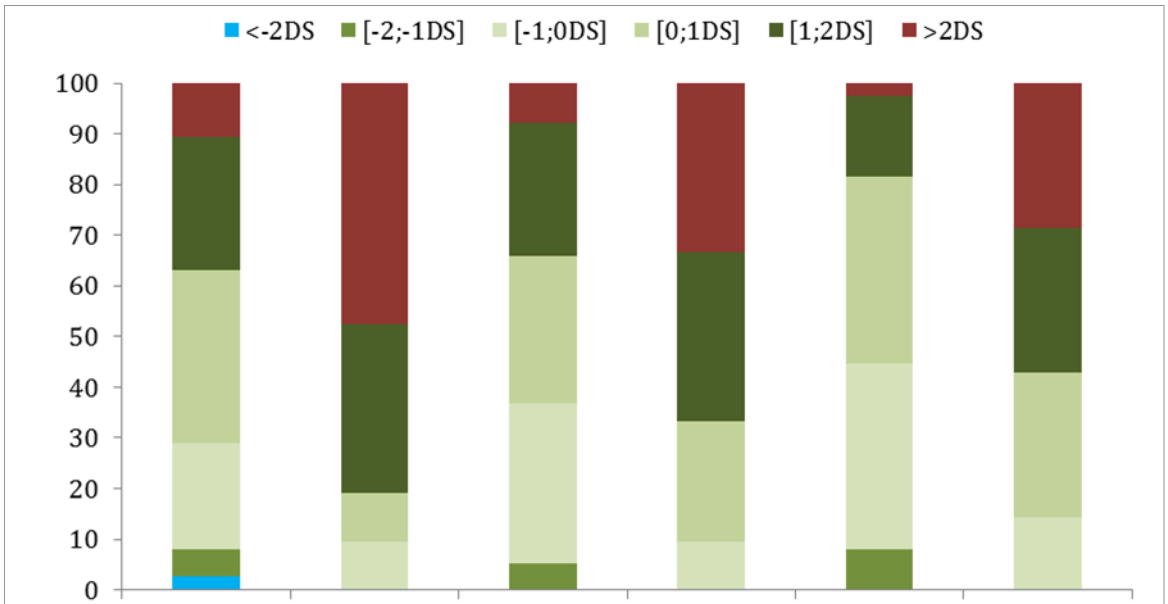


Figure 26. Répartition des taux d'IGF-II (kit MEDIAGNOST RIA et ELISA) chez les patients SRS par LOM 11p15 (ronds noirs) ou par mupd7 (carrés oranges), avant et pendant le traitement par rGH, en fonction des valeurs normales proposées par le fabricant. La médiane correspond à la ligne au centre et les lignes supérieure et inférieure figurent les 97,5ème et 2,5ème percentiles. Les répartitions selon la méthode de dosage sont disponibles en Annexe, section 6.1.



%	IGF-I		IGF-BP3		IGF-I/IGFBP-3	
<-2DS	3	0	0	0	0	0
[-2;-1DS]	5	0	5	0	8	0
[-1;0DS]	21	10	32	10	37	14
[0;1DS]	34	10	29	24	37	29
[1;2DS]	26	33	26	33	16	29
>2DS	11	48	8	33	3	29

Figure 27. Comparaison de la répartition (en %) des IGF-I, IGFBP-3 et des ratios IGF-I/IGFBP-3 selon les intervalles de déviation standard chez les patients SRS par LOM 11p15 avant et pendant traitement par rGH. Il y a une augmentation des valeurs hautes avec le traitement par rGH.

Enfin, nous avons figuré les résultats individuels des concentrations d'IGF-I, d'IGFBP-3 et du ratio IGF-I/IGFBP-3 chez 14 patients avec LOM 11p15, avant et après traitement par rGH (figure 28). Le délai moyen après l'instauration du traitement par rGH était de 1,0 an (0,2-2,1). Les données individuelles concernant les concentrations d'IGF-I, d'IGFBP-3, du ratio IGF-I/IGFBP-3 et de l'ALS de 17 patients avec LOM 11p15 ou mupd7 avant et après traitement par rGH sont présentées séparément pour les filles et les garçons (figures 29 et 30).

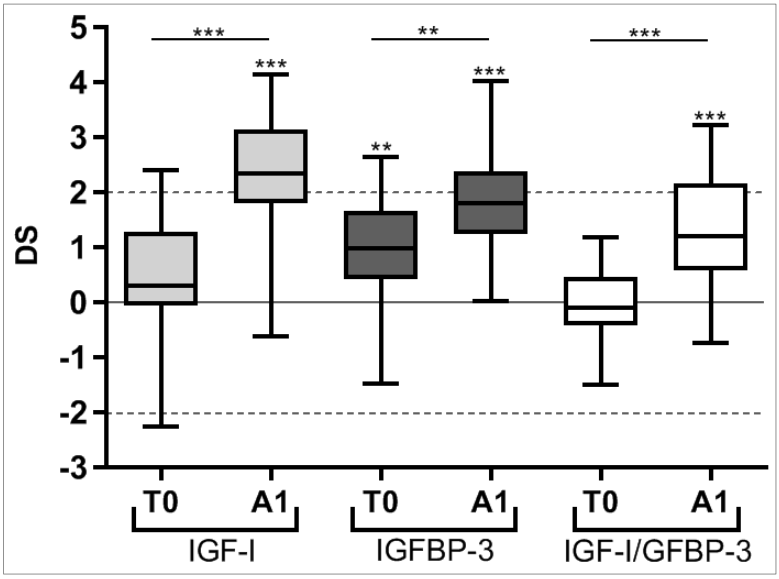


Figure 28. Comparaison des IGF-I, des IGFBP-3 et des ratios IGF-I/IGFBP-3 chez les patients avec LOM 11p15 (n= 14) avant ou pendant traitement par rGH (T0 : prélèvement avant rGH et A1 : prélèvement à un an après le début du traitement par rGH) (Bidlingmaier *et al.* 2014; Friedrich *et al.* 2014). Comparaison statistique des distributions (présentées avec moyenne, minimum et maximum) à la répartition normale (figurée au-dessus de chaque distribution) et entre elles, avant et pendant traitement (figurées au-dessus des ponts).

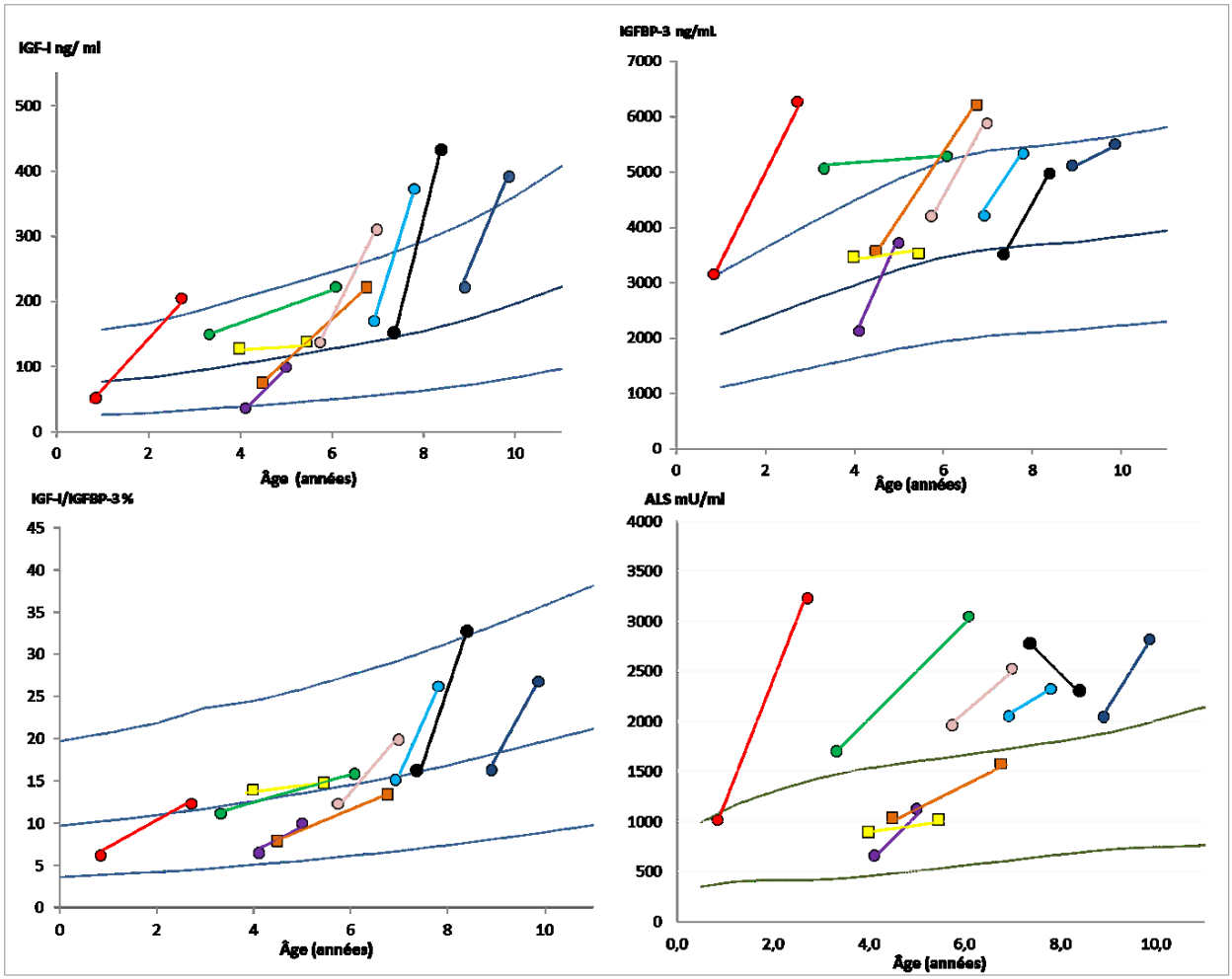


Figure 29. Évolution des taux d'IGF-I, IGFBP-3, ALS et du ratio IGF-I/IGFBP-3 chez 9 garçons avec SRS par LOM 11p15 (ronds, n=7) ou mupd7 (carrés, n=2). Chaque couleur correspond à un patient. Le premier point correspond aux dosages avant le traitement par rGH et le second au dosage réalisé en moyenne un an après l'instauration du traitement. Les taux d'IGF-I et d'IGFBP-3 augmentent avec le traitement, mais les ratios sont en règle normaux sauf pour le patient en noir qui est au dessus de 2DS, seul ce patient a une taux d'ALS qui baisse avec le traitement.

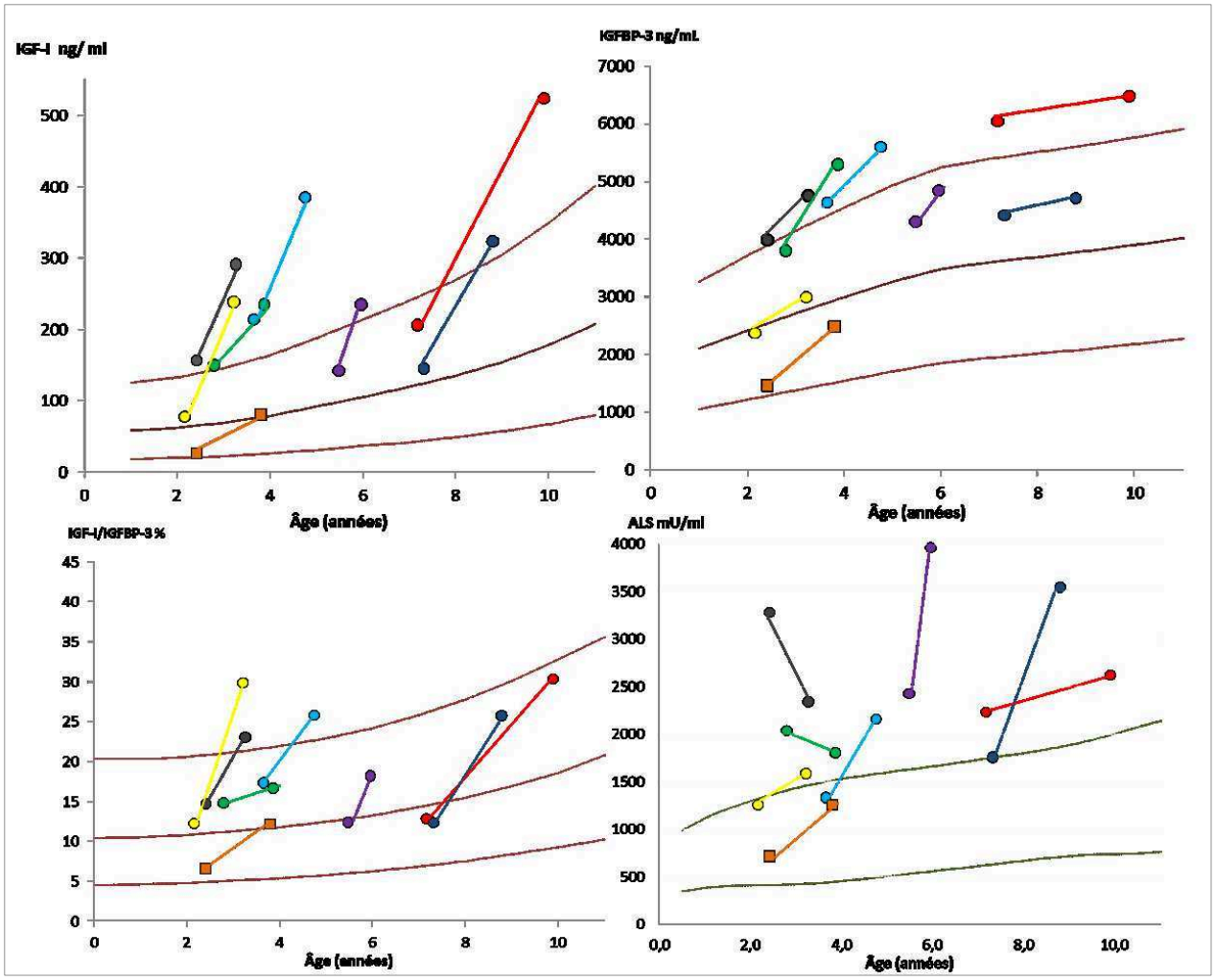


Figure 30. Évolution des taux d'IGF-I, IGFBP-3, ALS et du ratio IGF-I/IGFBP-3 chez 8 filles avec SRS par LOM 11p15 (ronds, n=7) ou mupd7 (carrés, n=1). Chaque couleur correspond à un patient. Le premier point correspond aux dosages avant le traitement par rGH et le second au dosage réalisé en moyenne un an après l'instauration du traitement. Les taux d'IGF-I et d'IGFBP-3 augmentent avec le traitement. Chez 3 filles (sur 7 avec LOM 11p15) le ratio IGF-I/IGFBP-3 augmente au-delà de 2DS. L'évolution des taux d'ALS avec le traitement est très variable.

2.1.2.4 Discussion

Nous confirmons donc sur une large cohorte la tendance de ces patients ayant un SRS à avoir des concentrations spontanément élevées d'IGF-I, ce qui avait déjà été rapporté sur de plus petits effectifs ou chez des patients avec un diagnostic clinique de SRS non prouvé sur le plan moléculaire (Binder *et al.* 2006, 2008). Ce phénomène est identifié uniquement chez les patients présentant des anomalies de la région 11p15, puisque les patients avec mupd7 avaient des taux bas d'IGF-I spontanément (avant traitement par rGH), correspondant à ce que l'on attend en raison de leur état nutritionnel. De même, nous avons identifié des concentrations d'IGFBP-3 et des ratios IGF-I/IGFBP-3 modérément élevés spontanément chez les patients avec anomalie de la région 11p15. Ce premier résultat est donc en faveur d'une résistance à l'IGF-I chez ces patients présentant un retard de croissance malgré des taux d'IGF-I total et probablement libre

(reflétés par le ratio IGF-I/IGFBP-3) élevés avant la mise sous traitement par hormone de croissance. Le résultat original de ce travail réside dans la mise en évidence de taux d'ALS élevés chez ces patients, et ce de façon spontanée, ce qui n'avait jamais été montré. Ces taux élevés d'ALS restent cependant difficiles à interpréter. Ils sont en faveur d'une augmentation d'IGF-I circulant sous forme de complexe ternaire chez ces patients. L'IGFBP-3 faisant également partie de ce complexe, les taux élevés d'IGFBP-3 rendent compte de l'augmentation des complexes secondaires et ternaires. Une première hypothèse serait donc celle d'une baisse des complexes secondaires, la majorité de l'IGF-I et de l'IGFBP-3 étant complexée avec l'ALS sous forme de complexes ternaires à la demi-vie plasmatique très longue. Ainsi, l'IGF-I biodisponible serait diminué par baisse de l'IGF-I mobilisable à partir des complexes secondaires. Un dosage de l'IGFBP5 pourrait également être intéressant du fait de sa capacité à lier ALS et IGF-I en complexe ternaire pour avancer dans la compréhension. L'IGF-I considéré comme libre (indirectement à partir du ratio IGF-I/IGFBP-3) pourrait être en fait lié sous forme de complexe ternaire ALS/IGFBP-5/IGF-I et donc voir sa biodisponibilité d'autant diminuée, sans pour autant que la quantité des complexes secondaires soient diminués. Pour aller plus loin, et du fait de la possible liaison de toutes les IGF-BP avec IGF-I circulant, il serait intéressant de mesurer la concentration de ces autres IGF-BP (bien qu'IGFBP-3 et -5 soient les plus abondantes dans la circulation sanguine). Sur le plan technique ces analyses, même si elles sont possibles pour la majorité des IGFBPs, sont d'interprétation difficile du fait de l'absence de normes publiées. C'est en effet un écueil important, qui rend impossible une évaluation quantitative précise de ces IGFBP, mais également de l'ALS. La détermination de valeurs normales selon l'âge et le sexe est donc un prérequis nécessaire à ces analyses. Dans ce cas d'excès d'IGF-I complexé avec d'autres IGFBP, le ratio IGF-I/IGFBP-3 surévaluerait l'IGF-I libre et en serait donc un mauvais reflet.

Une autre hypothèse serait celle d'une baisse de la protéolyse des complexes secondaires et ternaires, diminuant ainsi la biodisponibilité d'IGF-I qui resterait sous forme liée de manière plus prolongée. C'est en effet ce type de profil biologique (IGF-I, IGFBP-3, ALS élevés) que l'on retrouve chez les patients ayant des anomalies de PAPP-A2, protéine responsable du clivage des complexes secondaires et ternaires (Dauber *et al.* 2016). Les ratios molaires IGF-I/IGFBP-3 n'ont pas étés reportés chez ces patients. Nous pourrions donc envisager de doser PAPP-A2 dans le sérum des patients avec SRS pour tester cette hypothèse. Comme précédemment évoqué, l'absence de normes publiées représentera un obstacle majeur à l'interprétation de ces analyses. Par ailleurs, l'IGFBP-3 dosée comprend la forme « intacte » mais également celle protéolysée dont la capacité de liaison à l'ALS et à l'IGF-I est diminuée mais non nulle (Devi *et al.* 2000). Il serait donc intéressant d'évaluer la quantité d'IGFBP-3 intacte chez ces patients et de calculer le rapport molaire IGF-I/IGFBP-3 intacte qui devrait rendre compte de manière plus précise de la proportion de complexes secondaires et ternaires stables. Donc, bien que sur le plan théorique le

ratio IGF-I/IGFBP-3 permette d'évaluer la proportion d'IGF-I libre circulant, il ne rend pas compte de la proportion de chaque forme circulante d'IGF-I qui conditionne la biodisponibilité d'IGF-I. La détermination de cette biodisponibilité constitue un enjeu important qui permettrait d'ajuster au mieux les recommandations d'adaptation des doses de traitement par rGH chez ces patients.

Par ailleurs, le traitement par hormone de croissance augmentait de manière très significative les taux circulants d'IGF-I, d'IGFBP-3 ainsi que le ratio IGF-I/IGFBP-3. Ces patients avec SRS présentent donc une bonne sensibilité hépatique à la GH. La plupart des patients avaient un ratio IGF-I/IGFBP-3 qui restait dans les normes, néanmoins, le fait que les ratios IGF-I/IGFBP-3 étaient au-dessus des normes pour certains patients en cours de traitement pourrait témoigner d'un excès d'IGF-I libre. On notera que, bien que dans des effectifs réduits, les ratios IGF-I/IGFBP-3 avaient tendance à être plus élevés chez les filles que chez les garçons (3/8 contre 1/9 au-delà de 2DS). Ces résultats, sont en faveur d'un possible dimorphisme sexuel dans le degré de résistance à l'IGF-I qu'il sera important d'évaluer sur une plus grande cohorte. Les données publiées sur l'évolution du ratio IGF-I/IGFBP-3 lors du traitement par rGH chez les enfants nés PAG sont contradictoires et les publications concernent des petits effectifs. Ainsi, Ballerini *et al.* ont montré une élévation du ratio IGF-I/IGFBP-3 à un et deux ans de l'instauration d'un traitement par rGH chez 25 patients nés PAG (Ballerini *et al.* 2017). Ce profil biologique est différent de celui identifié chez 24 patients nés PAG chez qui les ratios IGF-I/IGFBP-3 semblaient tous rester dans les normes au cours du traitement par hormone de croissance (Gaddas *et al.* 2019)(figure 31). Il reste donc à déterminer si le ratio IGF-I/IGFBP-3 est un bon reflet de la biodisponibilité d'IGF-I chez les patients né PAG et ceux avec SRS. Un élément de réponse sera apporté par la corrélation de leur vitesse de croissance avec leur ratio IGF-I/IGFBP-3. Néanmoins, là aussi la vigilance dans l'interprétation de ces données s'impose car ces ratios varient avec le statut nutritionnel, le sexe, le stade pubertaire et l'âge chez les individus sains (Juul *et al.* 1995). Nous prévoyons donc l'analyse des ratios IGF-I/IGFBP-3 de cette cohorte à la lumière de ces données cliniques chez nos patients, en comparaison à deux groupes de sujets: des individus sains et des patients nés PAG sans étiologie retrouvée, appariés en âge et en sexe.

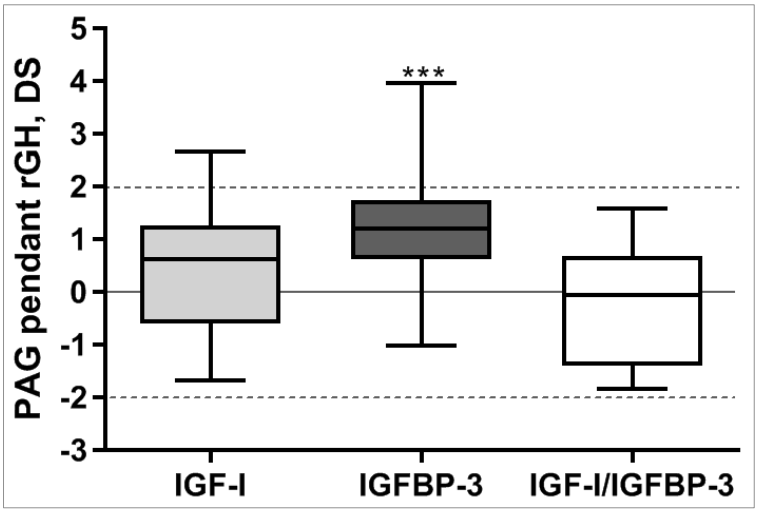


Figure 31. Concentrations d'IGF-I, d'IGFBP-3 et ratio IGF-I/IGFBP-3 chez 24 patients nés PAG pendant traitement par rGH. Données extraites de (Gaddas *et al.* 2019). Comparaison statistique des distributions (présentées avec moyenne, minimum et maximum) à la répartition normale (figurée au-dessus de chaque distribution).

Par ailleurs, nous démontrons que les taux circulants d'IGF-II ne sont pas abaissés chez ces patients, comme cela avait déjà été montré dans différentes publications, malgré la perte de méthylation d'*H19/IGF2*:IG-DMR. Ces résultats sont concordants avec les travaux précédemment publiés chez ces patients (Binder *et al.* 2006, 2008). Ceci est probablement dû au fait que, la sécrétion hépatique d'IGF-II étant sous la dépendance du promoteur P1 du gène *IGF2* qui n'est pas soumis à empreinte parentale, il s'exprime à partir des deux allèles comme pour les sujets sans anomalie de méthylation du domaine. Cela rejette l'hypothèse d'une augmentation de l'IGF-I sous forme complexée du fait de la baisse d'IGF-II circulant et donc d'une augmentation des sites de liaison des IGFBP pour IGF-I.

Sur le plan physiopathologique, les mécanismes régulant cette augmentation d'IGF-I, d'IGFBP-3 et d'ALS restent à élucider. En effet, aucun lien direct entre l'anomalie de méthylation de l'*H19/IGF2*:IG-DMR et l'élévation sérique de ces facteurs ne semble évident. Le fonctionnement en réseau des différents gènes soumis à empreinte parentale et leur impact possible sur des gènes non soumis à empreinte pourrait cependant permettre de telles interactions. L'étude de l'expression des gènes des IGFBPs, d'IGF-I et de l'ALS dans les hépatocytes des patients serait extrêmement intéressante mais non réalisable éthiquement en pratique. Les modèles murins de KO d'*Igf2* étant les plus satisfaisants pour évaluer les anomalies en 11p15 présentées par les patients pourraient nous renseigner sur ces questions. De même, les modèles cellulaires en cours de développement à partir de cellules pluripotentes induites différenciées en hépatocytes sont une voie de recherche intéressante bien que non au point actuellement du fait de modification de la méthylation des ICR dans les phases de dédifférenciation-refifférenciation (Bar *et al.* 2017).

2.2 Chevauchement clinique et moléculaire dans deux syndromes de restriction de croissance fœtale

2.2.1 Syndrome de Temple et syndrome de Silver-Russell

Dans ce second projet, nous nous sommes intéressés aux mécanismes sous-tendant le chevauchement clinique entre les syndromes de Temple (TS) et de Silver-Russell qui avait déjà été mis en évidence sur des petites séries de patients (Poole *et al.* 2013; Azzi *et al.* 2015a; Kagami *et al.* 2017). En effet, plusieurs équipes ont identifié des anomalies de la région 14q32.2 chez des patients adressés pour une suspicion clinique de SRS, avec un score clinique de Netchine-Harbison (NH-CSS) de plus de 4 sur 6. Nous avons donc voulu décrire précisément les caractéristiques cliniques communes entre les deux syndromes et leurs spécificités. Pour ce faire, nous avons étudié les présentations cliniques des patients pour lesquels une disomie uniparentale maternelle du chromosome 14 (n = 8), une perte de méthylation du centre d'empreinte *MEG3/DLK1*:IG-DMR (n = 17) ou une délétion de la région 14q32.2 (n = 3) avaient été identifiées dans le laboratoire. Nous avons ainsi pu montrer que 72,7% de ces patients avaient un score NH-CSS d'au moins 4 sur 6 compatible avec un diagnostic clinique de SRS. Les patients avec TS présentaient également une hypotonie néonatale, une prise de poids importante dans la petite enfance, un décalage des acquisitions fréquent mais modéré et une puberté précoce de manière très fréquente, bien que ces caractéristiques cliniques soient également présentes chez les patients avec SRS.

Enfin, l'étude systématique de la méthylation de dix autres ICR a mis en évidence une proportion très importante (autour de 60%) d'anomalies de la méthylation (MLID) à au moins un des autres ICR étudiés chez les patients avec une perte de méthylation du centre d'empreinte *MEG3/DLK1*:IG-DMR. Nous n'avons cependant pas identifié d'anomalies génétiques au niveau des ICR ou de facteurs agissant en *trans* pouvant interférer dans la mise en place ou le maintien de la méthylation chez ces patients. Les chevauchements cliniques entre les différents syndromes pourraient être expliqués par ces anomalies multiples de la méthylation.

Ce travail a fait l'objet de la publication n°4 (Geoffron *et al.* 2018).

2.2.1.1 Discussion

Par cette étude, nous avons confirmé le chevauchement clinique entre ces deux syndromes par la proportion importante (72,7%) de patients avec un score clinique positif pour le diagnostic de SRS au sein d'une cohorte de patients avec un TS. De plus, contrairement aux précédents travaux publiés, nous avons retrouvé une majorité de patients porteurs d'une anomalie épigénétique (hypométhylation de *MEG3/DLK1*:IG-DMR, 60%) par rapport aux disomies uniparentales maternelles du chromosome 14 ou aux délétions (Ioannides *et al.* 2014; Kagami *et al.* 2017). Ces différences tiennent probablement aux différentes stratégies de diagnostic utilisées par les équipes. En effet, notre laboratoire de diagnostic étant l'un des seuls en France actuellement à étudier la méthylation de *MEG3/DLK1*:IG-DMR, nous avons peut-être une surreprésentation de ces diagnostics, puisque les délétions et les disomie maternelles peuvent être identifiées avec des techniques de génétique conventionnelle dans d'autres laboratoires. Du fait de l'important chevauchement clinique entre le SRS et le TS, il semble opportun de réaliser une analyse de la méthylation de *MEG3/DLK1*:IG-DMR en seconde intention chez un patient suspect de SRS et sans anomalie des chromosomes 7 et 11 (Wakeling *et al.* 2016). Enfin, nous avons identifié une proportion importante (60%) de patients avec des anomalies multiples de la méthylation (MLID) dans d'autres centres d'empreinte chez les patients avec une perte de méthylation du centre d'empreinte *MEG3/DLK1*:IG-DMR en 14q32.2. Ces résultats suggèrent donc une possible anomalie de la régulation en cis ou en trans que nous n'avons pas identifiée pour l'instant dans cette cohorte. Néanmoins, il serait intéressant de rechercher des mutations des facteurs impliqués dans le maintien post-zygotique de la méthylation chez les mères des patients avec MLID, de même que d'évaluer leur histoire obstétricale (fausses couches précoces, môles hydatiformes, hypofertilité, recours à la procréation médicalement assistée et type de protocole utilisé).

Récemment, nous sommes allés plus loin dans la mise en évidence de profils d'expression géniques comparables chez des patients porteurs d'anomalies dans différentes régions soumises à empreinte parentale (Abi Habib *et al.* 2019). En premier lieu, nous avons pu confirmer qu'en cas d'anomalie de la méthylation de *MEG3/DLK1*:IG-DMR chez les patients avec un TS, il existait une expression biallélique des gènes de la région 14q32.2 (normalement exprimés uniquement à partir de l'allèle maternel). De plus, nous avons mis en évidence une baisse d'expression d'*IGF2* dans les fibroblastes des patients avec anomalie de la méthylation de *MEG3/DLK1*:IG-DMR, alors que la méthylation de la région 11p15 était normale (figure 32A.). L'effet des ARN non codants à expression maternelle de la région 14q32.2 sur l'expression d'*IGF2* a été confirmé dans des fibroblastes contrôles par des expériences de *small interfering* (si)RNA et de surexpression de ces gènes (figure 33). Ainsi, la surexpression de *MEG3* et *MEG8* (dans les fibroblastes des

patients avec TS) était capable d'induire *in vitro* une baisse d'expression d'*IGF2*, tandis que leur inactivation (séparément et conjointement) induisait une augmentation de l'expression d'*IGF2*.

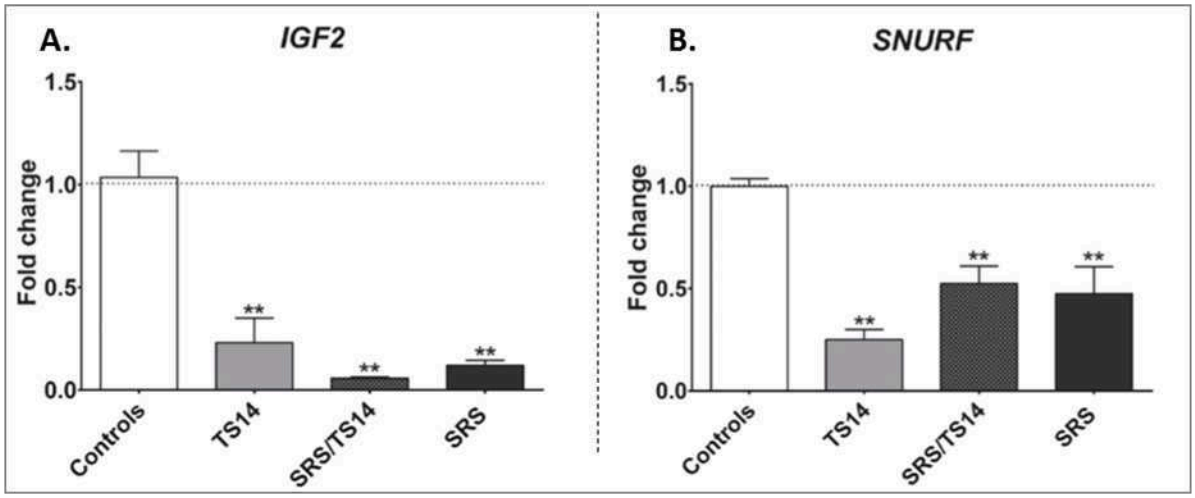


Figure 32. A. Baisse d'expression d'*IGF2* ou B. de SNURF dans les fibroblastes de patients avec syndrome de Temple (TS14), syndrome de Silver-Russell (SRS) ou les deux syndromes (hypométhylation d'*H19/IGF2*:IG-DMR et de *MEG3/DLK1*:IG-DMR) par rapport aux contrôles. Les données sont représentées en moyenne ± erreur standard, pour 5 différents passages, chez 4 patients avec TS14, 5 patients SRS et un patient SRS/TS14 comparés à 5 contrôles (Abi Habib *et al.* 2019).

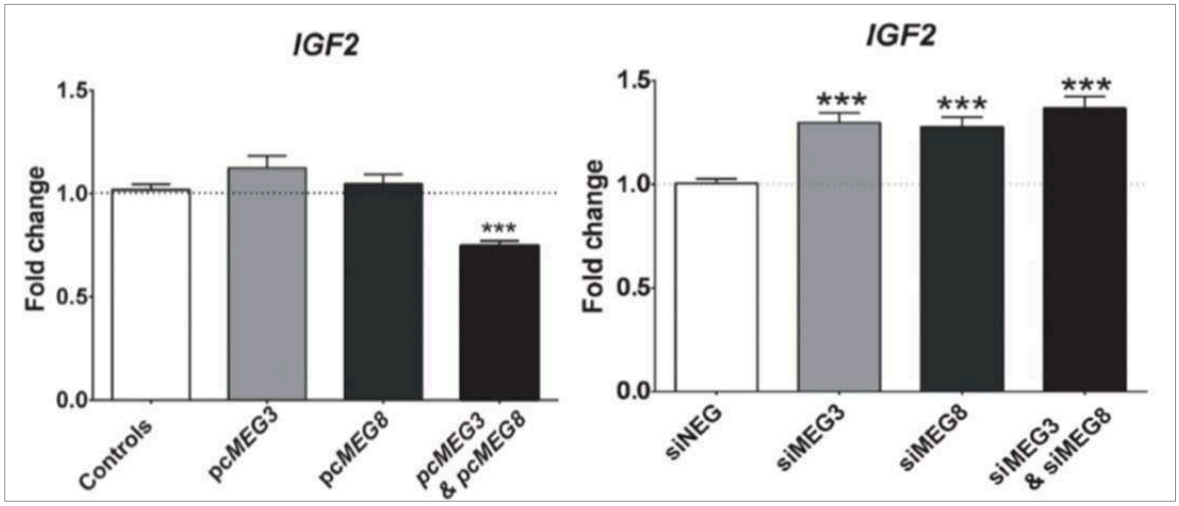


Figure 33. Modifications de l'expression d'*IGF2* dans des fibroblastes contrôles après surexpression (à gauche) et inactivation par siRNA (droite) de *MEG3* et *MEG8* dans des fibroblastes de contrôle. Les données sont représentées en moyenne ± erreur standard, pour 4 expériences de transfection indépendantes (Abi Habib *et al.* 2019).

De plus, ce travail a montré un impact de *MEG3* et *MEG8* sur *SNURF,* qui est un gène exprimé à partir de l'allèle paternel dans la région 15q11-q13 impliquée dans le syndrome de Prader-Willi (SPW) (figure 32B). Ces résultats ont donc confirmé *in vitro* les observations cliniques qui mettent en avant un chevauchement clinique entre les patients avec TS et SPW (Kagami *et al.*

2017). Enfin, une analyse par RNAseq à partir de fibroblastes de ces patients SRS ou TS retrouvait des profils d'expression comparables entre les patients avec SRS ou TS. Les baisses communes d'expression portaient majoritairement sur des gènes impliqués dans la croissance et l'inflammation (Publication dans son intégralité en Annexe (Abi Habib *et al.* 2019).

Ces deux études, ont permis de caractériser le chevauchement clinique et moléculaire entre les syndromes de Temple et de Silver-Russell de manière objective et en particulier de mettre en évidence l'atteinte de l'expression d'*IGF2* dans les deux syndromes. Ces résultats sont donc en faveur de proposer une prise en charge commune aux patients atteints de ces deux syndromes, tout en étant conscient de certaines de leurs spécificités. Les notions de MLID et d'IGN sont donc explorées ici de manière indépendante, mais ce sont deux processus qui peuvent contribuer au chevauchement phénotypique des différentes maladies liées à l'empreinte parentale. Ils renforcent ainsi les connaissances sur le réseau des gènes soumis à empreinte parentale. En effet, ces résultats sont concordants avec certains travaux effectués chez le porc qui ont mis en évidence une colocalisation d'*Igf2* avec d'autres gènes soumis à empreinte, et principalement *Dlk1* dans les hépatocytes (Lahbib-Mansais *et al.* 2016). De plus, ce travail a montré que les défauts d'expression des gènes soumis à empreinte observés dans les deux syndromes perturbaient de façon comparable l'expression de gènes non soumis à empreinte. Les mécanismes biologiques qui contrôlent cette régulation et leur dynamique seront essentiels à découvrir pour envisager d'éventuels avancées thérapeutiques chez les patients dont la pathologie implique la régulation de ces gènes. Une piste privilégiée à l'heure actuelle est l'implication des ARN non codants dans cette régulation. La difficulté d'étude de ces facteurs est la non spécificité des séquences (principalement pour les miR) qui rendent cependant l'étude et l'interprétation de leur expression complexe.

3 PERSPECTIVES

Dans ce travail, nous avons exploré la physiopathologie du système des IGFs dans différents modèles de restriction de croissance fœtale secondaires à des anomalies génétiques ou épigénétiques de différents acteurs de ce système. De plus, du fait de notre expérience de cliniciens, nous pensons que ces travaux permettront de mieux comprendre les mécanismes de résistance au traitement par hormone de croissance chez les enfants nés petits pour l'âge gestationnel (PAG) pour améliorer leur prise en charge thérapeutique. En effet, ce mécanisme restant inconnu, les taux sériques d'IGF-I au-delà des normes pour le sexe et l'âge (supérieurs à 2DS) ne correspondent pas aux objectifs habituels de surveillance d'un traitement par rGH, bien qu'il soit admis que pour les patients nés PAG, les taux d'IGF-I puissent être spontanément élevés et ne soient pas à prendre en compte pour l'adaptation du traitement par rGH (Johannsson *et al.* 2018). Il y a donc un enjeu important à comprendre ce phénomène de résistance, afin d'être plus sereins sur le fait que ces taux élevés ne sont pas fiables pour l'adaptation du traitement par rGH chez ces patients, et idéalement, il faudrait développer d'autres indicateurs rendant compte de la bioactivité de l'IGF-I afin d'optimiser l'adaptation du traitement par rGH chez les patients ayant un SRS.

Cette résistance à l'IGF-I est préexistante au traitement par rGH chez les patients avec SRS, comme en témoignent les taux spontanément élevés d'IGF-I que nous avons rapportés. Notre hypothèse de l'existence d'une baisse d'activité de l'IGF1R s'est révélée peu convaincante devant les résultats que nous avons obtenus avec un test fonctionnel validé, bien que d'autres expériences soient envisageables pour poursuivre les investigations dans cette voie. Cependant, la mise en évidence d'une augmentation concomitante à celle d'IGF-I de sa protéine de liaison principale (IGFBP-3) et de l'ALS, pourrait être en faveur d'une baisse de la biodisponibilité d'IGF-I plutôt que d'un phénomène de résistance. Pour conforter cette hypothèse, nous pourrions quantifier le degré d'activité d'IGF1R induit par le sérum, par une méthode développée par une équipe allemande (KIRA) qui utilise directement les sérums des patients sur des lignées cellulaires transfectées avec *IGF1R* et qui quantifie le degré de phosphorylation d'IGF1R par une technique ELISA (Chen *et al.* 2003). Cette activation est ensuite rapportée aux concentrations sériques d'IGF-I des patients pour obtenir une valeur d'IGF-I dit « bioactif ». Cette méthode a déjà été utilisée chez les patients avec un syndrome de Prader-Willi pour montrer une baisse d'IGF-I bioactif au cours du traitement par hormone de croissance (Bakker *et al.* 2015). Cependant, les ratios IGF-I/IGFBP-3 n'étaient pas corrélés à l'IGF-I biodisponible chez les patients avec SPW en période pubertaire et seules des données acquise en cours de traitement étaient disponibles. Une première étape importante serait de valider le ratio IGF-I/IGFBP-3 comme reflet de la biodisponibilité d'IGF-I dans une population contrôle en étudiant sa corrélation avec la vitesse de croissance et l'IGF-I biodisponible. Idéalement, il faudrait pouvoir également intégrer le stade pubertaire et l'état nutritionnel pour faire de ce marqueur un outil

fiable. De même, il faudrait étudier son évolution avec la mise en place du traitementpar rGH pour, *in fine,* adapter de manière personnalisée les modalités du traitement. Une autre méthode de quantification serait d'utiliser, là encore, les sérums des patients et d'effectuer des tests de prolifération *in vitro* sur des fibroblastes de contrôle (scratch test par exemple (Li *et al.* 2016)). Cependant, ces différentes techniques, si elles permettaient d'avancer dans la compréhension des mécanismes de résistance à l'IGF-I, ne pourraient être utilisées en routine clinique pour évaluer le degré de résistance propre de chaque patient, indépendamment de ses taux d'IGF-I élevés. Il est donc crucial d'analyser les données cliniques précises (vitesse de croissance, maturation osseuse, stade pubertaire, adrénarche) en rapport avec les informations biologiques obtenues (notamment le ratio molaire IGF-I/IGFBP-3), pour évaluer la signification physiologique de ces données biologiques. Comme évoqué ci-dessus, le manque de normes publiées pour les dosages tels qu'ALS, IGFBP-5, PAPP-A2 ou IGFBP-3 intact est un obstacle important à l'interprétation des analyses et doit être une première étape pour l'interprétation quantitative précise des données.

Reste à comprendre le lien possible entre l'hypométhylation d'*H19/IGF2*:IG-DMR chez ces patients et une baisse de l'activité d'IGF-I quels qu'en soient le ou les mécanismes responsables (diminution d'activité d'IGF1R et/ou de la biodisponibilité d'IGF-I).

Les résultats obtenus dans la seconde partie de notre travail pourraient apporter quelques voies de recherche pour répondre à cette question. En effet, la mise en évidence des conséquences d'anomalies dans des régions soumises à empreinte parentale sur la régulation de l'expression d'autres gènes (soumis à empreinte parentale ou non) ouvre un large champ d'étude. Les ARN non codants (longs ou miRNA ou snoRNA) sont des acteurs potentiels très intéressants. En effet, les régions soumises à empreinte parentale en sont riches et de plus en plus de travaux montrent leur implication dans des processus de régulation de l'expression de gènes en *trans*. La difficulté de leur étude réside dans la faible spécificité de leur séquence (d'autant plus vrai pour les miRNA) qui rend leur action très ubiquitaire (comme la grande famille des *let7-lin28)*.

Enfin, la caractérisation d'anomalies génétiques des différents facteurs intervenant dans la mise en place et le maintien de la méthylation des ICR à des étapes clés du développement reste primordiale pour identifier les rôles précis de chacun d'entre eux. Ces mécanismes de MLID bien que fréquents dans les pathologies liées à l'empreinte ne concernent pas tous les patients et leur conséquence clinique reste à caractériser. De ce fait, l'organisation en réseau des gènes soumis à empreinte entre eux et avec des gènes à expression biallélique semble une piste majeure, notamment dans l'exploration de la programmation fœtale.

La majorité de nos travaux a été réalisée dans les fibroblastes des patients, du fait de la facilité d'accès et de culture de ce tissu. Il serait essentiel de pouvoir étudier le cartilage de croissance qui tient le rôle central dans la croissance staturale. La difficulté d'accès et d'étude *in vitro* de ce tissu rend cet abord très complexe. Néanmoins, des approches indirectes sont envisageables. Tout d'abord, l'étude dans les modèles murins est intéressante, et a permis à certaines équipes d'étudier l'expression des gènes soumis à empreinte dans les différentes zones du cartilage et à différents temps de la croissance staturale (Andrade *et al.* 2010; van Meurs *et al.* 2019). Une autre approche serait celle d'induire des cellules pluripotentes à partir de cellules de patients et de les différencier secondairement en chondrocytes. La difficulté actuelle est de pouvoir maintenir les anomalies de méthylation aux cours des différentes étapes de l'induction de la pluripotence et de redifférenciation (Bar *et al.* 2017). De plus, la différenciation en chondrocyte est une étape intéressante mais ne permettra pas une étude structurelle du cartilage de croissance comme cela est possible *ex vivo.* Enfin, nous envisageons une approche indirecte qui est celle de l'étude de la structure et de la minéralisation des dents déciduales de ces patients. En effet, de par leur similitude en termes de structure et de minéralisation avec l'os, les dents déciduales sont un excellent témoin indirect de la composition osseuse (Opsahl Vital *et al.* 2012). De plus, les dents préservent de manière définitive tous les évènements ayant impacté leur structure de la période prénatale aux premières années de vie et permettent donc de retracer finement l'historique et la cinétique de ces évènements (Coyac *et al.* 2018). Enfin, l'induction de la différentiation des cellules stromales/mésenchymateuses pulpaires en cellules de la lignée ostéoblastique et chondrocytaire permettrait l'étude des gènes soumis à empreinte parentale dans ces cellules (Acuña-Mendoza *et al.* 2013; Novais *et al.* 2019). Ces travaux seront l'objet de la suite de mon activité de recherche, avec pour objectif de caractériser la méthylation dans ces cellules aux différents stades de différenciation chez des contrôles, puis chez des patients avec SRS par anomalie de la méthylation en 11p15. J'étudierai également l'expression des gènes impliqués dans l'IGN soit dans une approche ciblée soit dans une approche globale (RNAseq). Enfin, le test fonctionnel de l'activité d'IGF1R pourra être développé sur ces cellules.

Sur la figure 34, j'ai représenté schématiquement une vision intégrée des données issuse des travaux précédents et de nos résultats, ainsi que les hypothèses de travail pour la suite de la compréhension de ces mécanismes faisant intervenir régulation épigénétique et système des IGFs. Ainsi, IGF-II/*IGF2* est un acteur central faisant le lien entre réseau de gènes soumis à empreinte et régulation hormonale de la croissance et a un rôle prépondérant dans la programmation foetale.

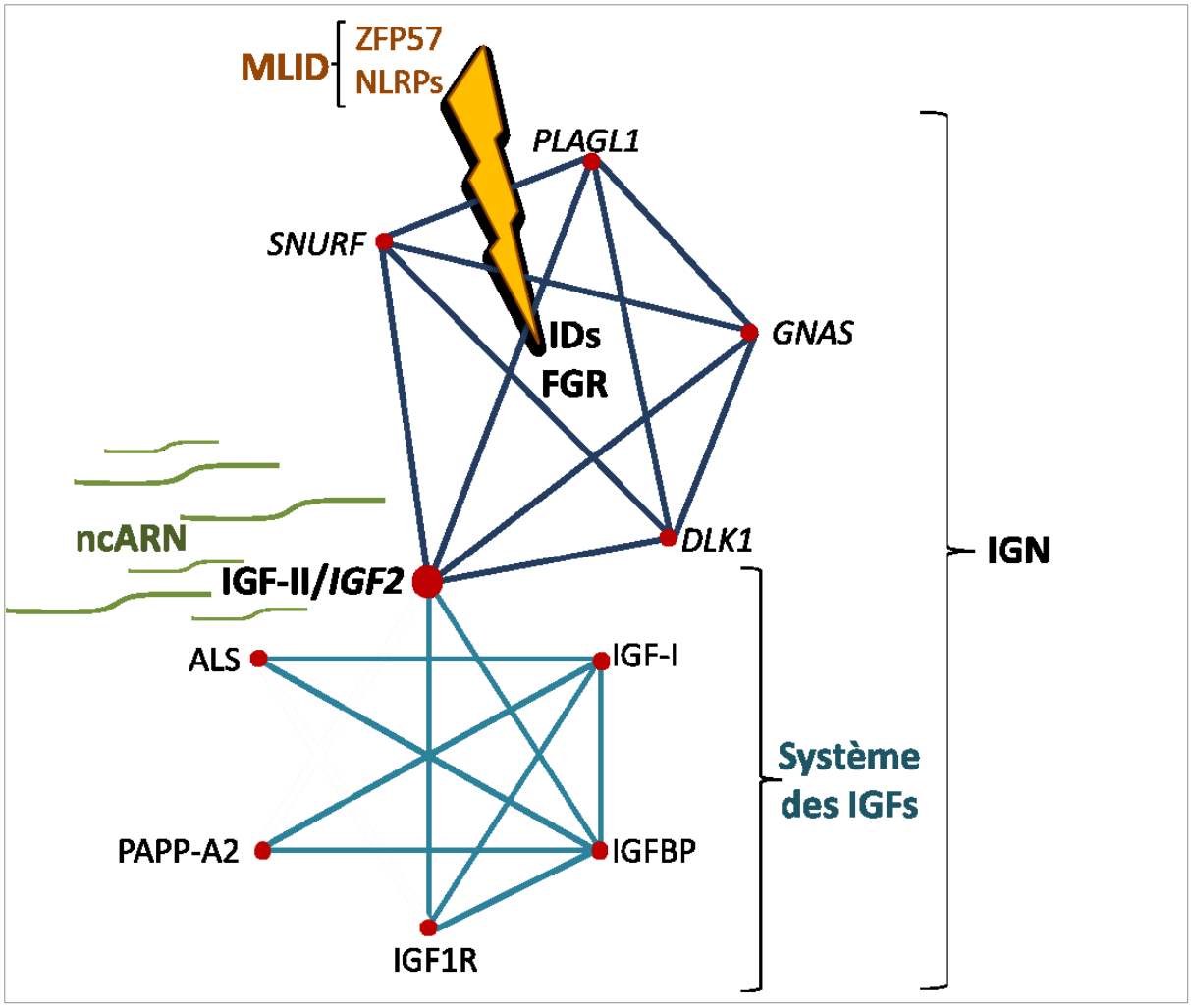


Figure 34. Schéma intégrant les différentes données et hypothèses de travail. Les gènes soumis à empreinte sont au centre d'un réseau (IGN) et peuvent être modifiés par des anomalies de protéines impliquées dans la mise en place et le maintien de la méthylation (MLID). IGF-II/*IGF2* est l'acteur clé, il peut-être responsable de restriction de croissance fœtale (FGR) dans le cadre de pathologie soumise à empreinte parentale (ID) au même titre que *DLK1, GNAS, SNURF* ou *PLAGL1.* Il est également un des membres du système des IGF impliqué dans la croissance fœtale et post-natale. Les acteurs du système des IGFs pourraient faire partie intégrante de l'IGN et les ARN non codants (ncARN) être un moyen de réguler cette expression.

Enfin, la physiopathologie des conséquences métaboliques à moyen et long terme de la restriction de croissance fœtale n'a pas été abordée dans ce travail mais reste essentielle. Là aussi, les pathologies liées à l'empreinte parentale sont de bons modèles d'étude puisque de plus en plus de données cliniques abondent dans le sens de troubles métaboliques (insulino-résistance, diabète de type II, obésité) à l'âge adulte chez certains de ces patients (Yzydorczyk *et al.* 2017). La constitution et l'analyse de cohortes nationales et internationales de ces patients et leur suivi à l'âge adulte devraient apporter une meilleure connaissance du retentissement d'une programmation fœtale altérée dans ces pathologies, et sera l'objet de mon travail de recherche clinique. Les mécanismes sous-tendant cette programmation devront être élucidés pour envisager une amélioration des conséquences péjoratives à long terme chez ces patients.

4 PUBLICATIONS

4.1 Publication n°1

New clinical and molecular insights into Silver-Russell syndrome.

Éloïse Giabicani, Irène Netchine, Frédéric Brioude.

Current Opinion in Pediatrics. 2016 Aug;28(4):529-35.

New clinical and molecular insights into Silver–Russell syndrome

Eloïse Giabicani[a,b,c], *Irène Netchine*[a,b,c], *and Frédéric Brioude*[a,b,c]

Purpose of review
The purpose of review is to summarize new outcomes for the clinical characterization, molecular strategies, and therapeutic management of Silver–Russell syndrome (SRS).

Recent findings
Various teams have described the clinical characteristics of SRS patients by genotype. A clinical score for the definition of SRS and for orienting molecular investigations has emerged. Insulin-like growth factor 2 (a major fetal growth factor) has been implicated in the pathophysiology of SRS, as the principle molecular mechanism underlying the disease is loss of methylation of the 11p15 region, including the imprinted *insulin-like growth factor 2* gene. Maternal uniparental disomy of chromosome 7 and recently identified rare molecular defects have also been reported in patients with SRS. However, 40% of patients still have no molecular diagnosis.

Summary
The definition of SRS has remained clinical since the first description of this condition, despite the identification of various molecular causes. The clinical issues faced by these patients are similar to those faced by other patients born small for gestational age (SGA), but patients with SRS require specific multidisciplinary management of their nutrition, growth, and metabolism, as they usually present an extreme form of SGA. Molecular analyses can confirm SRS, and are of particular importance for genetic counseling and prenatal testing.

Keywords
fetal growth, IGF2, imprinting disorders, Silver–Russell syndrome

INTRODUCTION

In 1953, Silver *et al.* [1] first described the phenotype of two children with low birth weights presenting marked hemihypotrophy and growth failure. One year later, Russell [2] described five patients born small for gestational age (SGA) with a very small infarcted placenta (in four of the five patients) and specific craniofacial features combining a triangular face with a high bossed forehead and an updrawn philtrum and mouth. Furthermore, all the patients in the series described by Russell *et al.* had severe anorexia and some also displayed hemihypotrophy and insulin resistance. In 1961, Black [3] grouped together these very similar phenotypes as Silver–Russell syndrome (SRS) – OMIM #180860. More than 60 years after its initial description, we now know much more about this uncommon syndrome, thanks largely to breakthroughs in the genetic and epigenetic fields, but its characterization remains incomplete.

MOLECULAR BASIS

As the identification of the main molecular mechanism underlying SRS in 2005, *insulin-like growth factor 2 (IGF2)* has been further implicated in the pathophysiology of SRS. The role of *IGF2* in fetal development has been highlighted by knockdown experiments in rodents, in which heterozygous animals with knocked down *IGF2* expression were found to be 40% smaller than their wild-type littermates [4]. Furthermore, a phenotype of growth retardation was observed only after paternal transmission, consistent with the imprinted nature of the *IGF2* gene [5]. Imprinted genes are expressed from only one of the two alleles, according to parental origin. Imprinted genes are involved in the control of growth, development, and metabolism. They are

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KEY POINTS

- NH-CSS, revised: six easily scored items, with children considered to have an SRS phenotype if they present at least four of the six items.
- New molecular strategies to guide the investigation of patients with clinical SRS, beginning with analyses of the methylation of the DMRs in the 11p15, 7p, and 7q regions.
- Multidisciplinary clinical guidelines for the management of this group of patients, from birth to adulthood as the result of the first expert consensus meeting on SRS will be available soon.
- Identification of new genetic and epigenetic causes of SRS to improve genetic counseling.
- International collaboration to improve diagnosis, molecular investigations, clinical guidelines, and future research on this rare disease.

grouped in clusters and their monoallelic expression is controlled by differentially methylated regions (DMRs). The epigenetic marks in these DMRs are acquired during gametogenesis, and normal embryo development is dependent on their maintenance after fertilization and during embryogenesis [6,7]. In humans, the 11p15.5–p15.4 region contains several imprinted genes strongly implicated in the control of fetal growth. *IGF2* is located in the telomeric domain of 11p15.5 and is expressed from the paternal allele. This gene has several promoters, only some of which are imprinted; these promoters are activated in a tissue-specific manner, accounting for the complex pattern of regulation observed for *IGF2* expression [8]. *IGF2* is expressed in a biallelic manner in the liver, but only weakly in the fibroblasts of patients with imprinting control region 1 (ICR1) loss of methylation (LOM) [9,10▪], these differences potentially accounting for the normal serum concentration of IGF2 in SRS patients.

The *H19* gene (encoding a long noncoding RNA), which is located in the same domain, is expressed from the maternal allele. The expression of both these genes is controlled by an imprinting control region, the H19/IGF2 IG-DMR (also known as ICR1 or IC1). ICR1 is a CpG-rich region that is methylated on the paternal allele. A second domain (the centromeric domain) contains other imprinted genes, including the cyclin-dependent kinase inhibitor 1C gene *(CDKN1C),* encoding a strong cell cycle inhibitor, which is expressed from the maternal allele. The expression of the genes in the centromeric domain is regulated by another CpG-rich region, the *KCNQ1OT1* TSS DMR (also known as ICR2, IC2, KvDMR1, or *LIT1)*, which is methylated on the maternal allele (Fig. 1) [6]. Abnormal methylation and/or duplication within the 11p15 region can lead to SRS or its clinical mirror, Beckwith–Wiedemann syndrome (BWS) (see Phenotype).

PHENOTYPE

The reported incidence of SRS varies from 1/3000 to 1/100000, depending on the definition used [11,12], making it difficult to determine the most relevant value. Indeed, some studies include only genetically proven cases of SRS, whereas others include patients born SGA with some features of SRS. Various teams have, therefore, been working toward a precise clinical definition of SRS, for the harmonization of research cohorts and, at the individual scale, to orient genetic investigations and clinical management [13–15]. Most of these studies began with the objective of distinguishing SRS from other causes of

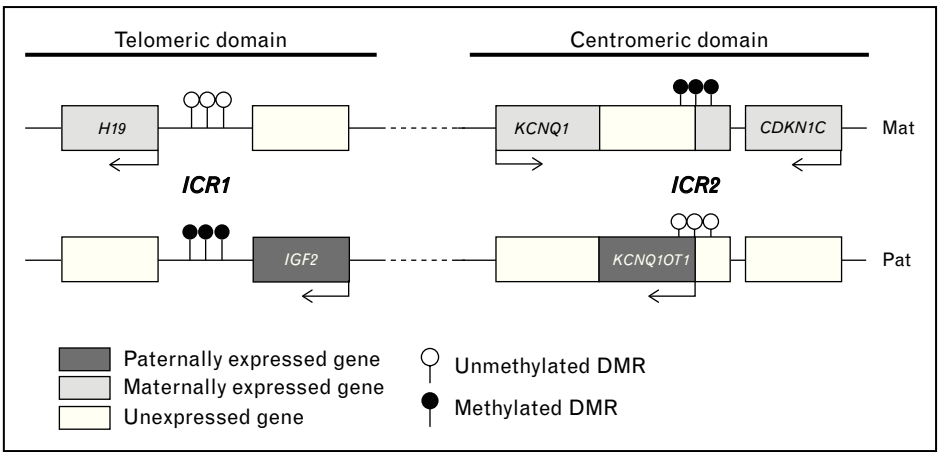

FIGURE 1. Representation of the 11p15.5 region in humans. The 'lollipops' indicate the two DMRs. Black circles indicate the methylated allele, whereas white circles indicate the unmethylated allele. DMRs, differentially methylated regions.

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SGA [16]. The most recently published score is the Netchine–Harbison clinical score system (NH-CSS), which was revised in 2015 on the basis of a prospective study [16,17▪]. According to this score, a clinical diagnosis of SRS is retained for patients displaying at least four of the following: SGA at birth, relative macrocephaly at birth, a protruding forehead in early life, body asymmetry, problems feeding and/or a low BMI at 2 years, and postnatal growth failure (Fig. 2). Other associated features include clinodactyly of the fifth finger, second–third toe syndactyly and shoulder dimples. Prominent heels and pervasive developmental disorder are more frequently observed in patients with UPD(7)mat [18]. Congenital malformations have been shown to be more prevalent in SRS patients with 11p15 LOM, and these malformations include genital abnormalities (cryptorchidism, hypospadias, Mayer–Rokitansky–Kuster–Hauser), renal, and cardiac defects [16,17▪,19,20].

MOLECULAR DEFECTS IN SILVER–RUSSELL SYNDROME

The first molecular cause of SRS to be identified was maternal uniparental disomy (UPD) — a cytogenetic defect corresponding to a double contribution of the maternal chromosome and a lack of contribution of the paternal chromosome – of chromosome 7 (UPD(7)mat), which is identified in 5–10% of patients with SRS [21]. In 2005, Gicquel *et al.* [10▪] identified LOM at the paternal ICR1 DMR as the main molecular cause of SRS, identified in up to 50% of patients with clinically diagnosed SRS patients (ICR1 LOM). This LOM leads to the biallelic expression of *H19* and a loss of *IGF2* expression, corresponding to the molecular mirror of BWS, an overgrowth syndrome in which 5–10% of patients display a gain of ICR1 methylation. Most of the molecular defects detected in patients with ICR1 LOM display mosaicism, consistent with a postzygotic event [6].

In addition to these two main mechanisms, a number of rare genetic defects have been described: (1) copy number variation (CNV) within the 11p15.5 domain, mostly involving maternal duplications encompassing the centromeric domain and the *CDKN1C* gene [22]; (2) Rare paternal deletions of enhancers in the telomeric domain, leading to lower levels of *IGF2* expression, resulting in SRS, have been described [23]; (3) gain-of-function *CDKN1C* mutations: such mutations were initially associated with IMAGe syndrome (OMIM #614732), a rare condition overlapping with SRS and combining growth retardation, metaphyseal dysplasia, adrenal insufficiency, and genital abnormalities. Brioude *et al.* [24] identified a gain-of-function mutation of the *CDKN1C* gene in a familial case of SRS with no evidence of adrenal insufficiency or bone dysplasia; (4) loss-of-function *IGF2* mutation: exome sequencing in a family including three patients with the SRS phenotype led to the recent identification of

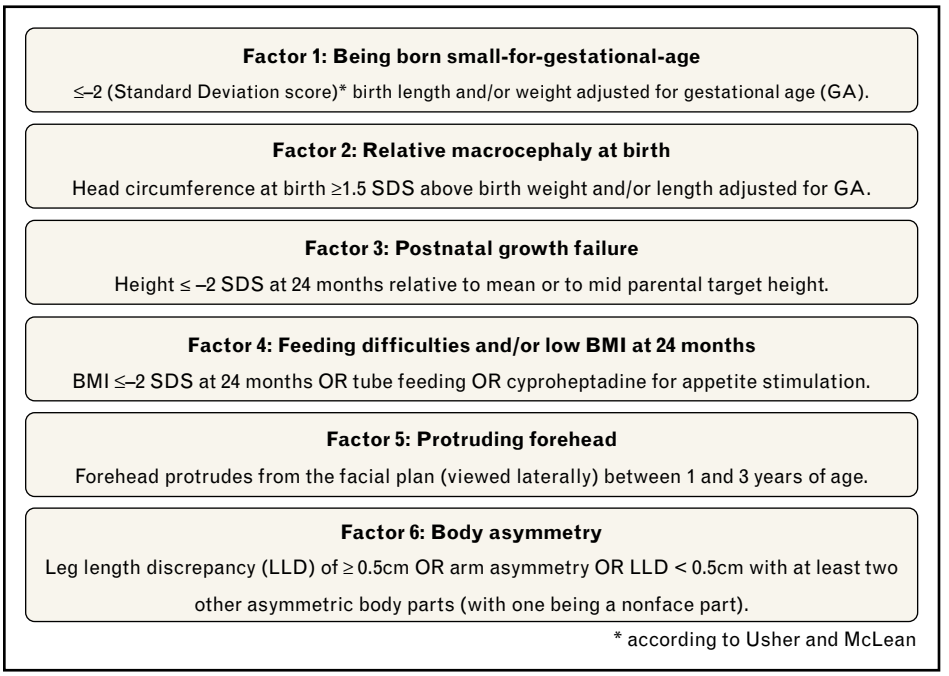

FIGURE 2. The Netchine–Harbison clinical scoring system [17▪]. Patients with four or more items of the score are suspected to have Silver–Russell syndrome and should undergo molecular testing.

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this *IGF2* mutation. No other mutation of this gene has yet been identified [25▪]; (5) CNV outside the 11p15 region and maternal UPD of other chromosomes have been identified in cohorts of patients with clinically diagnosed SRS. However, the prevalence of these defects in patients with SRS has yet to be determined [17▪]; and (6) abnormalities of chromosome 14 [imprinted locus *delta-like 1 homolog (DLK1)/maternally expressed gene 3)*]: maternal UPD of chromosome 14 UPD(14)mat, paternal deletions, and LOM at the *DLK1/GTL2* IG-DMR have recently been detected in patients with clinically diagnosed SRS [17▪,26▪]. Such defects were initially identified in Temple syndrome (OMIM #616222), a condition closely resembling SRS in which patients present pre and postnatal growth failure, neonatal hypotonia, small hands and feet, precocious puberty, and obesity [27▪].

Despite the recent identification of new molecular mechanisms, a known molecular abnormality is detected in only about 60% of patients with an NH-CSS score over 4.

MOLECULAR TESTING STRATEGIES

Molecular testing strategies have recently been reviewed in the context of a 'best practice' protocol [28]. In cases of a positive clinical diagnosis of SRS, molecular testing should be carried out, at least to assess the methylation of the DMRs of the 11p15.5 region and chromosome 7p and 7q. Methylation analyses can identify epimutations, UPDs, and deletions/duplications. However, sensitive techniques must be used, to facilitate the detection of defects displaying low rates of mosaicism.

If tests for molecular defects are negative, additional strategies should be used, depending on the clinical presentation. Differential diagnoses should be ruled out, particularly for patients with microcephaly, and appropriate molecular analyses should be performed. For patients with a clinical diagnosis of SRS (at least four of the six clinical criteria) and no molecular defects in either 11p15.5 or chromosome 7, comparative genomic hybridization or single nucleotide polymorphism array analyses could be performed to rule out rare forms of CNV. Additional techniques such as single nucleotide polymorphism arrays could be used to rule out the possibility of rare maternal UPD at other imprinted loci. Methylation of the *DLK1/MEG3* IG-DMR on chromosome 14 could be assessed, particularly for patients with features of Temple syndrome. Finally, the sequencing of *CDKN1C* or *IGF2* is indicated only in rare situations in which the family history is consistent with an imprinted gene defect (i.e., maternal or paternal transmission of the disease for *CDKN1C* and *IGF2* mutations, respectively). Given the possibility of mosaicism, molecular testing of a second sample of different origin (mostly fibroblasts) may be indicated, particularly for patients with body asymmetry.

CLINICAL MANAGEMENT

Clinical management is complex, and requires a specific multidisciplinary approach (endocrinologist, gastro-nutritionist, neurologist, orthopedic, ear, nose and throat, and maxillofacial surgeons, geneticist, psychologist) involving doctors with experience in the treatment of patients with this rare disease.

Nutrition and metabolism

SRS patients experience severe nutritional problems, beginning in the first few days of life. They often require nutritional support in early childhood, but gastrostomy should be used only for children with severe hypoglycemia or complete anorexia. Treating oromotor and/or sensory issues impacting oral intake can improve food intake. These patients also frequently suffer from atypical esogastric reflux, without vomiting, that may persist into late childhood and require long-term treatment with proton pump inhibitors. Constipation is also very frequent and patients often require long-term laxative treatment [29]. We have found that the BMI of SRS patients is very sensitive to increases in calorie intake and care should be taken to avoid the overfeeding of these patients, which might lead to cardiovascular and metabolic diseases in adulthood, as in other patients born SGA [17▪,30,31]. The nutritional goal is to maintain a BMI low for age in these children and to detect any rapid weight gain. Indeed, these children are very lean in appearance but appear to have an abnormal distribution of fat and muscle mass, with excess fat mass even if only slightly overfed.

Like other children born SGA, SRS patients tend to display metabolic disorders. Hypoglycemia and ketonuria are frequent, probably because of a number of different factors, including relative macrocephaly (increasing glucose requirement), anorexia and poor food intake resulting in poor glycogen storage, and low muscle mass [18,32]. In cases of fasting (acute diarrhea, anorexia, surgery with anesthesia, etc.), parents and doctors need to be aware of these problems, and intravenous glucose should be administered to prevent hypoglycemia. Few data are available for adults with SRS, but there have been studies of such patients displaying insulin resistance or type 2 diabetes mellitus, even without being overweight [33▪].

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Growth: Historical records suggest that SRS patients attain an adult height of about −3 Standard Deviation score (SDS) in the absence of recombinant growth hormone (rGH) treatment [30]. rGH treatment, prescribed for the indication of SGA, is reasonably effective, with a mean total height gain of +1.2 to +1.4 SDS for doses of 35–70 μg/kg/d [34–36]. These results are similar to those obtained for nonsyndromic SGA, which depend principally on the age and height of the patient at treatment initiation, and on the pubertal growth spurt. Insulin-like growth factor 1 (IGF-I) levels in response to rGH treatment are difficult to interpret in patients with SRS. Children with 11p15 LOM have significantly higher IGF-I levels (even when malnourished) than those with UPD(7)mat and other children born SGA, suggesting that they may display some IGF-I resistance [37]. The mechanism underlying these high serum IGF-I levels in SRS patients remains unknown.

As described in Prader–Willi syndrome [38], rGH treatment may have beneficial effects on glucose regulation and body composition in SRS patients and can diminish the occurrence of hypoglycemia. Like other patients born SGA, SRS patients tend to display early puberty, in terms of either chronological age or height reached by puberty, with rapid progression, resulting in an acceleration of bone age and a poor pubertal growth spurt [30]. The roles of exaggerated adrenarche or true precocious puberty in this aggressive bone maturation remain unclear. The acceleration of bone maturation has a negative impact on adult height, even in patients treated with rGH. One study of patients born SGA (including SRS patients) showed that it was beneficial to block puberty with gonadotrophin-releasing hormone agonists in patients who were short at the start of puberty, regardless of age at puberty onset [39]. No metabolic disturbance was observed in the patients treated [40]. In a recent longitudinal study, a group of children with SRS were treated with a combination of gonadotrophin-releasing hormone agonists and rGH [35]. These patients achieved adult heights similar to those observed in the children born SGA without SRS. Aromatase inhibitors may be useful for preventing rapid bone maturation in SRS patients exposed to sex hormones, but few data are available concerning the efficacy and tolerance of this treatment. A randomized clinical trial in France is currently evaluating anastrozole tolerance and the effect of this drug on bone age maturation in patients (including both girls and boys) with SRS and Prader–Willi syndrome, during adrenarche, before the onset of central puberty (NCT01520467 – clinicaltrials.gov).

Neurodevelopmental aspects

SRS patients may present delayed motor development, probably because of their lack of muscle mass and their relatively heavy heads. Furthermore, patients with UPD(7)mat are more likely to have developmental verbal dyspraxia, to display autistic traits and to develop myoclonic dystonia because of a lack of expression of the sarcoglycan epsilon (*SGCE*) gene, an imprinted gene expressed exclusively from the paternal allele, mutations of which have been shown to cause myoclonic dystonia [17▪,18,41]. Most patients attend normal schools, but some assistance may be required, particularly for SRS patients with UPD(7)mat.

Maxillofacial features

Almost all patients display microretrognathism, because of maxillary and mandibular hypoplasia. These malformations can lead to a narrowing of the upper airways, and some patients display obstructive sleep apnea. Speech therapy and mandibular distraction may be required in some cases, to improve pronunciation, chewing, and esthetic appearance [42].

Adulthood

Few clinical data are available for adult SRS patients. One of the first patients described by Searle and Johnson [33▪] is now 69 years old. He reached a final height of 154.5 cm (−3.1 SDS), and he still finds it difficult to keep his weight above 52 kg. He has normal cognitive function, but presents type 2 diabetes mellitus, hypercholesterolemia (since childhood), partial hypogonadotropic hypogonadism, and he underwent orthodontic treatment as a teenager. Follow-up to check for the various metabolic abnormalities is important during adulthood, although studies of larger adult cohorts of adult SRS patients are required to improve our knowledge and for the formulation of appropriate guidelines for adult SRS patients.

PRENATAL DIAGNOSIS

Demand for the prenatal testing of imprinting disorders is increasing, particularly in cases of early intrauterine growth retardation. Data for suspected prenatal cases of SRS and BWS from several expert centers have recently been reported [43▪]. Both false-positive and false-negative results have been reported, probably because of inappropriate sampling times or mosaicism. The indication for prenatal molecular testing should, therefore, be discussed carefully by the parents, physician, and

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molecular geneticist. In particular, any decisions regarding the outcome of the pregnancy to be taken on the basis of a positive or negative result of the molecular test should be considered beforehand, and the parents and the physician should be aware of the possibility of false-positive or false-negative results.

GENETIC COUNSELING

Genetic counseling can be difficult for imprinting diseases and should be left to physicians with training and experience in these rare disorders. Most cases of SRS are sporadic, with a theoretically low rate of recurrence within a given family. This is the case for SRS because of 11p15.5 ICR1 LOM or UPD(7)mat. In cases of SRS because of CNV, both the content of the duplicated/deleted region and the sex of the carrier should be taken into account [22]. For example, maternal transmission of a duplication of the entire 11p15.5 domain (telomeric and centromeric domains) leads to SRS, whereas paternal duplication of the same domain leads to BWS. Mutations of the *CDKN1C* gene lead to a pathological phenotype only if transmitted maternally, whereas mutations of the *IGF2* gene lead to a pathological phenotype only if transmitted paternally, in accordance with the imprinted status of these two genes [24,25▪].

CONCLUSION

Many studies characterizing the phenotype and genotype of patients with SRS have been carried out in recent years. The identification of new genetic and epigenetic abnormalities resulting in similar phenotypes, which can now be better identified with the NH-CSS, has led to the retention of clinical diagnosis for SRS. An expert consensus meeting took place at the end of 2015, with the support of international pediatric endocrinology societies, and clinical and molecular guidelines should be published soon. Nevertheless, important issues, such as the metabolic profile of patients and its change over time, IGF-I insensitivity profile, the management of puberty, and the genetic or epigenetic abnormalities in the 40% unexplained cases, remain to be addressed. International collaboration between scientists, physicians, and patient support groups will be required to progress and to bridge the gaps in our knowledge of this rare disease.

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Conflicts of interest

There are no conflicts of interest.

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4.2 Publication n°2

Roles of type 1 insulin-like growth factor (IGF) receptor and IGF-II in growth regulation: evidence from a patient carrying both an 11p paternal duplication and 15q deletion.

Éloïse Giabicani, Sandra Chantot-Bastaraud, Adeline Bonnard, Myriam Rachid, Sandra Whalen, Irène Netchine, Frédéric Brioude.

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CASE REPORT
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Roles of Type 1 Insulin-Like Growth Factor (IGF) Receptor and IGF-II in Growth Regulation: Evidence From a Patient Carrying Both an 11p Paternal Duplication and 15q Deletion

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We report an original association of complex genetic defects in a patient carrying both an 11p paternal duplication, resulting in the double expression of *insulin-like growth factor 2 (IGF2)*, as reported in Beckwith-Wiedemann syndrome, and a 15q terminal deletion, including the *type 1 IGF receptor gene* (*IGF1R*), resulting in haploinsufficiency for this gene. The patient was born with measurements appropriate for her gestational age but experienced growth retardation in early childhood, allowing a better comprehension of the IGF system in the pathophysiology of growth. It is possible that IGF-II plays a key role in fetal growth, independently of IGF1R signaling, and that its role is less important in post-natal growth, leaving IGF-I and growth hormone as the main actors.

Keywords: Beckwith-Wiedemann syndrome, IGF1 receptor, IGF-II, fetal growth restriction, imprinting disease, 11p duplication

BACKGROUND

Fetal and postnatal growth is a complex process, involving genetic, endocrine, and environmental factors. The insulin-like growth factor (IGF) system includes two ligands (IGF-I and IGF-II), two receptors [the IGF receptor type I (IGF1R) and the mannose-6-phosphate cation independent (M6PCI) receptor or IGF2R] (1). Circulating IGF-I is mainly produced by the liver and its production is stimulated by growth hormone (GH) after birth. *IGF2* is located in the 11p15 region in humans and is an imprinted gene expressed only from the paternal allele (**Figure 1**) (2). During fetal life, *IGF2* exhibits monoallelic expression and IGF-II acts as an auto/paracrine factor. After birth, circulating IGF-II is produced from both alleles by the liver, whereas *IGF2* is expressed from one allele in most other tissues and acts as a paracrine factor (3, 4). The role of circulating IGF-II after birth in humans is unclear. IGF-I and IGF-II both act through the IGF1R, which is a ubiquitously expressed tyrosine kinase receptor.

In humans, molecular anomalies of the 11p15 region have been observed in two rare diseases characterized by abnormal fetal and postnatal growth: Beckwith-Wiedemann syndrome (BWS, OMIM #130650) and Silver Russell syndrome (SRS, OMIM #180860). BWS is characterized by fetal and postnatal overgrowth, macroglossia, exomphalos, organomegaly, lateralized overgrowth, and an increased risk of embryonic tumors during early life (5). In contrast, SRS is characterized by fetal and postnatal growth retardation, with a relatively conserved head circumference at birth, hemihypotrophy, feeding difficulties, and a protruding forehead (6, 7). The 11p15 region contains two domains: the telomeric domain, containing *IGF2,* only expressed from the paternal allele, and the maternally expressed *H19* gene (a long non-coding RNA); and the centromeric domain, which includes the maternally expressed *cyclin-dependent kinase inhibitor 1C* (*CDKN1C)* gene (a negative regulator of the cell cycle, which reduces fetal growth) (**Figure 1**) (2, 8, 9). The expression of *H19* and *IGF2* is controlled by an imprinting center, called the *H19*/*IGF2* intergenic differentially methylated region (IG-DMR) (previously called IC1), which is methylated on the paternal allele. *CDKN1C* expression is controlled by a second imprinting center called *KCNQ1OT1*:TSS-DMR (or IC2), which is methylated on the maternal allele. Abnormal methylation of IC1 or IC2 or uniparental disomy can lead to abnormal expression of *IGF2* and/or *CDKN1C*, resulting in abnormal fetal/postnatal growth (2, 6). Duplications of 11p15 have been rarely reported, with either overgrowth or growth retardation, depending on the gene content and the parental origin of the duplication (9–11).

IGF1R is located on chromosome 15q26 and spans 315kb. *IGF1R* disruption (OMIM#270450) is usually responsible for fetal and postnatal growth retardation, with paradoxically high levels of plasma IGF-I (defining IGF-I resistance) and can be associated with microcephaly, variable levels of cognitive impairment, micrognathia, and feeding difficulties (12, 13). The phenotype is highly heterogeneous. In most cases, the anomaly is present in a heterozygous state, but rare homozygous or compound heterozygous mutation carriers have been reported (13–15).

We report here a patient with postnatal growth retardation and a complex chromosomal rearrangement, including a distal 15q26.3-qter deletion, encompassing the telomeric part of *IGF1R*, and a mosaic paternal duplication of the entire 11p15 region. Although the 11p duplication should have led to BWS, the patient presented with growth retardation, microcephaly, and intellectual disability, which is in accordance with the *IGF1R* disruption phenotype. We discuss the impact of these two rare genetic defects on the growth phenotype, which highlights the major role of IGF1R in IGF-II signaling.

CASE PRESENTATION

Clinical Aspects

The patient was sent to a reference tertiary center because of intellectual disability. She was born after 36 weeks of amenorrhea (WA), with birth parameters appropriate for gestational age (AGA). Her birth weight was 2380 g [−0.6 standard deviation score (SDS)]. Her birth length was not recorded, but at 1 month of age (equivalent to 40 WA), it was 50 cm (in the normal range). Her head circumference at birth was 30 cm (−2.4 SDS). She was born from unrelated healthy parents of Romanian origin. The mother is 162 cm (−0.2 SDS) and the father 170 cm (−0.8 SDS) tall; both had birth parameters AGA. The target height was 159.5 cm (−0.7 SDS) and there was no familial history of short stature. The growth curve is shown in **Figure 2**. At the age of 8 years and 9 months, her height was 117.8 cm (−2.1 SDS), weight 24.5 kg [body mass index (BMI) of 17.7 kg/m² (1.2 SDS)], and head circumference 47.5 cm (−3.2 SDS). She had no clinical signs of BWS according to the consensus clinical scoring system proposed in 2018 (5). Only two out of four items for the clinical scoring system for *IGF1R* defects were present (16). She presented with strabismus and interventricular communication, with no cardiac failure. She acquired motor skills with normal timing but experienced an early delay in language and cognitive development and required specialized education.

Biological Aspects

At the age of 3 years, her serum IGF-I level was in the upper range of the norm (145 ng/ml, 0.9 SDS), with elevated basal GH (45 mUI/L). At 8 years and 9 months, her hormonal status was as follows: IGF-I 345.3 ng/mL (2.1 SDS), IGF-binding protein (IGF-BP3) 4,638 ng/mL (normal range from 2,146 to 5,801 ng/mL), acid-labile subunit 2,145 mU/mL (normal range from 813 to 1,729 mU/mL) and IGF-II 710 ng/mL (normal range from 433 to 997 ng/mL). These data are in favor of IGF-I resistance with high levels of IGF-I and ALS.

Molecular Aspects

Karyotype analysis revealed a mosaic karyotype 45,XX,dic(15;21)(q26.3;q10)[4]/46,XX[20] with two cell lines: (1) a 45,XX cell line with a dicentric chromosome dic(15;21) due to an apparently balanced mosaic structural rearrangement involving one chromosome 15q and one chromosome 21p (**Figure 3A** left) and (2) a 46,XX cell line. The karyotypes of the parents were normal.

Single-nucleotide polymorphism (SNP) array revealed a more complex unbalanced autosomal structural rearrangement than expected from the karyotype analysis: (1) no copy number variation for chromosome 21, (2) a 3 Mb homogeneous heterozygous 15qter deletion, and (3) a 13.4 Mb mosaic 11pter duplication in approximately 30% of the cells analyzed (**Figure 3B** from top to bottom) (10).

Subsequent fluorescent *in situ* hybridization (FISH) analysis with specific subtelomeric 11p, 15q, and 21q probes showed a supernumerary signal for 11p at the end of a deleted 15qter chromosome in ∼30% of cells, accounting for an unbalanced translocation t(11p;15q), with a normal signal on the two telomeres of 21q (**Figures 3A** right, **E**). In approximately 20% of cells, one signal for the chromosome 21q telomere was located at the end of the deleted 15qter chromosome with a normal signal for the two 11p telomeric probes, accounting for an unbalanced translocation t(15q;21q) (**Figures 3A** left, **C**). In the remaining cells, with a normal signal on telomeres 11p and 21q, only one signal from the telomeric 15q probe was detected, corresponding

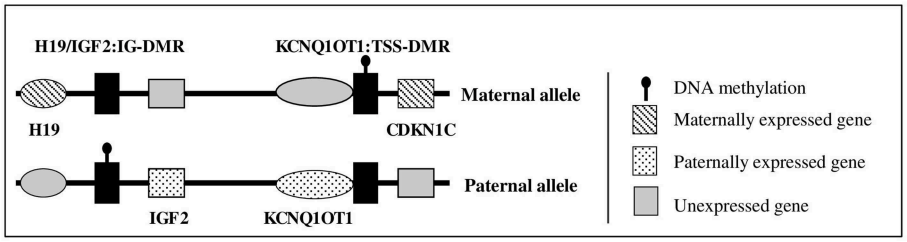

FIGURE 1 | Schematic representation of 11p15 region gene expression.

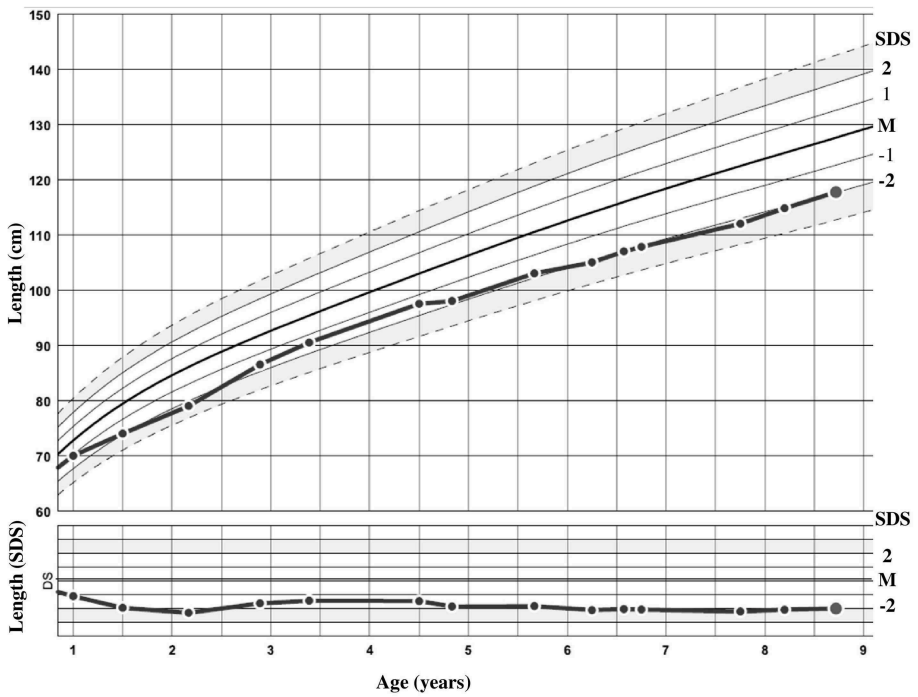

FIGURE 2 | Growth curve of the patient in centimeters and standard deviation score (SDS).

to a 15q deletion (**Figures 3A** middle, **D**). Thus, the karyotype of the patient was finally amended to ISCN 2016 as *de novo* mos 45,XX,dic(15;21)(q26.3;q10)/46,XX.ish.der(15)(11;15)(p15.2;q26.3)(RP11-889I17+,D15S936-)/46,XX.ish.del(15)(q26.3)(D15S936-).arr[hg19]11p15.5p15.2(204,062-13,618,804)x3[0.3],15q26.3(99,434,357-102,461,162)x1dn.

Thus, the patient's mosaic included three abnormal cell lines: the first (30%) with 46 chromosomes and a chromosome 15 derived from an unbalanced translocation between the chromosome 11p region and the terminal 15q26 region of chromosome 15; the second (20%) with 45 chromosomes and an unbalanced dicentric chromosome derived from both chromosomes 21 and 15, with a 15q26 terminal deletion, and the third (50%) with 46 chromosomes that only carried the 15q26 terminal deletion of one chromosome.

Methylation studies (using methylation-specific multiplex ligation dependent probe amplification after bisulfite treatment of DNA) of the 11p15 locus revealed a partial gain of methylation of the *H19/IGF2*:IG-DMR (methylation index of 55; normal range 46 à 51) and a partial loss of methylation of the *KCNQ1OT1*:TSS-DMR (methylation index of 43; normal range 48- 53), in favor of a paternal origin of the 11p duplication (17).

DISCUSSION

We report a patient with a complex chromosomal rearrangement which includes both *IGF2* and the *IGF1R*. In this case, two rare conditions coexist: a mosaic 11p15 paternal duplication, which usually leads to overgrowth (BWS), and a 15qter deletion including *IGF1R*, which usually leads to fetal and post-natal growth restriction. The major role of IGF-I and IGF–II as major actors in the control of fetal growth, through their binding to the IGF1R, has been known for years. Indeed, in humans, genetic defects of *IGF1* and the *IGF1R* or alterations in *IGF2* expression, through genetic or epigenetic mechanisms (SRS) leads to fetal

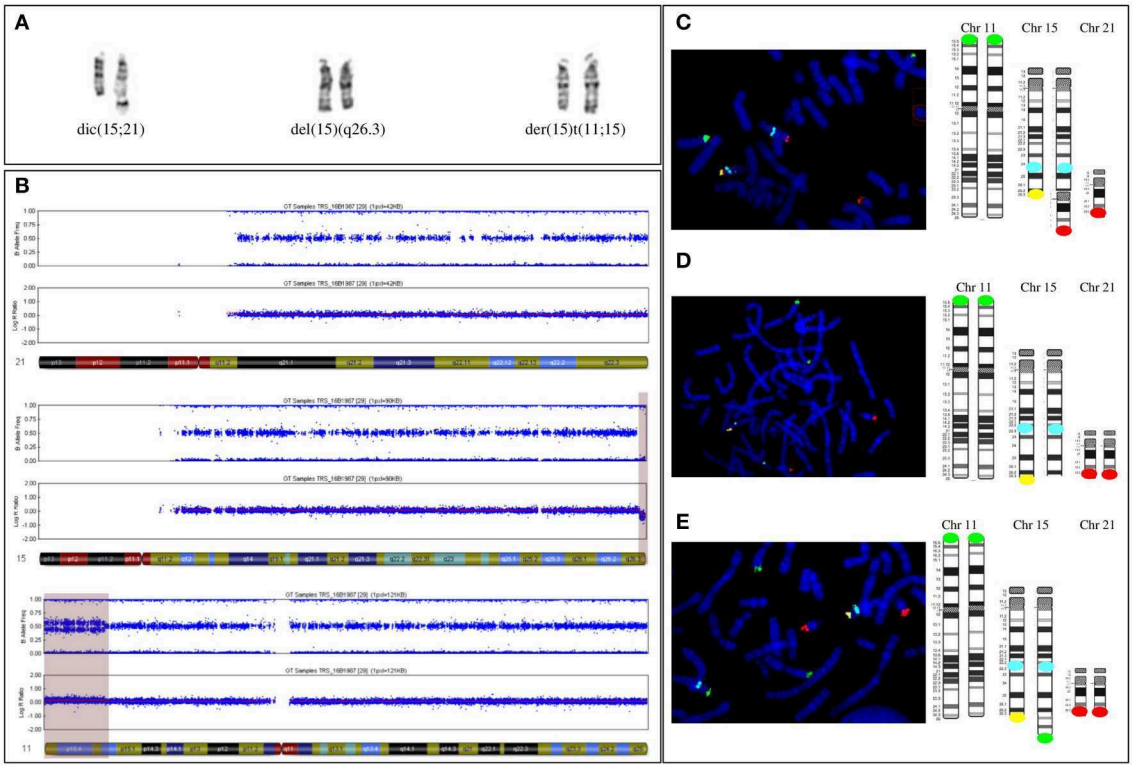

FIGURE 3 | (A) Paired homologous chromosomes15 with Derivative chromosome 15 (left couple, on the right) and apparently normal 15 G-banded chromosomes 15 (middle couple) and (right couple). The two apparently normal chromosomes were then shown to carry a 15q terminal deletion (middle couple, on the right) and be a derivative chromosome 11 (right couple, on the right). **(B)** The SNP array profile shows a normal chromosome 21 (top), a heterozygous homogeneous 15q terminal deletion (middle, light purple area), and a mosaic gain on chromosome 11p (bottom, light purple area). **(C)** FISH [with a specific 11p subtelomeric probe (green), specific 15q22 probe (aqua blue), specific 15q subtelomeric probe (yellow), and specific subtelomeric 21q probe (red)] and schematic representation showing chromosomes 11, 15, and 21 in the three cell lines. First cell line with an unbalanced 15q;21p translocation. **(D)** Second cell line with a deleted 15q chromosome. The derivative chromosome 15 is missing a yellow signal. **(E)** Third cell line with an unbalanced 11p;15q translocation. The derivative chromosome 15 has a supernumerary green signal and no yellow signal.

growth restriction (16–18). Conversely, overexpression of *IGF2* (BWS) or the *IGF1R* lead to overgrowth (19, 20).

Moreover, murine models of invalidation of these genes confirmed these observations, as knockout models for either *Igf1*, *Igf2,* or *Igf1r* present with growth restriction at birth (21, 22). Interestingly, the double knockout for *Igf1* and *Igf2* or *Igf2* and *Igf1r* are smaller at birth than the knockout for *Igf1r,* whereas double knockouts for *Igf1* and *Igf1r* are the same size as the *Igf1r* knockout mice. These data suggest that, conversely to IGF-I, which only interacts with the IGF1R, IGF-II may also act through other signaling pathways (22).

In humans, many findings have highlighted the role of IGF-I in stimulating postnatal growth: patients with GH deficiency or GH resistance [Laron (OMIM#262500) or Noonan (OMIM#163950) syndromes] have extremely low levels of IGF-I and present with growth failure after birth (12). Conversely, patients with acromegalogigantism (because of GH pituitary adenoma) have extremely high levels of IGF-I and tall stature.

The role of IGF-II in postnatal growth is unclear. Indeed, SRS patients with a loss of methylation at the *H19/IGF2*:IG-DMR present with persistent post-natal growth failure, despite normal circulating IGF-II levels (23, 24). Such normal circulating IGF-II levels may be due to the biallelic expression of *IGF2* from a non-imprinted promotor in the liver. However, *IGF2* is still imprinted in other tissues, and loss of methylation at 11p15 leads to the loss of *IGF2* expression in these tissues (4, 17). Thus, these plasmatic levels of IGF-II do not reflect the local levels and activity of IGF-II. 11p15.5 paternal duplications have been recently reviewed (10). In such duplications, either growth retardation (SRS), overgrowth (BWS), or a normal phenotype can be observed, depending on the extent of the duplication and the parental origin of the duplicated allele. Usually, duplications of the paternal telomeric domain of 11p15 leads to *IGF2* overexpression and thus BWS, whereas a gain of *CDKN1C* expression, due to maternal duplication of the centromeric domain of 11p15, leads to SRS (10). In our patient, we demonstrate that the duplicated 11p15 allele was of paternal origin and encompassed both IC1 and IC2. Thus, *IGF2* is likely overexpressed in the contingent of cells with the 11p15 duplication. However, in this patient plasmatic IGF-II levels were within the normal range which could be secondary to a different mosaicism in the liver with a lower rate of cells carrying the 11p15 duplication.

The growth retardation, microcephaly, developmental delay, and high plasma levels of IGF-I observed in our patient are more concordant with the *IGF1R* deletion phenotype. Here, fetal growth was not affected, despite *IGF2* overexpression and the coexisting *IGF1R* defect. These two opposite mechanisms may compensate each other and finally lead to normal birth parameters. Nevertheless, we should be cautious when speculating on the respective role of genetic anomalies impact here since we do not know the exact proportion of mosaicism in the different tissues. Information on placenta, for example, would have been of major interest since IGF-II and IGF1R play a major role on placental development and function (25). It reinforces the hypothesis that IGF-II can signal through a pathway that is independent of the IGF1R, at least during fetal life (22). Finally, *in vitro* studies have also shown a stronger affinity of IGF-I than IGF-II for IGF1R and IGF1R is activated with lower concentrations of IGF-I compared to IGF-II (26, 27).

The growth retardation our patient experienced suggests that the IGF1R defect prevails over IGF-II overexpression after birth. Indeed, post-natal growth retardation with a biological IGF-I resistance profile is concordant with the predominant dysfunction of the IGF1R. In patients with low *IGF2* expression (SRS), the fetal growth restriction is obvious, whereas their growth velocity is usually unaffected after birth (despite no catch-up) (23). Conversely a comparison of patients with BWS shows birth length to generally be greater in patients with *IGF2* overexpression (gain of methylation in IC1), whereas height during childhood is usually greater in patients with an isolated IC2 loss of methylation (no *IGF2* overexpression) (19). This favors a predominant role of IGF-II in fetal rather than in post-natal growth. For the patient reported here, another explanation for the normal measurements at birth may lie in the mosaicism presented by the patient, since all her circulating cells carried the *IGF1R* deletion, but only approximately 30% had the 11p duplication. This mosaicism may vary across various tissues, and the growth plate may have a different proportion of cells with the 11p duplication.

Patients with *IGF1* or *IGF1R* defects usually present with microcephaly, which distinguishes them from SRS patients, for whom head circumference is relatively conserved at birth. This would favor a major role of IGF-I in cerebral development over that of IGF-II (28). Another possibility is the absence of maternal imprinting of *IGF2* in brain, as several studies found either biallelic expression in different cerebral regions or, more recently a maternal expression (4, 21, 29). In the latter case, *IGF2* would not be overexpressed in the brain of the patient (because of the paternal origin of the duplication) conversely to the other tissues. Thus, *IGF1R* haploinsufficiency would have led to the microcephaly, in accordance with previous observations of *IGF1R* disruption.

CONCLUSION

We report here an original association of multiple chromosomal rearrangements involving *IGF2* and *IGF1R*, two critical genes involved in the regulation of fetal and post-natal growth regulation. It favors the predominance of IGF-II during fetal growth and IGF-I during post-natal growth.

ETHICS STATEMENT

A written informed consent was obtained from the parents of the participant for the publication of this case report.

AUTHOR CONTRIBUTIONS

EG, SC-B, and FB shared writing of the manuscript. AB, MR, and SC-B performed the molecular tests and analysis. SW and IN participated in the discussion and revisions during the writing process.

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4.3 Publication n°3

Diagnosis and Management of Postnatal Fetal Growth Restriction.

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Diagnosis and management of postnatal fetal growth restriction

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Fetal growth restriction (FGR) can result from multiple causes, such as genetic, epigenetic, environment, hormonal regulation, or vascular troubles and their potential interaction. The physiopathology of FGR is not yet fully elucidated, but the insulin-like growth factor system is known to play a central role. Specific clinical features can lead to the identification of genetic syndromes in some patients. FGR leads to multiple global health concerns, from the perinatal period, with higher morbidity/mortality, through infancy, with neurodevelopmental, growth, and metabolic issues, to the onset of puberty and later in life, with subfertility and elevated risks of cardiovascular and kidney diseases. Adequate follow-up and therapeutics should be offered to these patients. We first review the main molecular etiologies leading to FGR and their specificities. We then highlight the main issues that FGR can raise later in life before concluding with the proposed management of these children.

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Introduction

The definition of fetal growth restriction (FGR) is still debated and generally includes children with intra-uterine growth restriction (IUGR) and/or those born small for their gestational age (SGA). In 2001, an international consensus defined being SGA as having a birth weight and/or length >2 standard deviations score (SDS) below the mean for gestational age [1–3]. Nevertheless, this definition is still not universally accepted and numerous studies use low birth weight (LBW) (<2500 g), irrespective of the term, or a weight and/or length at birth below the 10th percentile [4,5]. The diagnosis of IUGR is made by ultrasound during pregnancy and an international consensus recently precisely defined FGR [6,7]. The diagnosis of IUGR is confirmed either by solitary criteria (fetal abdominal circumference or estimated fetal weight $\leq$ 3rd percentile) or contributory criteria (multiple abnormal Doppler measurements) [6]. Thus, IUGR and SGA are not strictly synonymous. Indeed, a fetus can experience FGR during a period of the pregnancy and then have normal dimensions at birth, even if perinatal growth was low for a period; on the contrary, a neonate can be SGA because of very late FGR missed by prenatal monitoring. Obstetricians are concerned about the perinatal morbidity and mortality that result from FGR, whereas endocrinologists consider these patients with potential long-term effects of FGR. Because of the conflicting definitions of FGR and the confusion between SGA and IUGR, published cohort studies are heterogeneous and their outcomes are difficult to compare.

Here, we will consider these children as being born SGA (weight and/or length $\geq$ 2 SD below the mean), because measurements at birth are more reliable than ultrasound determination of term and growth *in utero* and because publications mostly include or concern SGA patients. It is likely that neonates born appropriate for gestational age (AGA), but with FGR, may have similar long-term problems as those born SGA, despite the difficulty to diagnose and follow them.

Prevalence of SGA has not been well established. Indeed, in 2013, Lee *et al.* found an extremely variable rate of children born SGA in 138 low or middle-income countries, from 5.3 to 41.5% [8]. In industrialized countries, only the prevalence of LBW is known (estimated between 5 and 10%) and there are no global data reported concerning SGA, except for a few national registries [9,10].

Diagnostic orientation

Fetal growth is a complex physiological process, including genetic or epigenetic, endocrine, and environmental factors. Environment includes maternal factors, such as maternal nutrition or exposure to toxic or infectious elements. Fetal growth is also influenced by exchange between the mother and fetus through the placenta [7]. Here, we will focus on (epi)genetic and endocrine mechanisms that can affect fetal growth. However, this list cannot be exhaustive, as more than 150 genetic disorders have been associated with FGR (HPO:0001511, http://compbio.charite.de/phenomizer_orphanet/).

The IGF system has been known for years to be a main actor in the control of fetal growth. It includes two ligands (IGF-I and IGF-II), binding proteins (IGF binding protein 1–6 (IGFBP-1 to -6)) and the acid labile subunit (ALS), and two receptors [the IGF1R and the mannose-6-phosphate cation independent receptor (M6PCI)]. IGF-I and IGF-II can both promote fetal growth through activation of the IGF1R, whereas M6PCI is involved in the clearance of IGF-II. Insulin can also bind to the IGF1R to promote fetal growth [11]. During fetal life, IGF-I is not regulated by the somatotropic axis. Therefore, molecular defects upstream of the growth hormone receptor do not usually (or only slightly) affect fetal growth.

In rodents, inactivation of the *igf1*, *igf2*, or *igf1r* genes leads to FGR [12,13]. Inactivation of *igf2* only results in FGR when the inactive allele is transmitted by the male, because of the imprinted character of *igf2*. Imprinting is defined by the monoallelic expression of a gene depending on its parental origin. Imprinting is controlled by epigenetic mechanisms, especially DNA methylation of differentially methylated regions (DMRs), in which maternal and paternal alleles are differentially methylated. Approximately 100 genes are imprinted in humans, most of which are involved in growth, metabolism, or development [14].

In humans, mutations in the *IGF1* or *IGF1R* genes have long been identified in children with pre- and postnatal growth retardation. Aside from growth restriction, these children generally have a low head circumference and may have sensorineural defects and mental retardation [15–17]. Mutations of the

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paternally-inherited *IGF2* gene (located at chromosome 11p.15) have been described only very recently in patients with Silver–Russell Syndrome (SRS), in which children present with pre- and postnatal growth restriction, severe feeding difficulties, and a relatively normal head circumference [18]. *IGF2* mutations are rare in SRS patients, and most patients show hypomethylation of the paternal *H19/IGF2*:IG-DMR, leading to the loss of expression of *IGF2* [19]. More recently, mutations in oncogenes (*PLAG1* and *HMGA2*), known to be upstream regulators of *IGF2*, have been identified in children born SGA, confirming the essential role of this pathway in the control of fetal growth [20]. Conversely, hypermethylation at the maternal *H19/IGF2*:IG-DMR leads to Beckwith-Wiedemann syndrome, an overgrowth syndrome with an increased risk of embryonic tumors [21].

Several other loci are imprinted (www.geneimprint.com/) and (epi)genetic defects within these loci lead to several imprinted diseases frequently associated with growth and/or metabolic disorders (Table 1). Children with Temple syndrome (TS14) present a phenotype very close to that of SRS, with severe obesity and early puberty [22,23]. TS14 is due to genetic or epigenetic defects within the 14q32.2 locus, which includes the paternally expressed *Delta Like Non-Canonical Notch Ligand 1* (*DLK1*) gene. DLK1 participates in several differentiation processes, including adipogenesis, osteogenesis, and neuroendocrine differentiation, and plays an important role in fetal growth [24]. During pregnancy, maternal circulating DLK1 is of fetal origin, and fetus-derived DLK1 is necessary for maternal metabolic adaptation to pregnancy [25].

SRS is clinically defined based on a clinical scoring system [19]. However, many conditions can clinically overlap with SRS and TS14 [23]. It is critical to make an accurate diagnosis, because for some of these conditions, some treatments may be contraindicated, such as growth hormone replacement (see below). Differential diagnoses of SRS have been recently reviewed, with some specific traits besides FGR. In cases of FGR, head circumference and early feeding difficulties are cornerstone traits to distinguish between these various conditions (Table 1). Furthermore, relative macrocephaly will expose to a high risk of hypoglycemia, which should be carefully monitored.

Neonatal nutrition and metabolic consequences

The concept of the early origins of adult disease was first developed by David Barker in the late 1980s [31]. He observed that cardiovascular risk, as well as the risk of diabetes and metabolic syndrome in adulthood, was elevated in LBW individuals. This resulted in the Barker hypothesis, in which restricted *in utero* nutrition permanently alters tissue structures and functions, and hence metabolism, increasing the risk of cardiovascular and metabolic disorders in adulthood [32]. This hypothesis was subsequently challenged by the “rapid catch-up growth” hypothesis, suggesting that LBW *per se* does not increase the risk of metabolic syndrome, but only in LBW infants who experience rapid catch-up growth during the first years of life. Many studies have provided evidence for one or the other hypothesis. Recently, a systematic review showed that, although LBW increased the risk of metabolic syndrome later in life, rapid postnatal catch-up growth of LBW neonates was a more important factor than LBW alone for the development of metabolic syndrome. In this review, 79.6% of all cardiovascular risk factors (blood pressure, insulin resistance, hypertriglyceridemia, LDL and HDL cholesterol levels, etc.) reported in studies of the rapid catch-up growth hypothesis were statistically significant, whereas the corresponding figure was 58.5% for the effect of LBW alone [33]).

LBW may be due to various reasons, including preterm birth and IUGR. Infants born SGA, but with no evidence of FGR, do not have an increased metabolic risk. They remain lighter and shorter than those born AGA, have a similar percentage of body fat, and show no differences in metabolic or hormonal parameters up to two years of age [34]. LBW infants who are born preterm have an increased risk of neurological impairment and poor postnatal growth increases the risk of adverse neurological outcomes. Although adequate postnatal nutrition associated with catch-up growth confers potential advantages for later cognitive outcome, it may also increase the risk of metabolic disease later in life. A systematic review investigating the relationship between postnatal growth rates, cognitive outcomes, and the risk of disease later in life in preterm infants reported consistent positive associations between postnatal weight or head growth and neurocognitive outcomes. However, it also reported limited evidence linking postnatal weight gain to later adiposity and cardiovascular disease risk factors [35]. The authors concluded that more research is needed to determine how to optimize postnatal nutrition

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Table 1
Several syndromes with FGR and comparative phenotypes concerning head circumference and early feeding difficulties. Imprinted genes are labelled with an *. For imprinted genes, $: transmission can depend on the gender of the transmitter. # Extreme caution must be given to avoid hypoglycemia in this subgroup of patients.

	Silver Russell syndrome	Temple syndrome	3-M syndrome	Mulibrey syndrome	Floating Harbor syndrome	Bloom syndrome	Fanconi syndrome	*IGF1*/*IGF1R* mutations/ deletions
OMIM	#180860	#616222	#273750	#253250	#136140	#210900	#227650	#608747 #270450
Genes	Hypomethylation of *H19*/IGF2**: IG-DMR Maternal UPD of chr 7 Mutations of *IGF2*, PLAG1, HMGA2, or CDKN1C**	Maternal UPD of chr 14 Hypomethylation/ deletion of *DLK1*/MEG3*:IG-DMR*	Mutations of *CUL7, CCDC8, OBSL1*	Mutations of *TRIM37*	Mutations of *SRCAP*	Mutations of *BLM*	Mutations of more than 20 genes (see reference)	Mutations of *IGF1R* *IGF1*
Transmission	Usually sporadic Autosomal dominant for mutations[$]	Usually sporadic Autosomal dominant for deletions[$]	Autosomal recessive	Autosomal recessive	Autosomal dominant	Autosomal recessive	Autosomal recessive or X-linked	Autosomal dominant for *IGF1R* Autosomal recessive for *IGF1*
Head circumference at birth	Relatively conserved compared to birth weight/length [#]					Microcephaly		
Feeding difficulties during early infancy	+++	+++	Usually absent	Usually absent	+	+	Absent	+
References	[19]	[22,23]	[26]	[27]	[28]	[29]	[30]	[17]

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and growth in preterm infants to achieve a neurocognitive benefit, while minimizing the risk of later cardiovascular and metabolic diseases.

The effect of rapid catch-up growth may be considered separately for full-term SGA infants, as the risks and benefits may differ from those of preterm infants. Only two randomized interventional studies have evaluated the effect of early growth promotion in full-term SGA infants on neurodevelopment, fat mass, and blood pressure [36,37]. Full-term infants born SGA were randomized into two groups: nutrient-enriched formula, which promoted early growth (28% more protein than standard formula) or standard formula. There was no difference in neurocognitive development between the two groups at 18 months. Enriched formula increased total body fat mass at the age of five to seven years and rapid catch-up growth was associated with increased blood pressure at six to eight years [38,39]. There have also been many observational studies that reported an association between postnatal growth and metabolic outcome in full-term SGA infants, but they were performed across diverse geographical regions and studied various markers of metabolic and cardiovascular health. Overall, they reported a positive association between faster postnatal weight gain and increased adiposity in infancy until the age of 21 [40]. Nevertheless, SGA infants generally have less adipose tissue than AGA. Only two studies reported that abdominal fat mass at the age of one to four years was positively associated with faster growth [41,42]. Fat distribution may be more informative than total fat mass to determine whether LBW infants accumulate greater visceral adiposity, exposing them to an adverse metabolic outcome. Most observational studies have reported an association between faster postnatal weight gain in full-term SGA infants and elevated blood pressure. Despite those highest blood pressure levels, compared to AGA children, blood pressure in SGA infants remains in the normal range [40]. At the age of four years, SGA children who experienced rapid catch-up growth during the first year of life had preserved insulin sensitivity, but lower insulin secretion in response to glucose stimulation, suggesting impairment of β-cell function [43].

Many systematic reviews have shown that breast feeding may protect against the long-term risk of developing obesity, but no study has focused on the effects of breast feeding on infants born SGA. Recommendations for neonatal nutrition have been published to optimize the growth of preterm infants, but there is no standardized nutritional protocol for full-term SGA infants. In view of current data, enriched aggressive neonatal nutrition for those infants may not be appropriate, but regular growth monitoring is important. Based on the data of a prospective cohort including 1957 full-term SGA infants, the recommended optimal growth trajectory may be to reach approximately the 30th percentile in the first postnatal months and the 50th percentile by the age of seven months [44]. Further studies are needed to establish the optimal nutritional regimen for SGA infants that promotes safe short-term outcomes and minimizes long-term risks.

Growth

Most children born SGA reach normal height, but approximately 10% do not [10,45,46]. The adult height (AH) of those who do not catch-up is reduced by approximately 4.5 cm for men and 4 cm for women [47]. Most catch-up during the first year of life, but it can take longer for preterm infants or patients with very LBW [48]. Furthermore, growth is retarded during the first months of life of preterm infants and such postnatal growth failure is more severe for babies born more preterm [48]. The so-called extra-uterine growth retardation can catch-up later in life or result in reduced AH [49].

Little is known about the mechanisms involved in growth failure in children with FGR. Some syndromes can be identified in a few, usually by extensive molecular analysis. Thus, this group of SGA patients who do not catch-up represents a very heterogeneous population in most publications. The effect of recombinant growth hormone (rGH) has been well established in various randomized *versus* placebo clinical trials [50–52]. These studies resulted, in 2001 in the USA, in the recommendation to treat with rGH children born SGA who did not catch-up from two years of age with a daily dose of 70 μg/kg (American Food Drug and Administration). This indication is different in Europe, where rGH is indicated since 2003 in children born SGA who have a height SDS ≤ -2.5 at four years and are 1 SDS below their target height and have a growth velocity below the mean (European Agency for the Evaluation of Medicinal Products) with a daily dose of 33 μg/kg [3]. Ranke *et al.* reported a dose-dependent response to rGH in children born SGA, together with a greater benefit associated with

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the duration of treatment [53]. The authors showed that growth gain within the first year of treatment was highly predictable of the long-term response to rGH treatment. Another study provided support for starting rGH treatment early, reporting a better response for treatment started at least two years before pubertal onset [50]. Measurement of serum IGF-I levels during rGH therapy in children has been used as a marker for treatment adherence. It has also been used to titrate rGH doses to obtain the upper limit of normal IGF1 levels, improving growth velocity in children with GH deficiency and idiopathic short stature [54–56]. However, this approach is not recommended for FGR children, as they may have some degree of associated GH and IGF-I insensitivity and their IGF-I levels are not linked to their growth velocity [57]. Tolerance to rGH treatment has been evaluated in numerous studies and it does not appear to worsen long-term cardiovascular risk, even if it impairs glucose tolerance during treatment, which must be monitored [3,58,59]. A few studies even showed a protective effect on blood pressure and body composition [59,60].

In syndromic causes of FGR, such as SRS, catch-up growth is very rare, and the recent international consensus has recommended starting rGH treatment early (at approximately two years) with the lowest possible dose to allow catch-up growth [19,61]. This recommendation is justified by the beneficial metabolic effects of GH (glycemia regulation, fat/muscle mass repartition) in these patients, as shown for those with Prader–Willi syndrome [62,63]. The European Medicines Agency has not officially authorized this treatment, as rGH treatment is only authorized from four years of age in Europe, whereas it is authorized from two years in the USA. Basal IGF1 serum levels in the upper quartile of the normal age-related range, or higher, can be expected in children with SRS, especially those with 11p15 epimutations. Thus, IGF-I serum levels are not used to adjust rGH treatment in this group of children [19,64].

Other causes of FGR with no catch-up, associated with chromosomal instability, should be considered with caution. These include Bloom syndrome, Nijmegen breakage syndrome, and Fanconi disease, as well as other syndromic causes of FGR which can contraindicate the use of rGH. In cases of IGF-I resistance, higher doses of rGH can be justified, for example in patients with *IGF1R* anomalies, or treatment with recombinant IGF-I for patients with *IGF1* mutations.

Pubarche and puberty

Pubarche

Since the late 90s, many studies have reported a relationship between FGR and precocious pubarche. A study of pubarche in children born SGA in the ALSPAC cohort in the United Kingdom confirmed the link between high levels of Dehydroepiandrosterone sulfate (SDHEA) and LBW [65]. Indeed, rapid weight gain was associated with aggressive adrenarche. These data were confirmed in other cohorts [66–68]. Veening *et al.* reported that high levels of SDHEA persist throughout puberty in adolescents born SGA and that they have a shorter period of puberty and more aggressive adrenarche than adolescents born AGA [69].

Puberty

The onset of puberty occurs early in patients born SGA and evolves rapidly, enhancing the risk of short AH [70–72]. Numerous authors have established an association between metabolic problems, such as weight gain or insulin resistance, and the early manifestation of puberty or adrenarche [71,73,74].

The Dutch SGA study reported the benefit of treating patients born SGA who entered puberty when still small (140 cm) with Gonadotropin Releasing Hormone (GnRH) agonists concomitantly with rGH [75,76]. Patients born SGA, under rGH therapy during the prepubertal stage because of absence of sufficient postnatal catch-up, were given GnRH agonists for two years if the onset of puberty occurred when their height was below 140 cm. The authors compared the AH of the patients treated with GnRH agonists with those that did not require it (those not below 140 cm in height at the onset of puberty). The two groups had similar AHs due to a greater pubertal growth spurt in the patients who received GnRH agonists. The same group later showed that there was good long-term metabolic tolerance to the

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co-administration of rGH and GnRH agonists [77]. In accordance with these studies, the recent international consensus recommends considering a two-year course of personalized GnRH agonist treatment for SRS patients with a poor AH prognosis at the onset of puberty [19].

Neuro-developmental aspects

The assessment of neuro-developmental difficulties in patients born SGA is challenging because of the presence of confounding factors, such as maternal psychopathology, socio-economic status, or more widely, environmental factors, as well as occasionally the underlying mechanisms of FGR [78,79]. Furthermore, the patients studied are often not well characterized in terms of FGR and many studies have focused on LBW neonates with no information concerning the term at birth. Nevertheless, some studies have shown that FGR is associated with an increased risk of psychopathology [80–82]. A recent review that summarized 16 studies reported neuro-developmental delay during the three first years of life in children born SGA in 11 of these prospective studies [81]. The most important concern is attention deficit hyperactivity disorder, which is associated with LBW [82,83]. A recent cohort study highlighted the higher frequency of adult mental disorders, such as schizophrenia, among LBW neonates, as well as an increased risk of alcohol/drug abuse, anxiety, or somatoform disorders [84]. The physiopathology is still unknown, but some have suggested that glucocorticoid levels during pregnancy could play a crucial role in fetal growth and neurodevelopment [82]. The consequences, in terms of brain alterations, are well described by Miller *et al.* who suggest that brain sparing during FGR is not perfect and results in microstructural alterations of the brain, impairing both grey and white matter [85]. These data should encourage pediatricians to monitor for early psychomotor or language delay to encourage early management of this group of children.

Adulthood

The consequences of FGR can also continue into adulthood. Although neonates born SGA and followed for 26 years showed no long-term social or emotional consequences in a large cohort in the United Kingdom, there were slight differences [10]. More individuals born SGA were referred to special education as teenagers, occupied fewer managerial jobs at 26 years of age, and had lower incomes than adults born AGA [10].

Few studies have focused on fertility in adults born SGA. The first concerns arose in 1997 when Francois et al. reported an increased proportion of LBW males in a cohort of males consulting for subfertility [86]. These results were confirmed by an Italian study showing reduced testicular size, lower testosterone and inhibin B levels, and higher LH levels in 25 males born SGA than in a control group of males born AGA [87]. At the same time, Ibañez *et al.* reported an increased risk of polycystic ovary syndrome in LBW females who experienced precocious pubarche. They suggested a major role of insulin resistance in the pathogenesis of ovarian dystrophy and thus performed a double blind randomized, placebo-controlled pilot clinical trial, showing the positive effect of Metformin in insulin sensitivity and central adiposity in a small cohort of 23 patients born SGA with postnatal catch-up [88]. These data have never been replicated in a larger cohort and should be still considered experimental.

Another less well-known impact of FGR is the emergence of chronic kidney disease and hypertension in adults. The LBW and Nephron Number Working Group recently published a consensus statement on this issue [89]. They recapitulate that LBW, growth restriction, and preterm birth can result in low nephron numbers and be associated with an increased risk of hypertension, proteinuria, and kidney disease later in life [90]. Furthermore, rapid catch-up in the early years of life worsens nephron reduction in these children. The risk of end-stage kidney disease is elevated, with a hazard ratio of 1.41 (1.05–1.90) for adults born SGA at term and rises to 4.02 (1.79–9.03) for adults born SGA and preterm [91]. The recommendations in this consensus are: not to overfeed neonates born SGA during infancy to avoid rapid catch-up, obesity, and type 2 diabetes mellitus; to monitor for hypertension and kidney function during childhood; and to pay attention to kidney donors born SGA, who are more at risk to develop chronic kidney disease later on [89].

Please see Fig. 1 for a global overview of outcomes and the management of patients with FGR throughout their lives.

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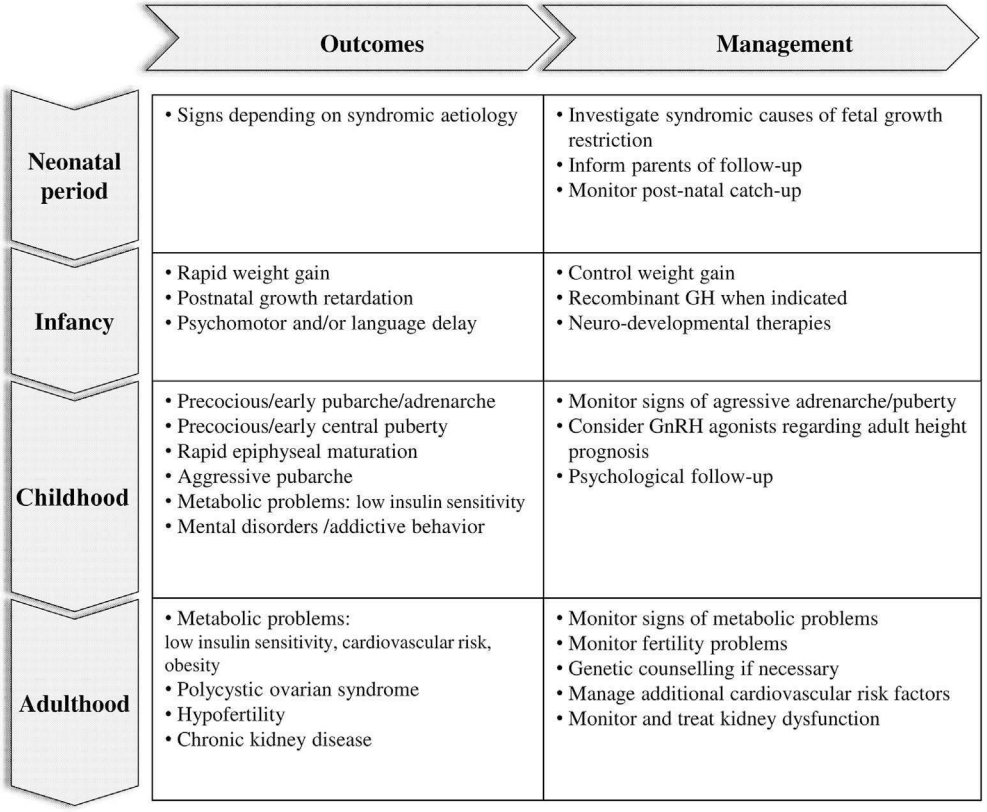

Fig. 1. Outcomes and their management in children born with a fetal growth restriction throughout their lives.

Summary

Fetal growth restriction (FGR) is multifactorial and can lead to adverse multi-systemic outcomes. The fetal programing and catch-up theories encourage close monitoring of the nutritional status of these patients. Genetic or epigenetic causes are responsible for this phenotype for some patients and they should be screened in the first years of life to propose and optimize management. Recombinant growth hormone treatment is now a well-recognized therapeutic option to improve adult height and global metabolic status. The onset of pubarche and puberty can occur early and rapidly progress, leading to a poor prognosis for adult height. Several studies on therapies, such as GnRH agonists, are available and encourage its use in specific and personalized situations. Finally, adults who experienced FGR should be closely followed, mostly for cardiovascular diseases, but also for fertility problems and chronic kidney disease. These potential late effects of FGR all justify early diagnosis, close follow-up, and adequate early intervention for these patients.

Conflicts of interest

FB: speaker or chair in meetings promoted by Ipsen Pharma, Lilly, Sandoz.

IN: speaker or chair in meetings promoted by Merck Serono, Sandoz and research support from Pfizer, Merck Serono.

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Practice points

- Syndromic genetic causes of fetal growth restriction (FGR) should be sought in light of dysmorphic, clinical, or biological parameters, because of its consequences on care.
- Attention should be given to rapid weight catch-up within the first two years of life. Rapid catch-up is a risk factor for cardiovascular and metabolic disease in adulthood.
- Growth hormone is a recommended treatment. Age at onset, height, and dose are different in the USA and in Europe.
- Pubarche and puberty occur early in this group of patients and can rapidly progress. Personalized GnRH agonist treatment can be considered to preserve growth in case of poor AH prognosis at the onset of puberty.
- Psychomotor and cognitive development should be assessed, and early intervention proposed if needed.
- Cardiovascular risk is elevated and metabolic disorders are frequent in patients with FGR and must be adequately followed. Exercise and good nutrition must be encouraged.

Research agenda

- Determine hormonal and non-hormonal mechanisms involved in fetal growth regulation.
- Determine the mechanisms leading from impaired fetal growth to metabolic disturbances.
- Determine optimized postnatal nutrition to achieve a neurocognitive benefit while minimizing the risk of later cardiovascular and metabolic diseases.
- Develop the personalized prediction of recombinant GH efficacy.
- Perform prospective randomized long-term evaluation of GnRH agonists and other bone maturation protection therapies.

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4.4 Publication n°4

Chromosome 14q32.2 imprinted region disruption as an alternative molecular diagnosis of Silver-Russell Syndrome.

Sophie Geoffron, Walid Abi Habib, Sandra Chantot-Bastaraud, Béatrice Dubern, Virginie Steunou, Salah Azzi, Alexandra Afenjar, Tiffanny Busa , Ana P Canton, Christel Chalouhi, Marie-Noëlle Dufourg, Blandine Esteva, Mélanie Fradin, David Geneviève, Solveig Heide, Bertrand Isidor, Agnès Linglart, Fanny Morice-Picard, Catherine Naud-Saudreau, Isabelle Oliver-Petit, Nicole Philip, Catherine Pienkowski, Marlène Rio, Sylvie Rossignol, Maithé Tauber, Julien Thevenon, Thuy-Ai Vu-Hong, Madeleine D Harbison, Jennifer Salem, Frédéric Brioude, Irène Netchine, Éloïse Giabicani.

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Chromosome 14q32.2 Imprinted Region Disruption as an Alternative Molecular Diagnosis of Silver-Russell Syndrome

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Abbreviations: aGNRH, gonadotrophin-releasing hormone analog; BMI, body mass index; GH, growth hormone; IG-DMR, intergenic differentially methylated region; LOM, loss of methylation; MLMD, multilocus methylation defect; NH-CSS, Netchine-Harbison clinical scoring system; SDS, standard deviation score; SGA, small for gestational age; SRS, Silver-Russell syndrome; TS, Temple syndrome.

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Context: Silver-Russell syndrome (SRS) (mainly secondary to 11p15 molecular disruption) and Temple syndrome (TS) (secondary to 14q32.2 molecular disruption) are imprinting disorders with phenotypic (prenatal and postnatal growth retardation, early feeding difficulties) and molecular overlap.

Objective: To describe the clinical overlap between SRS and TS and extensively study the molecular aspects of TS.

Patients: We retrospectively collected data on 28 patients with disruption of the 14q32.2 imprinted region, identified in our center, and performed extensive molecular analysis.

Results: Seventeen (60.7%) patients showed loss of methylation of the *MEG3/DLK1* intergenic differentially methylated region by epimutation. Eight (28.6%) patients had maternal uniparental disomy of chromosome 14 and three (10.7%) had a paternal deletion in 14q32.2. Most patients (72.7%) had a Netchine-Harbison SRS clinical scoring system ≥4/6, and consistent with a clinical diagnosis of SRS. The mean age at puberty onset was 7.2 years in girls and 9.6 years in boys; 37.5% had premature pubarche. The body mass index of all patients increased before pubarche and/or the onset of puberty. Multilocus analysis identified multiple methylation defects in 58.8% of patients. We identified four potentially damaging genetic variants in genes encoding proteins involved in the establishment or maintenance of DNA methylation.

Conclusions: Most patients with 14q32.2 disruption fulfill the criteria for a clinical diagnosis of SRS. These clinical data suggest similar management of patients with TS and SRS, with special attention to their young age at the onset of puberty and early increase of body mass index. (***J Clin Endocrinol Metab* 103: 2436–2446, 2018**)

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Impaired fetal growth is associated with an increased risk of perinatal morbidity and mortality and metabolic problems later in life, according to the Developmental Origins of Health and Disease theory (1). Imprinted regions are known to play an important role in fetal growth (2). Paternally expressed genes are mostly involved in growth promotion, whereas maternally expressed genes repress it. Most imprinted regions are methylated on the maternal allele. In humans, only two regions are methylated in the male germ line (3, 4), the 11p15 *H19/IGF2* intergenic differentially methylated region (IG-DMR) and the 14q32.2 *MEG3/DLK1*:IG-DMR, involved in Silver-Russell syndrome (SRS)/Beckwith-Wiedemann syndrome, and Temple syndrome (TS)/Kagami-Ogata syndrome, respectively.

SRS is characterized by fetal and postnatal growth retardation and feeding difficulties (5–9). Epimutation, resulting in the loss of methylation (LOM) of *H19/IGF2*:IG-DMR on the paternal allele, is identified in 50% of SRS cases (10–12). In this region, the imprinting center *H19/IGF2*:IG-DMR is methylated on the paternal allele, resulting in *IGF2* expression. When unmethylated, as on the maternal allele, it allows *H19* expression, a long noncoding RNA (Fig. 1). The key role of *IGF2* in prenatal growth is well-established. Maternal uniparental disomy for chromosome 7 [upd(7)mat] is seen in ~5% to 10% of patients with SRS (13). However, for 35% to 40% of patients with SRS, the molecular etiology remains unknown.

TS, first clinically and molecularly described in 1991, associates fetal and postnatal growth retardation, hypotonia, obesity, and early puberty (14). TS is caused by disruption of the 14q32.2 imprinted region, where *MEG3/DLK1*:IG-DMR is methylated on the paternal allele. *MEG3/DLK1*:IG-DMR methylation results in *DLK1*, *RTL1*, and *DIO3* expression, whereas long noncoding RNAs (MEG3 and MEG8), microRNAs, and small nucleolar RNAs are expressed when it is unmethylated (as on the maternal allele) (Fig. 1). In a meta-analysis of 51 patients with TS, the molecular anomalies identified consisted mostly of maternal uniparental disomy of chromosome 14 [upd(14)mat] (78.4%), epimutation of *MEG3/DLK1*:IG-DMR on the paternal allele (11.8%), and paternal deletion of the *MEG3/DLK1* domain (9.8%) (15). A cohort of 32 patients with

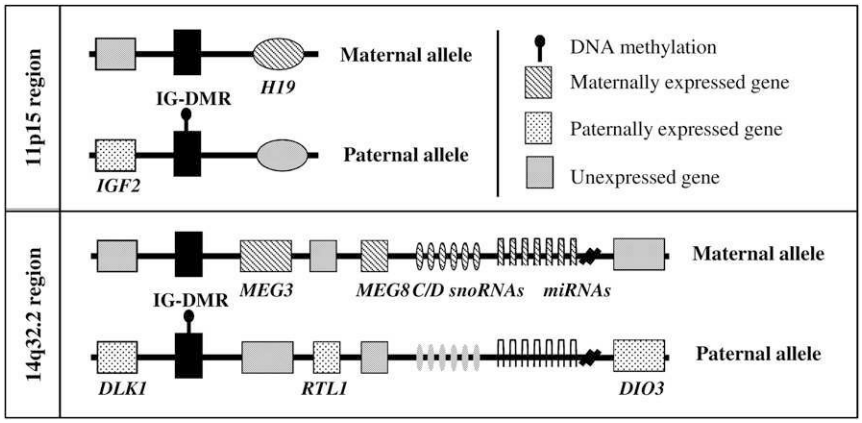

Figure 1. Scheme of normal 11p15 and 14q32.2 chromosomal regions. *DIO3*, iodothyronine deiodinase 3; *DLK1*, delta-like homolog 1; *H19*, long noncoding RNA; *IGF2*, insulin-like growth factor 2; *MEG3, MEG8*, maternally expressed genes 3 and 8; *RTL1*, retrotransposon-like 1;snoRNAs, small nucleolar RNAs; miRNAs, microRNAs.

14q32.2 anomalies has recently been reported and, again, most had upd(14)mat (71.9%), whereas only 18.8% had epimutations (16). Clinical overlap between SRS and TS has been previously highlighted in reports of patients presenting with a clinical diagnosis of SRS with no 11p15 disruption or upd(7)mat, but for whom chromosome 14q32.2 anomalies were identified (17–19). Thus, these syndromes overlap in terms of phenotype and may be caused by anomalies of imprinted regions sharing similar molecular organization, both methylated in the male germ line.

We identified 28 patients with chromosome 14q32.2 disruption in our molecular diagnostic laboratory. The purpose of this study was to clinically and molecularly characterize these patients to determine the clinical overlap with patients with SRS. Furthermore, we sought to identify the mechanism involved in the onset of 14q32.2 epimutation.

Patients and Methods

Study population

The study population consisted of 28 patients with chromosome 14q32.2 disruption. The molecular diagnosis of 25 patients was performed in our laboratory and three upd(14)mat were identified without methylation analysis in other diagnostic laboratories. All patients were either followed in our clinic or were referred by other clinical centers for molecular analysis. A clinical file, including extensive clinical data, growth charts, a detailed phenotypic description, and pictures was completed for all patients. Each patient had been examined by a geneticist and/or a pediatric endocrinologist. Written informed consent for participation was received either from the patients themselves or their parents, in accordance with French national ethics rules for patients recruited in France (Assistance Publique – Hôpitaux de Paris authorization no. 681) and with the institutional review board I00000204 of the Mount Sinai School of Medicine, New York, for patients recruited in the United States.

Clinical assessment and definitions

The Netchine-Harbison clinical scoring system (NH-CSS), recently adopted as the clinical definition of SRS by the first international consensus on this syndrome (9, 11), was applied to each of the 28 patients. This scoring system defines a suspicion of SRS if at least four of the six following criteria are met: (1) being born small for gestational age (SGA) [birth weight and/or birth length ≤ -2 standard deviation score (SDS) for gestational age], (2) postnatal growth failure (height at 24 $\pm$ 1 months ≤ -2 SDS or height ≤ -2 SDS from midparental target height), (3) relative macrocephaly at birth (head circumference at birth $\geq$1.5 SDS above birth weight and/or length SDS), (4) protruding forehead (forehead projecting beyond the facial plane on a side view as a toddler), (5) body asymmetry [leg length discrepancy $\geq$0.5 cm or arm asymmetry or leg length discrepancy $<$0.5 cm with at least two other asymmetrical body parts (one nonface)], and (6) low body mass index (BMI) (BMI ≤ -2 SDS at 24 months) and/or feeding difficulties defined by the use of a feeding tube and/or cyproheptadine for appetite stimulation. See the Supplemental Materials and Methods for auxologic methods.

Premature pubarche was defined by the appearance of pubic or axillary hair occurring before eight years in girls and nine years in boys (20). Precocious puberty was defined by breast development (thelarche) before age 8 years in girls and testicular enlargement before age 9 years in boys (21). Exaggerated adrenarche was defined by high levels of serum dehydroepiandrosterone sulfate for age (after other diseases were excluded) (22).

Molecular analysis

Methylation studies at both 11p15 *H19/IGF2*:IG-DMR and *MEG3/DLK1*:IG-DMR loci are described in the Supplemental Data. All patients had hypomethylation at *MEG3/DLK1*:IG-DMR. We distinguished three different mechanisms: upd(14)mat, deletion, and LOM by epimutation.

Single nucleotide polymorphism microarray analysis

We analyzed the DNA samples using Illumina CytoSNP-12 arrays (Illumina, San Diego, CA) to distinguish between *MEG3/DLK1*:IG-DMR epimutation, upd(14)mat, and large copy number variations. See the Supplemental Materials and Methods for details.

IG-DMR and exome variant sequencing

Whole-exome sequencing

Library preparation, exome capture, sequencing, and data analysis were performed by IntegraGen SA (Evry, France). The sequencing methods and bioinformatics analysis are detailed in the Supplemental Materials and Methods.

Statistical analysis

The characteristics of the population are described as percentages for qualitative variables or as SDS and mean (range) for

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continuous variables. For subgroup comparisons, we used the Wilcoxon Mann-Whitney test and the Fischer test.

Results

Patients

Our cohort was composed of 28 patients (17 girls). Three patients (10.7%) were conceived with the aid of medically assisted procreation: two *in vitro* fecundations and one intrauterine insemination. The median maternal age was 29.1 (19.8–41.5) years and paternal age was 30.7 (25.8-44.8) years.

Molecular diagnosis

Classical molecular anomalies found in SRS [*i.e.,* 11p15 epimutation and upd(7)mat] were ruled out for all but three patients with upd(14)mat not identified in our laboratory. All patients presented with chromosome 14q32.2 hypomethylation at the *MEG3/DLK1*:IG-DMR, which was secondary to upd(14)mat in eight (28.6%) patients or to a paternal deletion of *DLK1/MEG3* region in three (10.7%), whereas 17 (60.7%) had *MEG3/DLK1*:IG-DMR LOM caused by epimutation on the paternal allele. This was ascertained after ruling out a upd(14)mat or deletion of the *DLK1/MEG3* region by single nucleotide polymorphism microarray.

Clinical features

The median age at the end of the study was 7.5 (1.3 to 21.6) years. Birth parameters, postnatal growth, dysmorphic anomalies, psychomotor development, and associated malformations are summarized in Table 1. Dysmorphic features such as protruding forehead, prominent heel, tented appearance of the mouth, and acromicria are presented in Supplemental Fig. 1.

Eight of the 23 patients, for whom data were available, were treated with recombinant growth hormone (GH) therapy from a mean age of 4.7 (1.1 to 11.3) years according to the SGA indication and posology (23).

NH-CSS

Among patients for whom all items of the NH-CSS were available, 72.7% (16/22) had a score ≥4/6, and consistent with a clinical diagnosis of SRS. One item was missing in six patients, of whom two had an NH-CSS score of 4/5 (compatible with a diagnosis of SRS), one had a score of 3/5 and three had scores of 2/5, which does not fulfill the criteria for a clinical diagnosis of SRS (Supplemental Fig. 2). Among the six patients who did not fulfill the NH-CSS criteria, five (83.37%) had an epimutation and one a deletion (case 26).

Puberty and pubarche

We collected data on puberty and pubarche for all patients but one (one girl for whom data were not available). At the end of the study, 11 patients had gone into puberty, eight girls and three boys; the oldest among the other nonpubertal patients was an 8.7-year-old girl. Of these 11 patients, six (54.5%) had precocious puberty, including five (62.5%) girls and one (33.3%) boy. Four (66.7%) had epimutations and two (33.3%) had upd(14)mat. Puberty occurred early for the other three girls, before age 9 years, and was rapidly progressive, with menarche <1 year after breast development for two of them. Puberty also started early for the other two boys, at 10.0 and 10.2 years (Table 2). Six (54.5%) patients were treated with gonadotrophin-releasing hormone analogs (aGnRHs) to suppress puberty at a mean age of 7.9 (5.0 to 10.3) years. Six (54.5%) patients had exaggerated adrenarche; four among them were treated with cyproterone acetate.

Epiphyseal fusion occurred early in four patients, at 12.7 (11.2 to 13.8) years in girls (n = 3) and 13.8 years in one boy, without aGnRH treatment. The mean final height of the girls (n = 4) was 143.5 (141.0 to 145.0) cm, corresponding to −3.6 (−4.0 to −3.3) SDS, according to Sempé (24), with a mean pubertal growth spurt of 12.8 (10.0 to 17.0) cm without aGnRH and 25.5 cm for the girl who was treated. One boy had a final height of 150.0 cm (−3.9 SDS), with a pubertal growth spurt of 19.3 cm; the second had a final height of 169 cm (−0.8 SDS), far from his target height (+2.7 SDS). None of these patients received recombinant GH treatment.

All clinical data concerning puberty and pubarche of these 11 patients are summarized in Supplemental Table 1.

Metabolic outcomes

The age of adiposity rebound was precocious for 93.8% (15/16 for whom data were available) of the patients, with a mean at 2.1 (1.0 to 6.5) years. Twelve patients (75.0%) had adiposity rebound by the age of 2 years. Among patients with precocious adiposity rebound, only one needed enteral feeding. For this patient, nutrition intake is on the decrease but she experienced complete anorexia. For all other patients, BMI had spontaneously grown precociously. The BMI of all patients for whom puberty had started increased markedly (>1 SDS) before the onset of pubarche and/or central puberty.

Extensive molecular analysis

Methylation analysis of 18 imprinted loci

We studied the methylation levels at 18 imprinted loci, using TaqMan allele-specific methylated multiplex real-time quantitative polymerase chain reaction, as previously described (19), and methylation specific multiplex ligation-dependent probe amplification (for chromosomes 6, 7, and 14) for 23 patients (Fig. 2). We found that 58.8% of patients with epimutation of the *MEG3/DLK1*:IG-DMR had methylation defects within at least one of the other studied loci. The upd(14)mat group, as well as one patient with a paternal deletion (case

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Table 1. Main Clinical Features of the Cohort

	All Patients			Epimutations (1)		
	n (%)	Mean (Min–Max)	<−2 SDS (%)	n (%)	Mean (Min–Max)	<−2 SDS (%)
Birth						
Term (wk of amenorrhea)	28	37.5 (28.0–40.0)		17	37.2 (28.0–40.0)	
Birth length (SDS)	28	−2.3 (−5.2 to 0.8)	16 (57.1)	17	−2.3 (−5.2 to −0.9)	9 (52.9)
Birth weight (SDS)	28	−2.4 (−4.0 to −0.7)	24 (85.7)	17	−2.4 (−4.0 to −0.9)	14 (82.4)
Intrauterine growth retardation	25/28 (89.3)			15/17 (88.2)		
Birth head circumference (SDS)	25	−1.3 (−3.7 to 1.0)		15	−1.3 (−3.7 to 1.0)	
Relative macrocephaly	14/25 (56.0)			7/15 (46.7)		
Growth						
Height at 2 y (SDS)	25	−2.0 (−4.4 to −0.2)	14/25 (56.0)	15	−2.0 (−4.4 to −0.2)	9 (60.0)
BMI at 2 y (SDS)	24	−1.5 (−3.2 to 0.0)	8 (33.3)	15	−1.4 (−3.2 to 0.0)	5 (33.3)
Early feeding difficulties	17/28 (60.7)			9/17 (52.9)		
NH-CSS						
NH-CSS ≥4	16/22 (72.7)			9/14 (64.3)		
Clinical signs						
Protruding forehead	25/28 (89.3)			17/17 (100)		
Body asymmetry	7/26 (26.9)			6/17 (35.3)		
Acromicria	19/26 (73.1)			12/17 (70.6)		
Downturned mouth	18/24 (75.0)			12/15 (80.0)		
Low-set posteriorly rotated ears	14/25 (56.0)			11/16 (68.8)		
Clinodactyly	16/26 (61.5)			10/16 (62.5)		
Development						
Neonatal hypotonia	14/20 (70.0)			5/10 (50.0)		
Motor delay	16/25 (64.0)			8/15 (53.3)		
Speech delay	12/23 (52.2)			7/15 (46.7)		
Normal schooling	13/19 (68.4)			10/12 (83.3)		
Behavioral disorders	3/23 (13.0)			2/15 (13.3)		
Associated malformations						
Urogenital[a]	5/23 (21.7)			3/12 (25.0)		
Stomatology[b]	7/21 (33.3)			6/13 (46.2)		
Heart[c]	2/17 (11.8)			0/9 (0.0)		

(*Continued*)

25), had normal methylation levels at all other studied loci, as expected for patients with a cytogenetic defect of the 14q32.2 region. We did not perform these analysis for two patients with deletions and the three with upd(14)mat not identified in our laboratory.

Screening for regulatory cis*-element defects*

We searched for large deletions or duplications of the 14q32.2 region, using Illumina CytoSNP-12 arrays and multiplex ligation-dependent probe amplification to identify a potential *cis*-element defect within the *MEG3/DLK1*:IG-DMR and the *MEG3*:TSS-DMR that could lead to epimutations. No genetic defects were identified. We then looked for point mutations or small insertions/deletions by sequencing the entire *MEG3/DLK1*:IG-DMR and *MEG3*:TSS-DMR. We identified a homozygous variation (C>A) within the IG-DMR at NC_000014.8:g.101274313C>A in one patient. All other copy number variations that we found were already reported in public single nucleotide polymorphism databases, making it highly unlikely that they disturb any regulatory *cis*-elements of either DMR.

Screening for regulatory trans*-acting factors*

We carried out extensive mutation screening by whole exome-sequencing to look for *trans*-acting factor defects that could be involved in the establishment, maintenance, or reading of DNA methylation marks and/or related to imprinting disorders for all patients with epimutations. We first looked for heterozygous or homozygous missense/nonsense mutations or frameshifts insertions/deletions in a common defective gene or different genes from the same family. Several genes were mutated in at least four patients, but their function or cellular localization made them very unlikely to be involved in the hypomethylation of the *MEG3/DLK1*:IG-DMR. We then examined genes encoding proteins known to be involved in the establishment/maintenance of DNA methylation marks related to imprinting, along with some of their binding proteins. The variations found within genes that could be involved in the hypomethylation defect are presented in Table 3. We validated these variations by Sanger sequencing and determined their parental transmission.

Discussion

Here we report clinical and molecular data on a large cohort of 28 patients with 14q32.2 imprinted region disruptions. The underlying mechanism for most of the patients (60.7%) is epigenetic, with a hypomethylation at

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Table 1. Main Clinical Features of the Cohort (Continued)

Upd(14)mat (2)			(1) vs (2)	Paternal Deletion		
n (%)	Mean (Min–Max)	<−2 SDS (%)	*P*	n (%)	Mean (Min–Max)	<−2 SDS (%)
8	37.6 (36.0–39.0)		1	3	38.7 (37.0–40.0)	
8	−2.4 (−3.7 to 0.8)	6 (75.0)	0.37	3	−1.6 (−2.2 to −1.2)	1 (33.3)
8	−2.8 (−3.3 to −2.3)	8 (100)	0.22	3	−2.0 (−3.1 to −0.7)	2 (66.7)
8/8 (100)			1	2/3 (66.7)		
7	−1.5 (−2.0 to −0.7)		0.46	3	−0.7 (−0.6 to −0.7)	
5/7 (71.4)			0.73	2/3 (66.7)		
8	−1.8 (−4.1 to −0.6)	3 (37.5)	0.56	2	−2.5 (−2.6 to −2.4)	2/2 (100)
7	−1.8 (−2.9 to −0.6)	3 (42.8)	0.27	2	−1.7 (−1.9 to −1.4)	0
6/8 (75.0)			0.71	2/3 (66.7)		
5/5 (100.0)			0.71	2/3 (66.7)		
6/8 (75.0)			0.76	2/3 (66.7)		
1/6 (16.7)			1	0/3 (0)		
6/7 (85.7)			1	1/2 (50.0)		
5/7 (71.4)			1	1/2 (50.0)		
2/7 (28.6)			0.44	1/2 (50.0)		
5/8 (62.5)			1	1/2 (50.0)		
6/7 (85.7)			0.70	3/3 (100)		
5/7 (71.4)			0.72	3/3 (100)		
2/5 (40.0)			1	3/3 (100)		
3/5 (60.0)			1	0/2 (0.0)		
1/5 (20.0)			0.5	0/3 (0.0)		
1/8 (12.5)			1	1/3 (33.3)		
1/5 (20.0%)			0.64	0/3 (0.0)		
1/6 (16.7%)			0.44	1/2 (50.0)		

Abbreviations: max, maximum; min, minimum.

[a]Bicornurate uterus (n = 1), renal agenesis (n = 1), bilateral cryptorchidism (n = 2), nephrocalcinosis (n = 1).

[b]Multiple agenesis (n = 3), crowded teeth (n = 3), delayed tooth eruption (n = 2).

[c]Aneurysm of the interatrial septum (n = 2), interatrial communication (n = 1).

the paternal *MEG3/DLK1*:IG-DMR, whereas it has been reported to be between 11.8% and 18.8% in recent publications, consisting mostly of upd(14)mat (15, 16). We have described as many 14q32.2 epimutations as those already published (n = 17; Table 4).

Epimutations at 14q32.2 have been rarely reported, and the mechanisms responsible for their occurrence are unknown. We therefore extensively studied the molecular pattern of the 17 patients with epimutations. We first looked for genetic disruption of *MEG3/DLK1*:

Table 2. Characteristics of Pubarche and Puberty for 11 Patients >9 Years of Age

	Total	Girls (n = 8)	Boys (n = 3)
Pubarche			
Premature pubarche	3/8 (37.5%)	2/6 (33.3%)	1/2 (50.0%)
Exaggerated adrenarche	6/10 (60.0%)	4/7 (57.1%)	2/3 (66.7%)
Age at pubarche onset, y		8.6 (6.3–12.0)	9.0 (8.0–10.0)
Bone age advancement during puberty	9/9 (100.0%)	8/8 (100.0%)	1/1 (100.0%)
Central puberty			
Age at thelarche or testicle enlargement, y	—	7.2 (4.0–8.5)	9.6 (8.6–10.2)
Age at menarche, y[a]	—	10.2 (8.9–12.0)	—
Delay between thelarche and menarche, y[a]	—	1.9 (0.5–3.5)	—
BMI			
At pubarche onset (SDS)	2.1 (0.2–6.1)	2.1 (0.2-6.1)	2.3 (1.0–3.6)
At central puberty onset (SDS)	1.9 (−0.9 to 6.1)	1.8 (−0.9 to 6.1)	2.4 (1.1–3.6)

[a]Without GnRH analogs.

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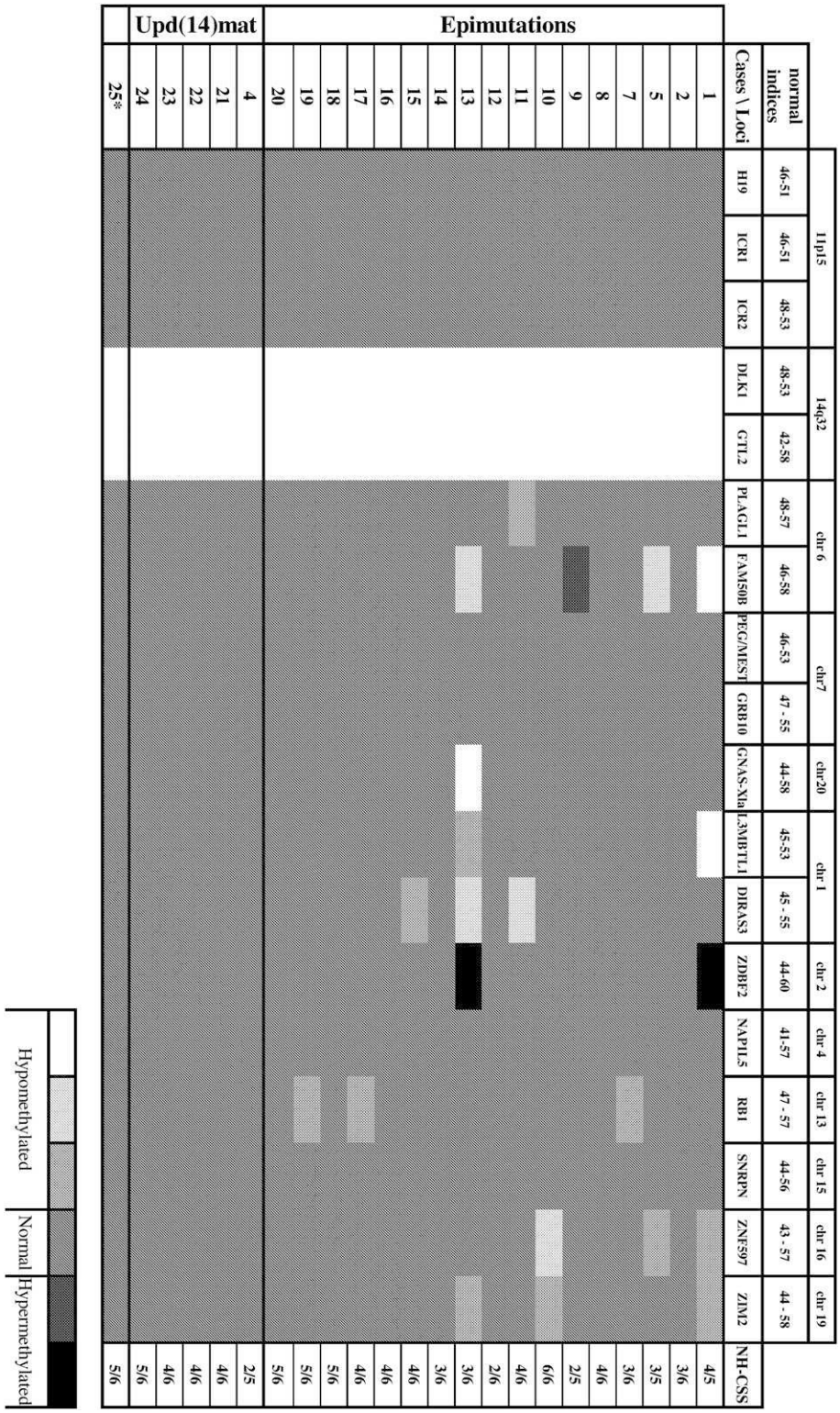

Figure 2. Multilocus methylation analysis for 23 patients together with their NH-CSS score. *Patient with a chr14 paternal deletion.

IG-DMR; however, we found no relevant sequence anomaly within this DMR. A large proportion of these patients (58.8%) had multilocus methylation defects (MLMDs). MLMDs have already been reported in patients with SRS with epimutations of *H19/IGF2*:IG-DMR and Beckwith-Wiedemann syndrome (another imprinted disorder leading to overgrowth secondary to abnormal methylation of the 11p15 region), although in much lower proportions, at 10% and 25%, respectively (25). The high proportion of our patients with MLMDs prompted us to look for genetic

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Table 3. Whole-Exome Sequencing for Proteins Involved in the Establishment and Maintenance of DNA Methylation at DMRs

Protein	Alternative Name(s)	Function	Phenotype From Protein Deficiency	Exome Sequencing Results	Patient	POLYPHEN-2 Prediction
KAP1	TRIM28, TIF1β, KRIP1	KRAB-associated protein 1, scaffold protein for heterochromatin factors	Stochastic loss of DNA methylation at multiple gDMRs	Paternal variation P429T (rs138696546)	Case 2	Possibly damaging
UHRF1	NP95, ICBP90	Binds to hemimethylated DNA, recruits DNMT1	Somatic loss of DNA methylation, including at imprinted loci	Paternal variation in *UHRF1BP1* (R827H)	Case 14	Possibly damaging
RBBP1; RBBP1L	ARID4A, ARID4B	RB binding proteins	Somatic loss of DNA methylation at *Snrpn* gDMR	Maternal variation in *ARID4A* (Y394C)	Case 13	Probably damaging
SETDB1	—	ZFP57/TRIM28 recruit SETDB1 to methylate H3K9me3, and the cofactor UHRF1 that recruits DNMT1o and DNMT1 to maintain 5mC within imprinted gDMRs	Lack of imprint establishment in germ cells	Compound heterozygous variations R1074C (rs147846533) and R1165Q (rs373907289)	Case 9	Both probably damaging

Abbreviations: gDMR, germinal differentially methylated region; POLYPHEN-2, Polymorphism Phenotyping, version 2.

anomalies in *trans*-acting factors involved in the regulation of specific parental methylation at imprinted loci. However, we did not find any commonly disrupted genes in these patients, and only four patients had variations within genes known to be involved in the establishment or maintenance of methylation. Two of these patients, for whom we identified two paternally inherited variants in the *KAP1* (rare variant) and *UHRF1BP1* (unreported variation) genes, showed no evidence of MLMD, making it unlikely that these candidate genes are involved in the hypomethylation process of *MEG3/DLK1*:IG-DMR. Another patient (case 13), with MLMD affecting six loci aside from 14q32.2, inherited an unreported maternal variation in the *ARID4A* gene, which has been shown to be involved in the maintenance of methylation at the *Snrpn* locus in mice. Finally, the fourth patient inherited a rare compound heterozygous variant of the *SETDB1* gene, which is also a key player in the methylation process of imprinted genes. This patient showed hypomethylation only at 14q32.2 and no evidence of MLMD. These variations, as well as the ~60% of MLMDs identified among these patients, and the absence of *cis*-regulatory element defects within either *MEG3/DLK1*:IG-DMR or *MEG3*:TSS-DMRs, strongly

Table 4. Comparison of Main Clinical Data Between Our Cohort of Patients With 14q32.2 Anomalies and Previously Published Cases

	Our Cohort, n (%)				Previous Studies, n (%)			
	All	Epi.	Upd(14)mat	Pat. Del.	All	Epi.	Upd(14)mat	Pat. Del.
	Geoffron *et al*				(5–14)	(5, 6, 10–13)	(5, 6–9, 13)	(5, 13, 14)
n	28	17	8	3	91	17	64	10
Intrauterine growth retardation	25/28 (89.3)	15/17 (88.2)	8/8 (100)	2/3 (66.7)	31/41 (75.6)	7/13 (53.8)	18/20 (90.0)	6/8 (75.0)
Relative macrocephaly	14/25 (56.0)	7/15 (46.7)	5/7 (71.4)	2/3 (66.7)	10/19 (52.6)	0/3 (0.0)	10/16 (62.5)	NA
Early feeding difficulties	17/28 (60.7)	9/17 (52.9)	6/8 (75.0)	2/3 (66.7)	35/42 (83.3)	14/15 (93.3)	16/22 (72.7)	5/5 (100)
Protruding forehead	25/28 (89.3)	17/17 (100)	6/8 (75.0)	2/3 (66.7)	18/34 (52.9)	6/9 (66.7)	10/20 (50.0)	2/5 (40.0)
Body asymmetry	7/26 (26.9)	6/17 (35.3)	1/6 (16.7)	0/3 (0)	3/19 (15.8)	0/3 (0.0)	3/16 (18.8)	NA
NH-CSS $\geq$4	16/22 (72.7)	9/14 (64.3)	5/5 (100)	2/3 (66.7)	10/15 (66.7)	0/2 (0.0)	10/13 (76.9)	NA
Neonatal hypotonia	14/20 (70.0)	5/10 (50.0)	6/7 (85.7)	3/3 (100)	71/80 (88.8)	17/17 (100)	46/55 (83.6)	8/8 (100)
Motor delay	16/25 (64.0)	8/15 (53.3)	5/7 (71.4)	3/3 (100)	54/72 (75.0)	12/16 (75.0)	34/48 (70.8)	8/8 (100)
Speech delay	12/23 (52.2)	7/15 (46.7)	2/5 (40.0)	3/3 (100)	29/54 (53.7)	8/14 (57.1)	14/33 (42.4)	7/7 (100)
Acromicria	19/26 (73.1)	12/17 (70.6)	6/7 (85.7)	1/2 (50.0)	62/74 (83.8)	14/15 (93.3)	42/51 (82.4)	6/8 (75.0)
Early puberty	11/11 (10)	8/8 (100)	3/3 (100)	NA	40/44 (90.9)	10/10 (100)	23/26 (88.5)	7/8 (87.5)
Obesity	15/16 (93.8)	9/10 (90.0)	4/4 (100)	2/2 (100)	33/57 (57.9)	9/12 (75.0)	20/35 (57.1)	4/10 (40.0)
Short stature	16/27 (59.3)	10/16 (62.5)	3/8 (37.5)	3/3 (100)	66/81 (81.5)	12/16 (75.0)	45/55 (81.8)	9/10 (90.0)

Abbreviations: All, all patients; Epi., epimutations; NA, not available; Pat. Del., paternal deletion.

Upd(14)mat: maternal uniparental disomy of chromosome 14. In our cohort, short stature was defined as length <-2 SDS at 24 mo; no definition for other cohorts. References 5–14 can be found in the Supplemental Data.

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suggest that these imprinting disturbances may be secondary to the dysfunction of one or several *trans*-acting factors. However, the potential involvement of the identified genetic variants in the LOM mechanism will require further investigation and/or identification of variants of these genes in additional patients.

Molecular analysis allowed us to distinguish among three types of anomalies in the 14q32.2 region: epimutations, upd(14)mat, and deletions. Identification of deletions in these patients is important because of the risk of recurrence if inherited from the father. Aside from the need for genetic counseling, the identification of deletions would also modify the therapeutic strategy. Indeed, some reports have highlighted an increased risk of thyroid cancer [papillar carcinoma (26)] in patients with a large deletion in the 14q32.2 region and/or mental disability in those with a *YY1* deletion, a gene within the same 14q32 region (27). Among the three patients with deletions in this study, two had an already identified recurrent deletion, leading to cognitive delay and thyroid cancer (Supplemental Figure 3). This cancer risk must be considered and carefully evaluated for patients who are candidates for recombinant GH treatment because of short stature.

We compared the clinical data from all published cases with our cohort (Table 4). We noticed the relatively high frequencies of NH-CSS items among the previously published patients (being born SGA, relative macrocephaly, feeding difficulties, short stature, and protruding forehead). Asymmetry was rare, as in our patients. In patients with SRS, asymmetry is also the less frequent sign when looking at the overall SRS group (57.3%) (14). As in our study, neonatal hypotonia, acromicria, obesity, and early puberty were frequent signs among patients with 14q32.2 anomalies. In our cohort, we found no substantial differences between the clinical features associated with epimutations and those associated with upd(14)mat.

The overlap between TS and SRS phenotypes has already been established in reported cases (17–19). Most of the patients in our cohort with a 14q32.2 disruption meet the criteria for a clinical diagnosis of SRS, as do other recently reported patients (16). Features classically reported in TS, such as neonatal hypotonia, acromicria, clinodactyly precocious puberty, obesity, and psychomotor delay, can also be identified in patients with SRS with 11p15 epimutations or upd(7)mat. Indeed, although certain classic characteristics are shared across the different molecular causes of SRS, others may be more common to one molecular cause than another (7, 9, 11). As such, patients with SRS can have a large spectrum of symptoms that are not included in the NH-CSS. The NH-CSS was developed to provide easy, rapid, and sensitive diagnosis of SRS but is not meant to be exhaustive.

As recently published in the first SRS international consensus (9), SRS is a clinical diagnosis, and molecular testing is useful for confirmation and stratification of the diagnosis. In our cohort, almost 3/4 of patients with 14q32.2 disruption fulfill the clinical criteria of the NH-CSS ($\geq$4/6) and therefore may be considered as falling within the SRS phenotype. As also proposed in the SRS consensus for *CDKN1C* and *IGF2* mutation, 14q32.2 disruption may be an alternative molecular diagnosis of SRS.

In this retrospective analysis, we focused on pubarche and puberty in the oldest patients. All but three patients of the cohort were born SGA and puberty was precocious or early for all patients. SGA patients are known to develop early and rapidly progressive puberty (22, 28). These data are concordant with our clinical experience (work under submission) with patients with SRS with 11p15 disruption or upd(7)mat, who frequently present with aggressive early adrenarche and puberty. GnRH analogs have been shown to be beneficial for patients born SGA of small stature at pubertal onset (with or without a clinical diagnosis of SRS), together with recombinant GH treatment (29, 30). This therapy is now considered to be a possible personalized treatment of patients with SRS (9). Adding aGnRH to recombinant GH treatment may also be considered for patients with 14q32.2 anomalies. Although the role of DLK1 in the regulation of puberty is still unknown, a recent familial report suggests a link between *DLK1* anomalies and pubertal onset (31).

All of our patients started pubarche or puberty after a substantial rise in BMI. *DLK1* expression has been implicated in the homeostasis of fat metabolism in studies in mice and humans, showing a role in preadipocyte differentiation, regulation of hypothalamic satiety, circulating leptin concentrations, peripheral adipose tissue activity, and muscle mass development (32–37). As in patients with SRS, the BMI of patients with 14q32.2 anomalies should be carefully followed to avoid an excessive and uncontrollable increase during infancy (38–40). The SRS international consensus recommends maintaining a ratio of weight/expected weight for height at 75% to 85% and BMI at 12 to 14 kg/m^2 until the age of 4 years in those with SRS (9). Likely, it is also very important for patients with 14q32 disruption to try to limit their weight gain in their early life to avoid the onset of obesity and metabolic complications usually observed in this group of patients.

When looking at the age at diagnosis, we identified two groups: one with early diagnosis (n = 17 before 3.2 years) and one with late diagnosis (n = 11 >8.5 years). The first group was typically diagnosed because of being born SGA and failure to thrive, whereas the second was diagnosed later because of precocious puberty and obesity. Because of the late diagnosis in these latter patients, which correspond

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to the classical presentation of TS described until now, none benefited from early care including active weight and pubertal management or recombinant GH therapy. Among a series of published cases with 14q32.2 disruption, only half were treated with an aGnRH during their precocious puberty and only about a fourth were treated with concomitant or isolated recombinant GH therapy (16). Using NH-CSS to screen these patients allows a more precocious diagnosis and the possibility of proposing an earlier specific management of their metabolic, growth, and pubertal issues. Further studies will be necessary to evaluate if patients with 14q32 disruption will benefit from this earlier management as do patients with SRS.

Conclusion

We provide clinical and molecular data to support that, as raised in the SRS international consensus, 14q32.2 disruption may be considered as an alternative molecular diagnosis of SRS. These patients should be managed with a close follow-up for early onset of obesity, pubarche, precocious puberty, and short stature. 14q32.2 disruption should be investigated in case of suspected SRS without 11p15 LOM or upd(7)mat, especially in the presence of more specific characteristics of TS such as neonatal hypotonia, acromicria, hyperphagia, and/or early obesity. In patients with *MEG3/DLK1*:IG-DMR hypomethylation, an additional molecular analysis must be carried out to identify any paternal deletion within the 14q32.2 region because of the different prognosis and management of these patients. Elucidation of the mechanisms that control epimutation should be a research priority because of the high prevalence of MLMD in these patients.

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6 ANNEXES

1- Résultats complémentaires (section Travail expérimental 2.1.2.3) :
   - par sexe pour les dosages d'IGF-I, IGFBP-3 et le ratio IGF-I/IGFBP-3 chez les patients avec SRS
   - par méthode (RIA ou ELISA) pour IGF-II

2- *Diagnosis and management of Silver-Russell syndrome: first international consensus statement.* Wakeling EL, Brioude F, Lokulo-Sodipe O, O'Connell SM, Salem J, Bliek J, Canton AP, Chrzanowska KH, Davies JH, Dias RP, Dubern B, Elbracht M, Giabicani E, Grimberg A, Grønskov K, Hokken-Koelega AC, Jorge AA, Kagami M, Linglart A, Maghnie M, Mohnike K, Monk D, Moore GE, Murray PG, Ogata T, Petit IO, Russo S, Said E, Toumba M, Tümer Z, Binder G, Eggermann T, Harbison MD, Temple IK, Mackay DJ, Netchine I. **Nature Reviews-Endocrinology.** 2017 Feb;13(2):105-124. (SIGAPS A. IF 20,3).

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6.1 Résultats complémentaires
(section Travail expérimental 2.1.2.3)

- par sexe pour les dosages d'IGF-I, IGFBP-3 et le ratio IGF-I/IGFBP-3 chez les patients avec SRS
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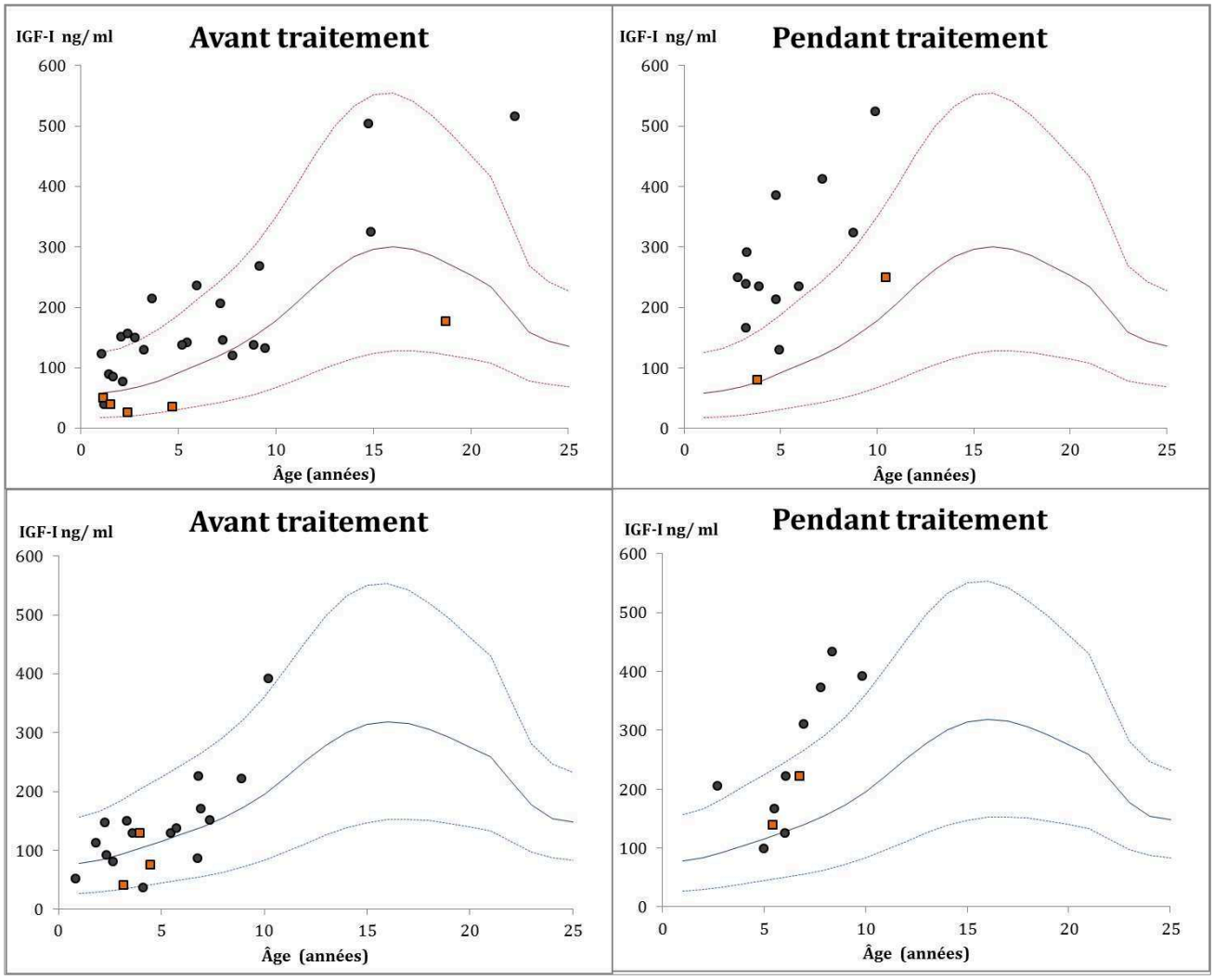

Figure 35. Répartition des taux d'IGF-I (mesurés en chemiluminescence automatisée) chez les patients SRS avec LOM 11p15 (ronds gris) ou par mupd7 (carrés oranges), avant et pendant traitement par rGH, en fonction des valeurs d'une population de référence. La médiane correspond à la ligne centrale, les lignes supérieure et inférieure figurent respectivement les 97,5ème et 2,5ème percentiles (Bidlingmaier *et al.* 2014). Les filles sont représentées sur les graphes supérieurs et les garçons sur les graphes inférieurs.

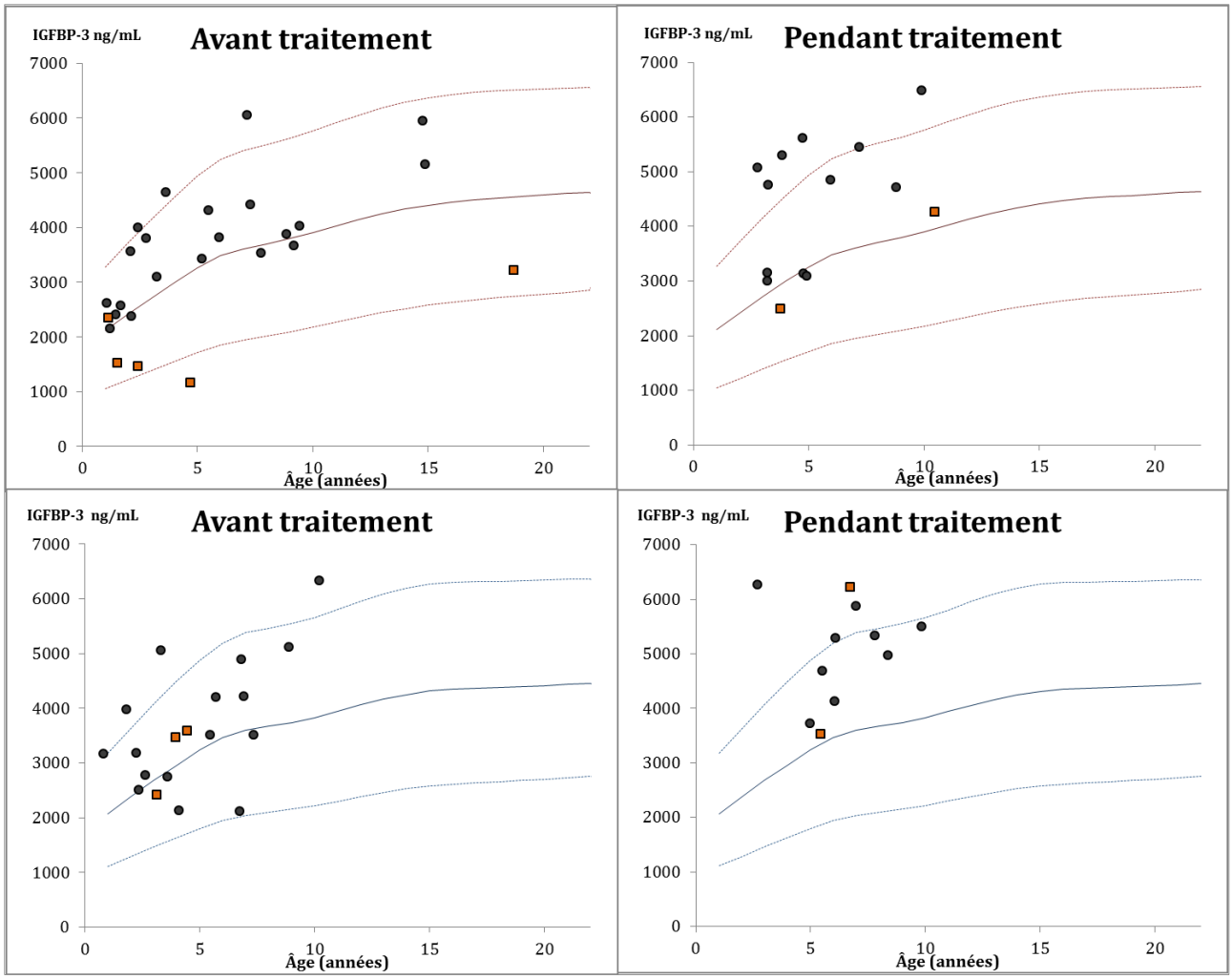

Figure 36. Répartition des taux d'IGFBP-3 (mesurés en chemiluminescence automatisée) chez les patients SRS avec LOM 11p15 (ronds gris) ou par mupd7 (carrés oranges), avant et pendant traitement par rGH, en fonction des valeurs d'une population de référence. La médiane correspond à la ligne centrale, les lignes supérieure et inférieure figurent respectivement les 97,5ème et 2,5ème percentiles (Friedrich *et al.* 2014). Les filles sont représentées sur les graphes supérieurs et les garçons sur les graphes inférieurs.

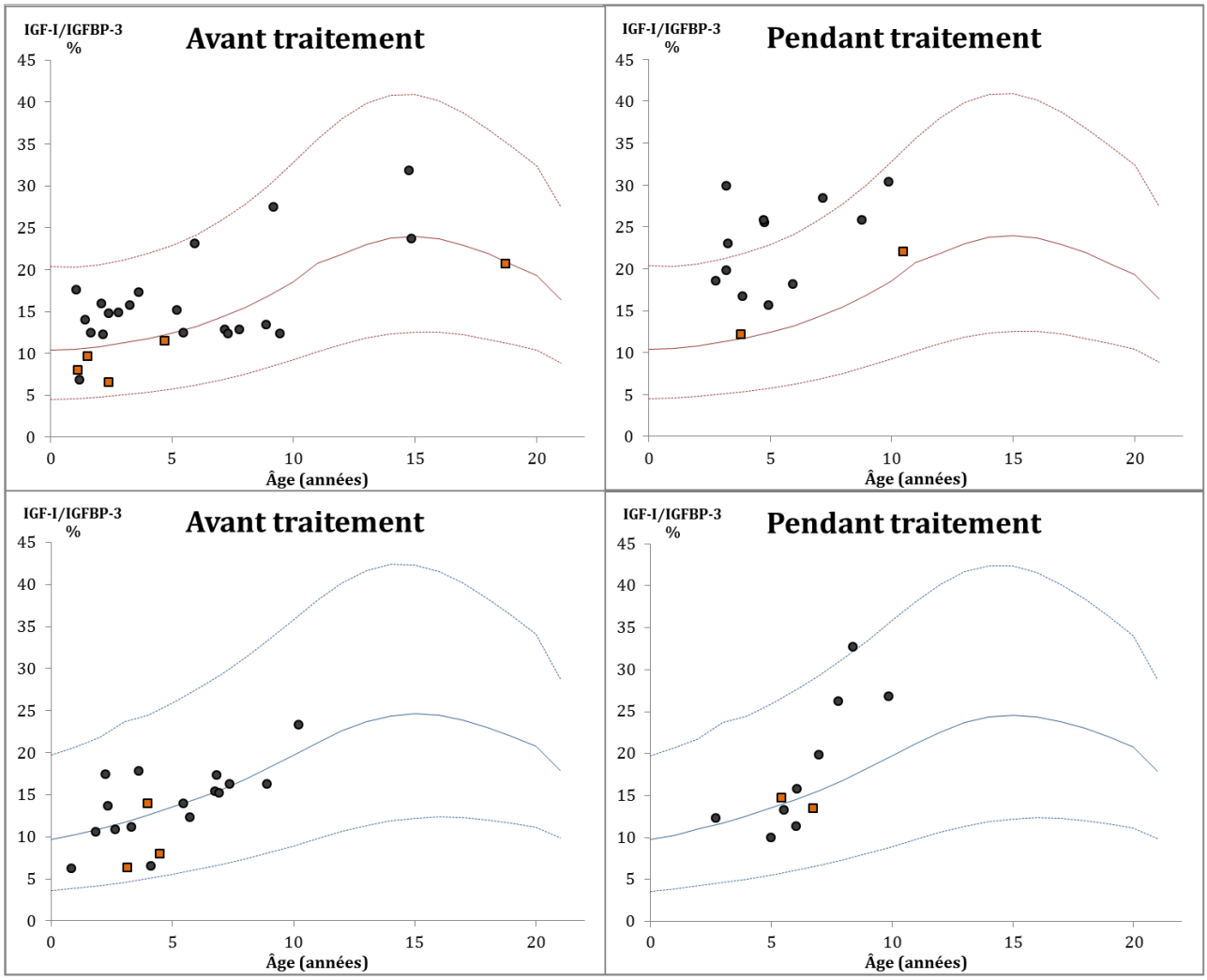

Figure 37. Répartition des ratios IGF-I/IGFBP-3 (mesurés en chemiluminescence automatisée) chez les patients SRS avec LOM 11p15 (ronds gris) ou par mupd7 (carrés oranges), avant et pendant traitement par rGH, en fonction des valeurs d'une population de référence. La médiane correspond à la ligne centrale, les lignes supérieure et inférieure figurent respectivement les 97,5ème et 2,5ème percentiles (Friedrich *et al.* 2014). Les filles sont représentées sur les graphes supérieurs et les garçons sur les graphes inférieurs.

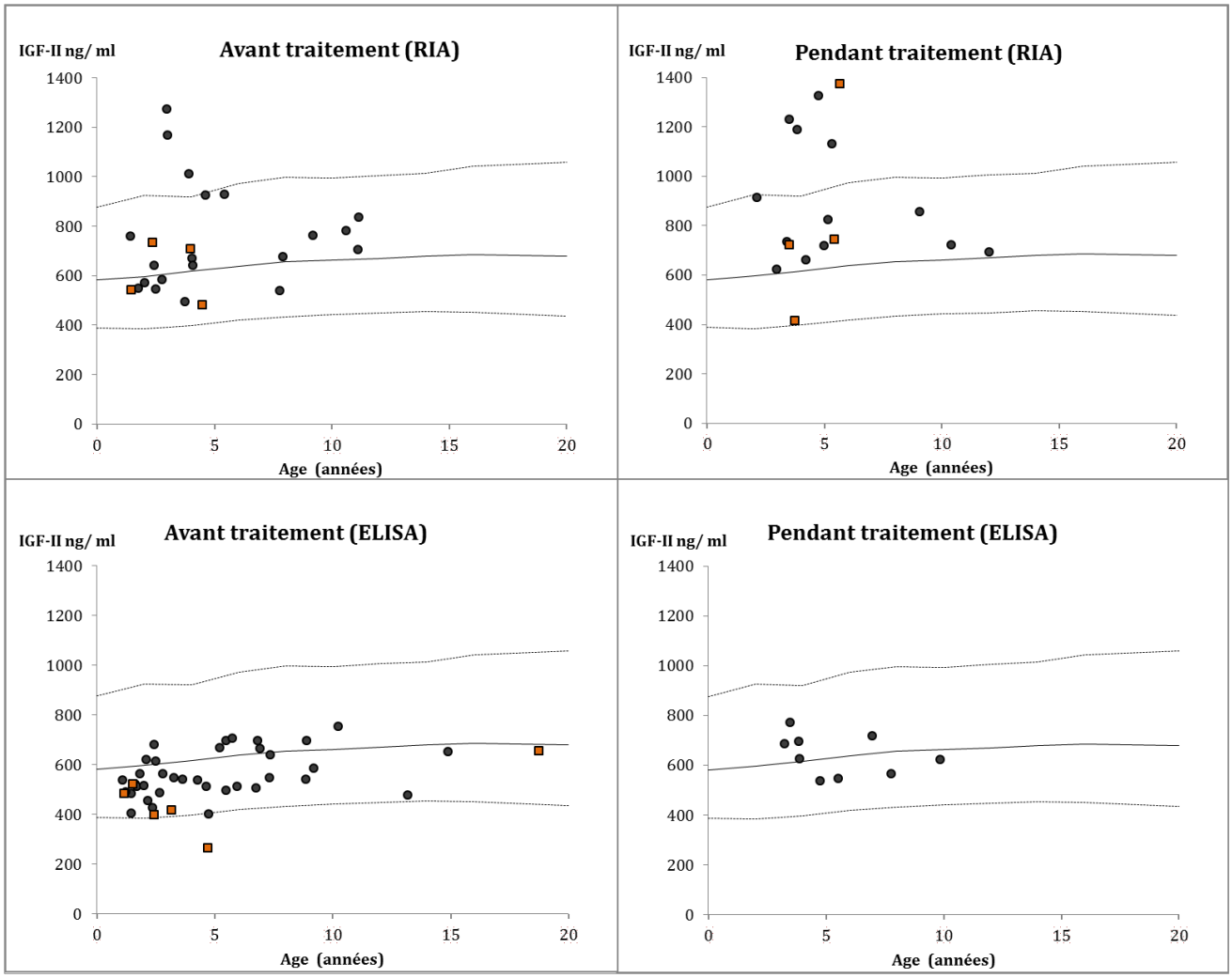

Figure 38. Répartition des taux d'IGF-II (kit MEDIAGNOST RIA en haut et ELISA en bas) chez les patients SRS par LOM 11p15 (ronds noirs) ou par mupd7 (carrés oranges), avant et pendant le traitement par rGH, en fonction des valeurs normales proposées par le fabricant. La médiane correspond à la ligne au centre et les lignes supérieure et inférieure figurent les 97,5ème et 2,5ème percentiles.

OPEN

EXPERT CONSENSUS DOCUMENT

Diagnosis and management of Silver–Russell syndrome: first international consensus statement

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Abstract | This Consensus Statement summarizes recommendations for clinical diagnosis, investigation and management of patients with Silver–Russell syndrome (SRS), an imprinting disorder that causes prenatal and postnatal growth retardation. Considerable overlap exists between the care of individuals born small for gestational age and those with SRS. However, many specific management issues exist and evidence from controlled trials remains limited. SRS is primarily a clinical diagnosis; however, molecular testing enables confirmation of the clinical diagnosis and defines the subtype. A 'normal' result from a molecular test does not exclude the diagnosis of SRS. The management of children with SRS requires an experienced, multidisciplinary approach. Specific issues include growth failure, severe feeding difficulties, gastrointestinal problems, hypoglycaemia, body asymmetry, scoliosis, motor and speech delay and psychosocial challenges. An early emphasis on adequate nutritional status is important, with awareness that rapid postnatal weight gain might lead to subsequent increased risk of metabolic disorders. The benefits of treating patients with SRS with growth hormone include improved body composition, motor development and appetite, reduced risk of hypoglycaemia and increased height. Clinicians should be aware of possible premature adrenarche, fairly early and rapid central puberty and insulin resistance. Treatment with gonadotropin-releasing hormone analogues can delay progression of central puberty and preserve adult height potential. Long-term follow up is essential to determine the natural history and optimal management in adulthood.

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Silver–Russell syndrome (SRS, OMIM #180860, also known as Russell–Silver syndrome, RSS) is a rare, but well-recognized, condition associated with prenatal and postnatal growth retardation. The syndrome was first described by Silver *et al.*[1] and Russell[2], who independently described a subset of children with low birth weight, postnatal short stature, characteristic facial features and body asymmetry. Almost all patients with SRS are born small for gestational age (SGA; BOX 1). The aetiology of intrauterine growth retardation and SGA is extremely heterogeneous. Children with SRS can be distinguished from those with idiopathic intrauterine growth retardation or SGA and postnatal growth failure by the presence of other characteristic features, including relative macrocephaly (defined as a head circumference at birth ≥1.5 SD score (SDS) above birth weight and/or length SDS), prominent forehead, body asymmetry and feeding difficulties[3–6].

Globally, estimates of the incidence of SRS range from 1:30,000 to 1:100,000 (REF. 7). In 2015, a study in Estonia[8] estimated an incidence of 1:70,000; however, only molecularly confirmed cases were included, which could have resulted in underdiagnosis. Overall, SRS is probably more common than some previous estimates have suggested, but the exact incidence remains unknown.

An underlying molecular cause can currently be identified in around 60% of patients clinically diagnosed with SRS[4]. The most common underlying mechanisms are loss of methylation on chromosome 11p15 (11p15 LOM; seen in 30–60% of patients) and maternal uniparental disomy for chromosome 7 (upd(7)mat; seen in ~5–10% of patients)[4,9,10]. However, the molecular aetiology remains unknown in a substantial proportion of patients.

Although considerable overlap exists in the clinical care of individuals born SGA and those with SRS, many management issues are specific to SRS. These include notable feeding difficulties, severe postnatal growth failure with no catch-up, recurrent hypoglycaemia, premature adrenarche, fairly early and rapid central puberty, insulin resistance and body asymmetry. Identification of the molecular cause in many patients has also raised questions about the management of individual molecular subtypes of SRS. As evidence from controlled trials is limited, a consensus meeting was organized to develop guidelines for the diagnosis and management of patients with SRS.

This Consensus Statement was produced on behalf of the COST Action BM1208 (European Network for Human Congenital Imprinting Disorders, http://www.imprinting-disorders.eu), European Society of Pediatric Endocrinology (ESPE), Pediatric Endocrine Society (PES), Asian Pacific Pediatric Endocrine Society (APPES) and Sociedad Latino-Americana de Endocrinología Pediátrica (SLEP).

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Methods

41 task force members from 16 countries, chosen for their publication record and expertise in SRS, collaborated to develop this consensus statement. They included paediatric endocrinologists, clinical geneticists, molecular geneticists, a gastroenterologist and five non-voting representatives from a parent support group. Participants included representatives nominated by the council and clinical practice committees from four international paediatric endocrine societies. All participants signed a conflict of interest declaration, and the consensus was supported by academic funding, without pharmaceutical support. A Delphi-like consensus methodology was adopted[11]. A comprehensive literature search was conducted using PubMed and the search terms "Silver Russell syndrome" and "Russell Silver syndrome". Additional relevant articles on SGA, differential diagnoses and growth hormone (GH) were also identified by PubMed searches when supplementary information was necessary. A comprehensive review of >600 articles formed the basis of discussion by three working groups. These groups focused on clinical diagnosis (working group 1: E.L.W., J.S., K.H.C., M.E., R.P.D., P.G.M., T.O., E.S., M.T. and I.K.T.), molecular testing (working group 2: F.B., J.B., K.G., M.K., D.M., G.E.M., S.R., Z.T., T.E. and D.J.G.M) and clinical management (working group 3: O.L.-S., S.M.O'C., J.H.D., A.P.M.C., B.D., E.G., A.G., A.C.S.H.-K., A.A.J., A.L., M.M., K.M., I.O.P., G.B., M.D.H. and I.N.), with 10, 10 and 16 members, respectively. Preparations for the consensus took place over 10 months, including two preparatory meetings and regular teleconference discussions between the working group members. At the final consensus meeting, propositions and recommendations were considered by participants and discussed in plenary sessions, enabling reformulation of the recommendations if necessary. Where published data were unavailable or insufficient, experts' clinical experiences and opinions were considered. Finally, all experts voted on the recommendations of each working group using the following system:

A. Evidence or general agreement allow full agreement with the recommendation
B. Evidence or general agreement are in favour of the recommendation
C. Evidence or general agreement are weak for the recommendation
D. There is not enough evidence or general agreement to agree with the recommendation

Depending on the proportion of votes received, the strength of the recommendation was recorded as follows:

+	26–49% of the votes
++	50–69% of the votes
+++	≥70% of the votes

Clinical diagnosis

SRS is currently a clinical diagnosis based on a combination of characteristic features. Molecular testing can confirm the diagnosis in around 60% of patients[4]. Molecular testing enables stratification of patients with SRS into subgroups, which can lead to more tailored management. However, molecular investigations are negative in a notable proportion of patients with characteristic clinical features of SRS. For these patients, an established clinical diagnosis enables access to appropriate support groups, treatment (including GH) and further research into the underlying incidence, natural history and aetiology of the SRS phenotype.

Box 1 | **Definitions**

Small for gestational age (SGA)
Weight and/or length less than −2 SDS for gestational age at birth, based on accurate anthropometry at birth (including weight, length and head circumference) and reference data from a relevant population[106].

Intrauterine growth retardation
Also known as intrauterine growth restriction, this diagnosis is based on at least two ultrasonography measurements at least 2 weeks apart, with fetal weight below the 10th percentile for gestational age. Intrauterine growth retardation might or might not result in a baby born SGA[161].

Silver–Russell syndrome (SRS)
A distinct syndromic growth disorder in which prenatal and postnatal growth failure are associated with other characteristic features, including relative macrocephaly at birth, protruding forehead in early life, body asymmetry and substantial feeding difficulties. Almost all children with SRS are born SGA. Postnatal catch-up growth is not seen in the majority of children with SRS.

However, the diagnosis of SRS can be difficult, as the condition varies widely in severity among affected individuals and many of its features are nonspecific[4–6]. Until now, no consensus has been reached on the clinical definition of SRS. Historically, this lack of consensus has probably led to underdiagnosis and overdiagnosis, particularly by clinicians unfamiliar with SRS.

Several clinical scoring systems for SRS have been proposed, which reflects the challenge in reaching a confident diagnosis[4,5,12–15]. All the systems use similar criteria, but vary in the number and definition of diagnostic features required for diagnosis. The relative sensitivity and specificity of these scoring systems have been compared in patients with confirmed molecular diagnoses[14,15].

Netchine–Harbison clinical scoring system

The Netchine–Harbison clinical scoring system (NH-CSS; TABLE 1), which was proposed by Azzi and colleagues in 2015,[15] is the only scoring system for the diagnosis of SRS that was developed using prospective data. Four of the six criteria are objective; protruding forehead and feeding difficulties remain subjective, but clear clinical definitions are given. Using the same cohort, the NH-CSS proved more sensitive (98%) than previous systems[4,14]. The NH-CSS also had the highest negative predictive value (89%), which gives a high degree of confidence that patients who have less than four of the six clinical criteria for diagnosis are truly unaffected by SRS. The system is easy to use in a busy clinical setting. The NH-CSS is also flexible enough to use even if data are incomplete, which is important as the diagnosis is often made in infancy, before information about postnatal growth and BMI is available.

Table 1 | **Netchine–Harbison clinical scoring system**

Clinical criteria	Definition
SGA (birth weight and/or birth length)	≤−2 SDS for gestational age
Postnatal growth failure	Height at 24 ± 1 months ≤−2 SDS or height ≤−2 SDS below mid-parental target height
Relative macrocephaly at birth	Head circumference at birth ≥1.5 SDS above birth weight and/or length SDS
Protruding forehead*	Forehead projecting beyond the facial plane on a side view as a toddler (1–3 years)
Body asymmetry	LLD of ≥0.5 cm or arm asymmetry or LLD <0.5 cm with at least two other asymmetrical body parts (one non-face)
Feeding difficulties and/or low BMI	BMI ≤−2 SDS at 24 months or current use of a feeding tube or cyproheptadine for appetite stimulation

Clinical diagnosis is considered if a patient scores at least four of six from these criteria. If all molecular tests are normal and differential diagnoses have been ruled out, patients scoring at least four of six criteria, including both prominent forehead and relative macrocephaly should be diagnosed as clinical Silver–Russell syndrome. *Protruding forehead is equivalent to 'prominent forehead' (REF. 164). LLD, leg length discrepancy; SDS, SD score; SGA, small for gestational age.

Similarly to other clinical scoring systems, the NH-CSS has a low specificity (36%)[15], which could result in false positive results when the diagnosis is just based on clinical findings. Relative macrocephaly at birth (defined as a head circumference at birth ≥1.5 SDS above birth weight and/or length SDS) and protruding forehead are the two features in the NH-CSS that best distinguish SRS from non-SRS SGA (see Supplementary information S1 (table))[4,15–18]. To maintain confidence in the clinical diagnosis if all molecular testing is normal, we recommend that only patients scoring at least four of six criteria, including both prominent forehead and relative macrocephaly, should be diagnosed as 'clinical SRS' (previously known as 'idiopathic SRS'); see the flow diagram for investigation and diagnosis of SRS (FIG. 1).

Diagnosis in late childhood or adulthood

All scoring systems for SRS have been developed and validated in paediatric cohorts. However, an increasing number of adults with a historical diagnosis of SRS are being seen by clinicians, particularly regarding their concerns about passing the condition on to their offspring (personal experience of working groups 1 and 3). In these patients, a clinical diagnosis is frequently challenged by lack of early growth data. An attempt should be made to obtain photographs of the individual aged 1–3 years, especially of the face in profile, as well as measurements at birth and in the first 2 years. No current evidence exists to support an alternative approach to diagnosis in adults.

Additional clinical features

In addition to the clinical features in the NH-CSS, several others are recognized in association with SRS, as shown in TABLE 2 and Supplementary information S1 (table). These characteristics are not specific to SRS, and might be present in children born SGA who do not have SRS, but at a lower frequency than in patients with SRS. However, a few features occur at a much higher rate in children with SRS than in those with SGA[4,15,16]. These features include low muscle mass, crowded or irregular teeth, micrognathia, down-turned mouth, clinodactyly and excessive sweating.

1 Recommendations

1.1 SRS should remain primarily a clinical diagnosis. Molecular testing is useful for the confirmation and stratification of diagnosis in SRS. Lack of a positive molecular result does not exclude the diagnosis of SRS. (A+++)

1.2 The flow chart (FIG. 1), based on the NH-CSS, should be adopted for the investigation and diagnosis of SRS. (A++)

1.3 In children aged <2 years, adolescents and adults, a reduced threshold for molecular testing might be required due to missing data. (A++)

Molecular diagnosis

Investigation and diagnosis

A positive molecular test result provides useful confirmation of the clinical diagnosis (FIG. 1). This result also enables stratification into a specific molecular subgroup that, in turn, can help guide appropriate management. However, many patients are referred for molecular testing with few, or atypical, features of SRS, which leads to low diagnostic yields and incurs unnecessary expense[19]. We, therefore, recommend the use of the flow chart in FIG. 1 to aid in the investigation and diagnosis of SRS.

Some patients, particularly those with upd(7)mat, have fewer typical clinical features of SRS than patients with 11p15 LOM[4,5,13,16,20,21]. In the cohort reported by Azzi and co-workers[15], one of the nine patients scoring three of six criteria (and therefore predicted 'unlikely to have SRS') had upd(7)mat. The threshold recommended in FIG. 1 for molecular testing (≥3 of six criteria) is, therefore, lower than that needed for a clinical diagnosis of SRS (≥4 of six criteria).

Conversely, in the same cohort, no positive molecular diagnoses were made in patients scoring less than three of six criteria[15]. Other studies have also excluded 11p15 LOM and upd(7)mat in patients born SGA with postnatal growth retardation but without additional features of SRS[4,10,22]. We, therefore, do not recommend testing for SRS in patients scoring less than three of six criteria. Of note, a small number of patients with body asymmetry have been reported to have 11p15 LOM

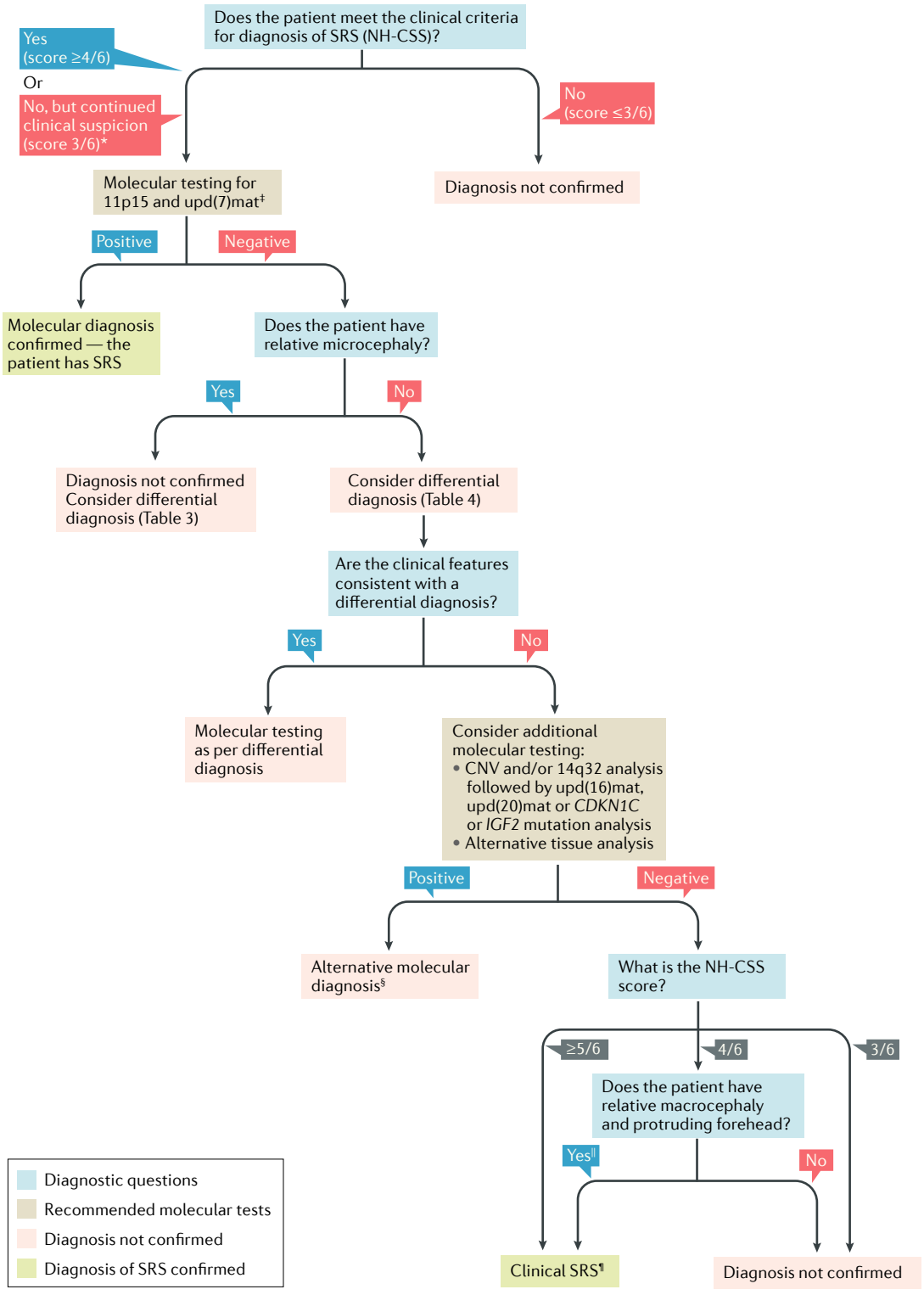

Figure 1 | **Flow chart for investigation and diagnosis of SRS.** Diagnostic questions are in blue boxes; recommended molecular tests are in beige boxes. Pink boxes: diagnosis not confirmed; green boxes: diagnosis of SRS confirmed. *Studies have excluded 11p15 LOM and upd(7)mat in patients with intrauterine growth retardation and postnatal growth retardation alone; some patients, particularly those with upd(7)mat or children under 2 years, score 3/6 (see text for details). ‡Arrange CNV analysis before other investigations if patient has notable unexplained global developmental delay and/or intellectual disability and/or relative microcephaly. §Insufficient evidence at present to determine relationship to SRS, with the exception of tissue mosaicism for 11p15 LOM. ‖Unless evidence of catch-up growth by 2 years. ¶Previously known as idiopathic SRS. CNV, copy number variant; LOM, loss of methylation; NH-CSS, Netchine-Harbison clinical scoring system; SRS, Silver–Russell syndrome.

Table 2 | **Additional clinical features of Silver–Russell syndrome**

Clinical feature	Frequency % (total no. patients)	Refs
Triangular face	94 (164)	16–18
Fifth finger clinodactyly	75 (319)	4,15–18,20
Shoulder dimples	66 (61)	15
Micrognathia	62 (115)	16,18,20
Low muscle mass	56 (103)	15,16
Excessive sweating	54 (106)	16,20
Low-set and/or posteriorly rotated ears	49 (266)	15–17,20
Down-turned mouth	48 (176)	15,16,18,20
High pitched or squeaky voice	45 (26)	16
Prominent heels	44 (61)	15
Delayed closure of fontanelle	43 (47)	18,20
Male genital abnormalities	40 (85)	15,16,18,20
Speech delay	40 (189)	16,17,20
Irregular or crowded teeth	37 (195)	16–18,20
Motor delay	37 (254)	4,16–18,20
Syndactyly of toes	30 (264)	15–17,20
Hypoglycaemia	22 (103)	4,20
Scoliosis and/or kyphosis	18 (227)	16,20,147

without associated growth retardation, probably due to tissue mosaicism[20,21,23]. These patients would score fewer than three of six criteria, which is insufficient to justify a clinical diagnosis of SRS in these patients.

Chromosome 11p15

Both SRS and the overgrowth condition Beckwith–Wiedemann syndrome are associated with molecular abnormalities of chromosome 11p15.5, which contains two imprinted domains (FIG. 2). Imprinting of the telomeric domain, which is strongly implicated in SRS[24,25], is controlled by the paternally methylated imprinting control region H19/IGF2 IG-DMR (H19/IGF2 intergenic differentially methylated region, previously known as IC1, ICR1 and H19 DMR). The centromeric domain contains the maternally expressed growth repressor *CDKN1C*; the imprinting of this gene is controlled by the maternally methylated imprinting control region KCNQ1OT1 TSS-DMR (previously known as IC2, ICR2, LIT1 or KvDMR1). FIGURE 3 summarises the more common molecular changes at chromosome 11p15 associated with SRS. Hypomethylation of the H19/IGF2 IG-DMR results in reduced paternal *IGF2* expression and increased maternal *H19* expression, which leads to growth restriction[9]. Numerous copy number variants (CNVs) involving the 11p15.5 region have been reported; the phenotype is dependent on CNV size, location and parental origin[24,26] (see Supplementary information S2 (table)).

Molecular testing must robustly and accurately measure DNA methylation of CpG dinucleotides at H19/IGF2 IG-DMR[27]. Assays involve either bisulfite analysis[28–30] or enzymatic methods, such as methylation-specific multiplex ligation-mediated PCR amplification (MS-MLPA) or Southern blotting[9,31]. The most common test in diagnostic use is MS-MLPA, which is economical on DNA, cost-effective and enables parallel analysis of copy number and DNA methylation[31,32]. Hypomethylation of H19/IGF2 IG-DMR is frequently incomplete and low levels of hypomethylation might elude detection. Methylation patterns might vary between different tissues and cells (leucocytes, samples from a buccal swab and skin fibroblasts)[21,33,34] and could explain cases of a negative molecular diagnosis using a blood sample.

Although copy number change can be detected by MS-MLPA, additional array analysis is useful for characterizing the size and gene content of any CNV identified.

Chromosome 7

Of individuals with SRS, ≤10% have upd(7)mat[35,36]. The SRS phenotype of upd(7)mat is thought to result from altered expression of an imprinted growth-regulatory gene (or genes)[37]. In addition, the duplication of pre-existing pathogenic mutations by isodisomy can lead to the clinical expression of recessive disorders (such as cystic fibrosis) in patients with upd(7)mat[38–40].

Candidate SRS regions have been suggested through identification of patients with segmental upd(7)mat or CNVs (see Supplementary information S3 (table)); the primary candidate SRS genes on chromosome 7 are currently *GRB10* (7p12.1) and *MEST* (7q32)[41–48].

Microsatellite analysis was the first diagnostic test for upd(7)mat[35,36]; however, this analysis cannot detect imprinting defects (epimutations) and requires DNA from at least one parent. DNA methylation analysis, including at least the imprinting control regions GRB10 alt-TSS-DMR and MEST alt-TSS-DMR, can identify upd(7)mat, epimutations, CNVs and segmental or whole-chromosome variations. DNA methylation analysis, for example by MS-MLPA, is economical on DNA, cost-effective and compatible with parallel analysis of 11p15 (REFS 30,49,50).

Additional testing

If testing of both 11p15 and chromosome 7 is negative, additional molecular testing can be considered.

CNVs. Over 30 different pathogenic CNVs have been described in patients with suspected SRS[15,51–53]. Patients with these CNVs usually have more severe developmental delay and/or intellectual disability than is typically seen in SRS[52,53]. Some patients fulfil the NH-CSS for diagnosis; others either do not meet the NH-CSS criteria, or insufficient data are given to use the criteria for their assessment. Although features of SRS can be present in individuals with a pathogenic CNV, clinical diagnosis of SRS is not helpful in these cases and management needs to be tailored specifically to the phenotypic consequences of the individual CNV.

While either array comparative genomic hybridization or single nucleotide polymorphism array can be used to detect CNVs, single nucleotide polymorphism array can also detect regions of segmental isodisomy[54].

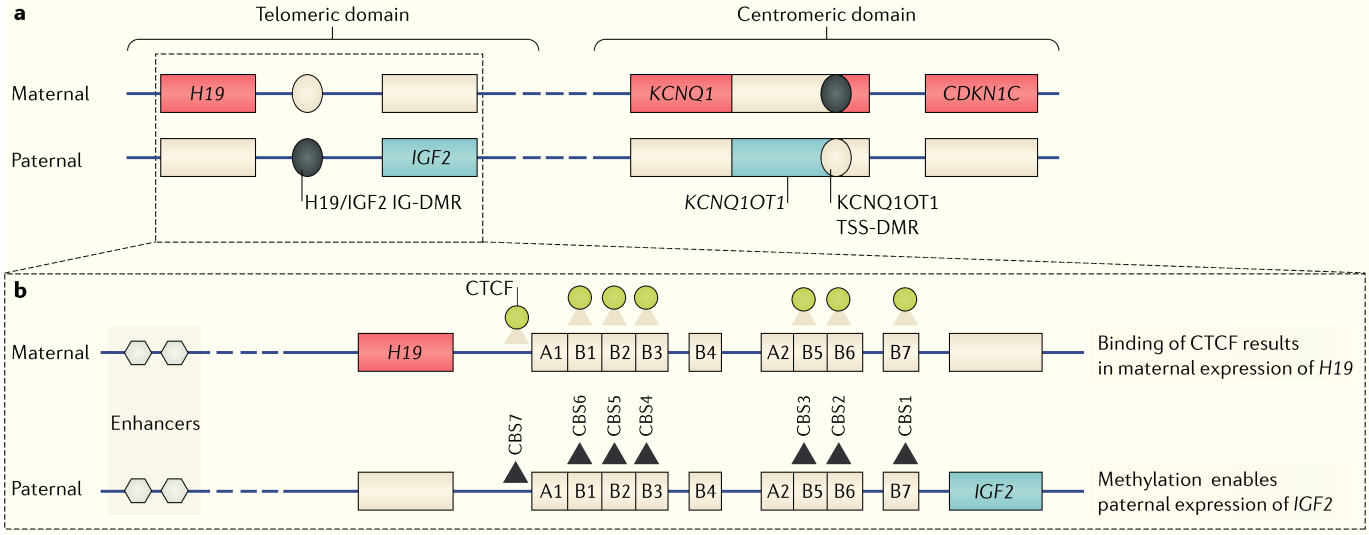

Figure 2 | **The imprinted domains of chromosome 11p15 that are implicated in Silver–Russell syndrome. a** | Representation of the 11p15 region, showing both centromeric and telomeric domains. Only the imprinted genes that are implicated in the pathophysiology of Silver–Russell syndrome are represented. Blue boxes indicate paternally expressed genes (the growth promoter *IGF2* and the long noncoding RNA (lncRNA) *KCNQ1OT1*). Red boxes indicate maternally expressed genes (the growth inhibitor *CDKN1C*, the ion channel *KCNQ1* and the noncoding RNA *H19*). Ovals indicate differentially methylated regions (DMRs). Dark grey ovals indicate methylated DMRs. Beige ovals indicate unmethylated DMRs. **b** | Structure of the H19/IGF2 IG-DMR (intergenic differentially methylated region). This DMR contains short repetitive blocks of sequence and harbours seven binding sites for the zinc finger protein CTCF (green circles). Multiple enhancer elements (grey hexagons) distal to *H19* are shared between *H19* and *IGF2*, and are able to increase expression of either. Binding of CTCF to the unmethylated maternal DMR blocks interactions between the *IGF2* promoter and enhancers downstream of *H19*, which results in maternal *H19* expression. Conversely, methylation of ICR1 on the paternal allele prevents CTCF binding, enabling interaction between the *IGF2* promoter and distal enhancers, and thus paternal *IGF2* expression[162,163]. Beige triangles indicate unmethylated CTCF binding sites. Dark grey triangles indicate methylated CTCF binding sites.

Chromosome 14q32 abnormalities. Molecular abnormalities at the paternally methylated imprinted locus on chromosome 14q32 include upd(14)mat, paternal microdeletions and hypomethylation of the DLK1/GTL2 IG-DMR (also referred to as MEG3-DMR, 14q32 DMR or IG-DMR). These result in Temple syndrome, which has clinical overlap with SRS[55,56], including being born SGA, postnatal growth retardation, hypotonia, delay in the development of motor skills and early puberty[55].

In cohorts of patients with SRS, a small number of patients have been found to have Temple syndrome: 1 of 127 patients[57], 2 of 85 patients[56] and 1 of 26 patients[15]. However, the true incidence of 14q32 abnormalities in patients meeting the NH-CSS criteria for diagnosis remains unknown.

upd(20)mat and upd(16)mat. Patients with both upd(20)mat and upd(16)mat have occasionally been detected among cohorts of patients investigated for prenatal and postnatal growth failure or SRS[15,57,58]. However, in a study published in 2015, none of eight patients with upd(20)mat had relative macrocephaly or asymmetry[59], two important criteria of the NH-CSS, which means that these patients might have eluded formal clinical diagnosis of SRS.

CDKN1C and IGF2 mutations. *CDKN1C* and *IGF2* are the coding genes on chromosome 11p15 that are responsible for the growth anomalies in SRS and Beckwith–Wiedemann syndrome. Maternally transmitted SRS was described in a four-generation family with a *CDKN1C* gain-of-function mutation[60], and paternally transmitted SRS in a family with an *IGF2* loss-of-function mutation[61]. However, no additional mutations have been reported to date in sporadic or familial cases of SRS[60,62,63]. Sequence analysis of either gene might be considered, particularly in familial cases of SRS where the inheritance pattern is consistent; however, coding variants in these genes are rare[60,62,63].

Multi-locus imprinting disturbance. A significant proportion (15–38%) of individuals with 11p15 LOM have multi-locus imprinting disturbance (MLID)[33,57,64–66]. High-density methylation arrays have revealed methylation changes involving both (maternally and paternally) imprinted and non-imprinted loci[67,68]. However, despite welcome advances in genome-wide methylation screening[67,69,70], standardization is required to ensure accurate description of MLID and comparison between cohorts.

The effect of MLID on clinical phenotype remains unclear. No difference in growth parameters was found at birth or at 2 years of age between patients with 11p15 LOM with and without MLID[64]. Although developmental delay and congenital anomalies were reported in patients with MLID, this finding might have been affected by ascertainment bias[57].

In principle, MLID might be caused by *trans*-acting genetic mutations that affect the acquisition or maintenance of imprints, but in practice, very few have been identified[71,72].

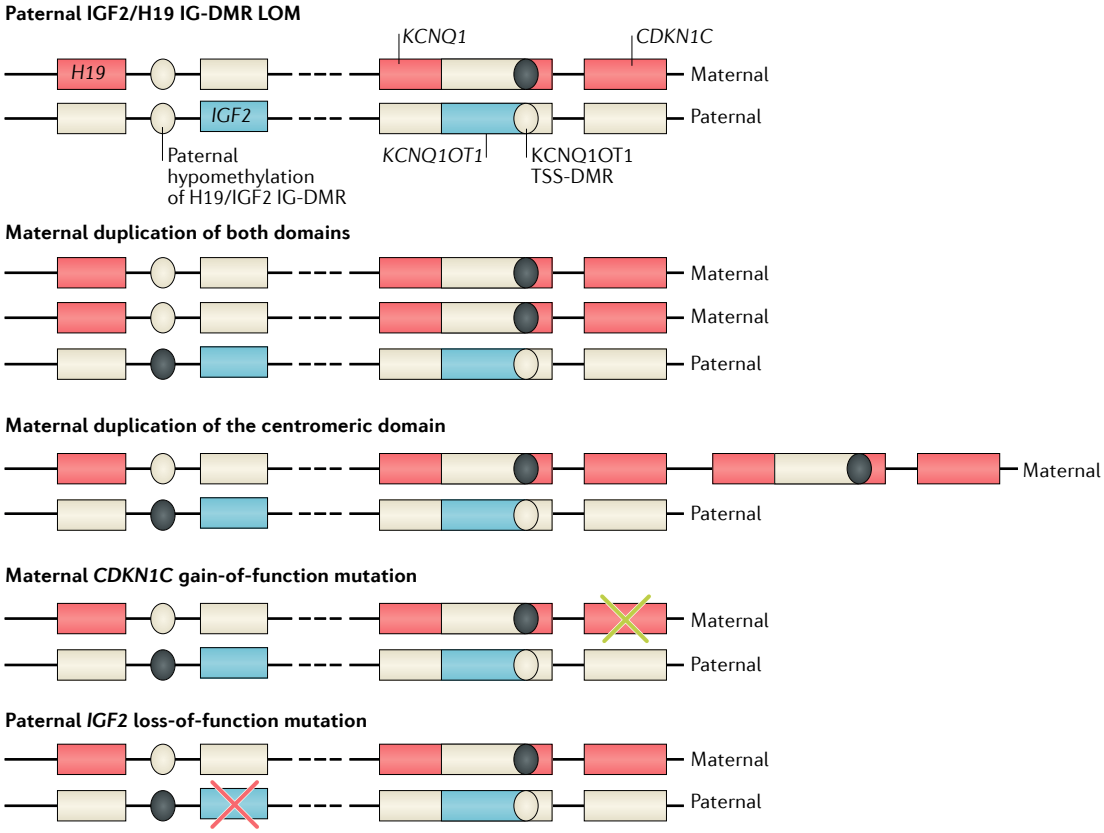

Figure 3 | **Mutations and epimutations of the imprinted region at chromosome 11p15 associated with Silver–Russell syndrome.** The structure of the 11p15 region is represented as in FIG. 2. Paternal hypomethylation of H19/IGF2 IG-DMR results in loss of paternal *IGF2* expression and gain of maternal *H19* expression, which leads to a growth restriction phenotype[9]. Less commonly, maternal duplication of the centromeric or both domains results in growth retardation due to increased dosage of *CDKN1C*; however, smaller copy number variants should be classified with caution due to the complex regulation of the region[27]. Rare familial cases have been associated with a maternal *CDKN1C* gain-of-function mutation (green cross)[60] or a paternal *IGF2* loss-of-function mutation (red cross)[61].

Overall, the effect of MLID on clinical phenotype and its relevance for genetic counselling remain uncertain. Further information is needed before recommending testing for MLID outside the research setting.

2 Recommendations

2.1 Molecular genetic testing should be performed by a health professional experienced in the field of imprinting disorders. Consistent and logical nomenclature should be adopted in publications and in test reporting. (A+++)

2.2 First-line molecular testing should include DNA methylation analysis of the H19/IGF2 IG-DMR and KCNQ1OT1 TSS-DMR. (A+++)

2.3 First-line molecular testing should include analysis of DNA methylation at the GRB10 alt-TSS-DMR and the MEST alt-TSS-DMR. (A+++)

2.4 In case of a positive test result at either 11p15 or chromosome 7, discrimination between epimutation, CNV and upd should be considered to estimate recurrence risk. (A+++)

2.5 After exclusion of changes in 11p15 and chromosome 7, a clinical decision should be sought about the direction of further testing. Depending on the clinical features and family history of the patient, further testing might include CNV analysis and DNA methylation analysis at chromosome 14q32. Testing might also be considered for very rare molecular anomalies, including upd(20)mat, upd(16)mat and mutations in *CDKN1C* and *IGF2*, as well as analysis of further tissues to detect somatic mosaicism. (A++)

2.6 When an underlying pathogenic CNV is identified, the diagnosis should focus on this finding, even if features of SRS are present. (A+)

(Epi)genotype–phenotype correlation

The frequency of individual features in specific SRS subgroups (11p15 LOM, upd(7)mat and clinical SRS) and patients with SGA but not SRS, where data are available, are shown in Supplementary information S1 (table). Genotype–phenotype studies of patients with SRS indicate considerable overlap in clinical phenotype between (epi)genotypes, and these are generally clinically indistinguishable. However, some features are more common in particular molecular subgroups[4,13,15–17,20,73].

Patients with 11p15 LOM tend to have a lower birth length and weight, more frequent body asymmetry and more frequent congenital anomalies than patients with

upd(7)mat[15,20]. Neurocognitive problems are more frequent in patients with upd(7)mat than in those with 11p15 LOM or clinical SRS[15,16,20] (see later section on neurocognitive problems).

Patients with 11p15 duplication encompassing H19/IGF2 IG-DMR and KCNQ1OT1 TSS-DMR have an SRS phenotype, but usually without asymmetry and with an increased likelihood of developmental delay[53]. Of 15 patients reported to have a 11p15 duplication encompassing H19/IGF2 IG-DMR and KCNQ1OT1 TSS-DMR, four were noted to have hearing loss[74].

Differential diagnosis

The differential diagnosis of children with short stature of prenatal onset includes many syndromic diagnoses and chromosomal rearrangements[75] (TABLES 3,4). Particular features should prompt consideration of diagnoses other than SRS. These include relative microcephaly (head circumference SDS below height and weight SDS), notable global developmental delay or intellectual disability (without a related explanation such as documented hypoglycaemia), absence of severe feeding difficulties and/or the presence of additional congenital anomalies, facial dysmorphism or other features atypical of SRS. Disproportionate short stature is suggestive of skeletal dysplasia. Photosensitive skin rash or recurrent bronchopulmonary infections should prompt investigation for chromosome breakage disorders. As SRS is generally sporadic, a family history of growth failure and/or consanguinity might suggest an alternative underlying diagnosis[76]. The clinical features of the most important and/or likely differential diagnoses are summarized in TABLES 3,4.

A correct diagnosis can have extremely important implications for management. Response to GH treatment, if given, varies depending on the underlying syndromic diagnosis. For instance, GH treatment is contraindicated in patients with chromosome breakage disorders, such as Bloom syndrome, due to the associated risk of malignancy[77]. GH treatment in patients with SHORT syndrome has been reported to precipitate insulin resistance and subsequent type 2 diabetes mellitus[78]. An incorrect diagnosis of SRS leading to the recommendation of GH treatment could, therefore, have adverse consequences in these patients.

Three patients (one with no history of fractures) have been reported with clinical features of SRS but a molecular diagnosis of osteogenesis imperfecta, with a *COL1A1* mutation[79,80]. Both SRS and osteogenesis imperfecta can cause prenatal onset of growth failure, relative macrocephaly, large fontanelle, blue sclerae and body asymmetry. Both diagnoses should, therefore, be considered in patients with features overlapping both conditions.

3 Recommendations

3.1 An alternative syndromic diagnosis, and specific investigation for this diagnosis, should be particularly considered in patients with any of the following: additional features atypical of SRS, family history of growth failure and/or consanguinity. (A+++)

3.2 Patients with features of SRS overlapping with osteogenesis imperfecta should have a skeletal survey to look for additional evidence for osteogenesis imperfecta, with consideration of *COL1A1/2* gene testing. (A++)

Management

SRS leads to a wide spectrum of abnormal physical characteristics and functional abnormalities. Multidisciplinary follow up and early, specific, intervention are necessary for optimum management of this group of patients.

4 Recommendation

4.1 Patients with SRS should receive multidisciplinary care in a centre of expertise in SRS in coordination with their local centre. The multidisciplinary team should be composed of paediatric subspecialists such as an endocrinologist (coordinator), gastroenterologist, dietician, clinical geneticist, craniofacial team, orthopaedic surgeon, neurologist, speech and language therapist and psychologist. (A+++)

Early feeding and nutritional support

The typical neonate with SRS has length SDS below weight SDS; but after birth, due to poor appetite, feeding difficulties and gastrointestinal problems, weight SDS drops below the length SDS[4,17,73,81]. Over time, progressive failure to thrive can result in a calorie-related length deficit[4,15,82].

Feeding difficulties and failure to thrive are considerably more frequent in patients with SRS than in children with SGA but not SRS[4,17]. Failure to thrive in children with SRS is probably due to a combination of factors, including feeding difficulties (poor appetite, oromotor issues and the resulting low caloric intake) as well as functional and structural gastrointestinal problems. Digestive problems or malnutrition occur in over 70% of patients with SRS[82], including severe gastrooesophageal reflux in 55%, which often results in persistent vomiting after the age of 1 year. Constipation is also common, particularly after age 2 years[82]. Cyproheptadine used as an appetite stimulant improves weight gain in other paediatric conditions[83,84]; however, specific studies of its use in SRS are needed before it can be recommended in these patients.

The main therapeutic goals for the first 2 years of life in patients with SRS are nutritional support, prevention of hypoglycaemia and recovery of any calorie-related length or height deficit, which should be addressed before initiation of GH therapy (see following sections on prevention of hypoglycaemia and GH therapy). However, careful monitoring is needed, especially during nonvolitional feeding, because rapid catch-up weight gain in children born SGA has been associated with an increased risk of metabolic and cardiovascular disease in later life[85].

Children with SRS have an abnormal body composition with low muscle mass, and are typically light for their length or height[3,15,86,87]. From our experience, the target for healthy nutritional status is narrow, and is dependent

Table 3 | **Differential diagnosis of Silver–Russell syndrome in patients with relative microcephaly**

Feature	Syndrome (OMIM number)					
	Bloom syndrome (#210900)	**Nijmegen breakage syndrome (#251260)**	**MOPD II (#210720)**	**Meier–Gorlin syndrome (#224690, #61380, #613803, #613804, #613805)**	***IGF1R* mutation or deletion (#147370, #612626)**	***IGF1* mutation (#147440)**
Birth weight SDS	Mean: −4.6	Mean: −1.6	Mean: −3.9	Mean: −3.8	−1.5 to −4.9	−2.5 to −4.5
Adult height range (cm)	• Male patients: 128–164 • Female patients: 115–160	• Male patients: 161–172 • Female patients: 150–165	Mean: 96	• Male patients: 136–157 • Female patients: 127–150	*IGF1R* mutation: 1 female patient (140), 2 male patients (133 and 170)	1 male patient: 117
Cognitive function	Usually normal	At pre-school age IQ normal or borderline; progressive deterioration to moderate ID	Variable: none or mild ID (majority), occasionally severe ID	90% normal IQ, occasionally mild or moderate ID	Variable: normal (~50%), mild ID (25%), moderate or severe ID (25%)	Severe ID
Facial features	Narrow face with underdeveloped malar area and mandible, fairly prominent nose, sun-sensitive telangiectasia in malar distribution	Receding forehead, prominent mid-face, small mandible, up-slanting palpebral fissures, long nose and philtrum, large ears	Prominent, long, broad nose with hypoplastic tip, low insertion of columella, prominent eyes in infancy, micrognathia	Microtia, narrow, beaked nose with low insertion of columella, small mouth, retrognathia	• *IGF1R* mutation: often normal; triangular face, micrognathia. • 15q26-qter deletion: micrognathia	No consistent features reported
Other features	Patchy areas of hypopigmented and hyperpigmented skin, feeding difficulties, high tumour risk (44% develop cancer by age 25 years), hypogonadism, type 2 diabetes mellitus, immunodeficiency, chromosomal instability with increased frequency of sister chromatid exchange	Severe, progressive microcephaly, immunodeficiency, cancer predisposition, chromosomal instability and rearrangements, café au lait spots, premature ovarian failure	Mean OFC at birth −4.6 SDS, progressive microcephaly, mesomelic limb shortening, progressive metaphyseal bone dysplasia, hip dysplasia, acanthosis nigricans, insulin resistance, cryptorchidism, intracranial aneurysm, dental anomalies, squeaky voice	Patellar hypoplasia, pulmonary emphysema, cryptorchidism, mammary hypoplasia (post-pubertal 100%), hypoplastic labiae	• *IGF1R* mutation: pectus excavatum, 5th finger clinodactyly, short fingers • 15q26-qter deletion: fifth finger clinodactyly, short fingers, talipes, congenital heart disease, renal anomalies	Sensorineural deafness
Inheritance and molecular abnormality	• Autosomal recessive • Mutations in *RECQL3* • High prevalence in Ashkenazi Jewish population	• Autosomal recessive • Mutations in *NBN* • High prevalence in Slavic population	• Autosomal recessive • Mutations in *PCNT*	• Autosomal recessive • Mutations in *ORC1, ORC4, ORC6, CDT1, CDC6*	*IGF1R* mutation: majority autosomal dominant; compound heterozygosity reported in two patients	• Autosomal recessive • Mutations in *IGF1*

ID, intellectual disability; MOPD II, microcephalic osteodysplastic primordial dwarfism type II; OFC, occipito-frontal circumference; SDS, SD score.

on individual innate muscle mass and even slight overnourishment (for example, weight >90% of ideal weight for length or height) can rapidly increase relative fat mass. Suggested targets for children aged 2–4 years preparing for GH therapy are: weight 75–85% of the 50th centile weight for length or height and/or BMI 12–14 kg/m^2, using height measurements on the longer side if notable leg length discrepancy is found (see following section on GH therapy). A weight below 70% of the ideal weight for length or height compromises growth velocity, despite GH treatment. For children >4 years old, the optimal target BMI will depend on their muscle mass. Two groups of patients are exceptions to this observation. Firstly, in patients with 11p15 LOM who have a very low muscle mass and considerable body asymmetry, a lower BMI might be adequate (11–12 kg/m^2). Secondly, for patients with upd(7)mat with near normal muscle mass, a higher BMI might be acceptable (14–15 kg/m^2).

5 *Recommendations*

5.1 For nutritional goals in the first years of life, we recommend nutritional repletion* with awareness of possible hazards of rapid postnatal catch-up leading to subsequent increased metabolic risk. (A+++)

5.2 Ask for and/or screen early for gut dysmotility (gastrooesophageal reflux, delayed gastric emptying and constipation) in all children. (A+++)

Table 4 | **Differential diagnosis of Silver–Russell syndrome in patients with relative normocephaly or macrocephaly**

Feature	Syndrome (OMIM number)				
	3-M syndrome (#273750)	**Mulibrey nanism (#253250)**	**SHORT syndrome (#269880)**	**Floating harbour syndrome (#136140)**	**IMAGe syndrome (#614732)**
Birth weight SDS	Mean: −3.1	Mean: −2.8 (range −4.0 to 0.5)	Mean: −3.3	Mean: −2.5	−2.0 to −4.0
Adult height range (cm)	115–150	136–150	Mean: 154	• Female patients: 98–156 • Male patients: 106–164	• 1 male patient: 160 • 1 female patient: 143
Cognitive function	Normal	Mild motor and speech delay only	Normal	Delayed speech. Intellect variable: normal to significant ID	Normal or mild ID
Facial features	Anteverted nares, full lips, mid-face hypoplasia, long philtrum	Triangular face, frontal bossing	Micrognathia, high broad forehead, triangular-shaped face, deep-set eyes, prominent nose, low-set posteriorly rotated ears, hypoplastic nasal alae, facial lipodystrophy, thin hair	Triangular face, deep-set eyes, long eyelashes, bulbous nose, wide columella, short philtrum, thin lips	Frontal bossing, low-set ears, flat nasal bridge, short nose
Other features	Prominent heels (also in upd(7)mat), short broad neck, pectus deformity, short thorax, winged scapulae, hyperlordosis, hip dysplasia, subtle radiographic changes (slender long bones, tall vertebral bodies)	Hepatomegaly, yellow spots on retina, progressive restrictive perimyocarditis, insulin resistance, high pitched voice, slender long bones with thick cortex and narrow medullar channels, shallow sella turcica, increased tumour risk (particularly Wilms and ovarian stromal tumours)	Rieger anomaly, dental delay, partial lipodystrophy, transparent skin, dimples on elbows and buttocks, herniae, fifth finger clinodactyly, hyperextensible joints, hypogonadism, high pitched voice, type 2 diabetes mellitus, nephrocalcinosis, thin gracile bones	Delayed speech development with expressive language delay, considerably delayed bone age, broad fingertips	Congenital adrenal hypoplasia, metaphyseal and/or epiphyseal dysplasia, male genital anomalies
Inheritance and molecular abnormality	• Autosomal recessive • Mutations in *CUL7*, *OBSL1*, *CCDC8*	• Autosomal recessive • Mutations in *TRIM37* • High prevalence in Finnish population	• Autosomal dominant • Mutations in *PIK3R1*	• Autosomal dominant • Mutations in *SRCAP*	Imprinted – maternally inherited mutations in *CDKN1C*

ID, intellectual disability, SDS, SD score.

5.3 Diagnose and treat any oromotor and/or sensory issues that affect oral intake of food. (A+++)

5.4 In patients with severe feeding failure who are unresponsive to standard care, anatomical or functional disorders of the gastrointestinal tract, such as malrotation, should be excluded. (A+++)

5.5 Avoid enteral feeding by nasogastric or gastrostomy tube in a child capable of eating where there is adequate nutritional repletion. (A+++)

5.6 In cases of extreme feeding difficulties or gastrooesophageal reflux, consider enteral feeding by gastrostomy tube (with or without fundoplication) or low-profile transgastric jejunostomy as a last resort to protect against hypoglycaemia and/or malnutrition. (A+++)

5.7 In the case of enteral feeding, prevent excessive weight gain in both volitionally and nonvolitionally fed children. (A++)

*Low muscle mass makes typical BMI targets excessive in this population. Targets currently used in some centres include: Waterlow score 75–85% (REF. 88); weight-for-length SDS −2 to −1 in first year of life; BMI target SDS between −2 to −1 after first year of life.

Prevention of hypoglycaemia

Young children with SRS, particularly under age 5 years, have low muscle and liver mass, a disproportionately large brain-for-body size and feeding difficulties, all of which increase their risk of fasting hypoglycaemia and its potential neurocognitive consequences. The incidence of hypoglycaemia in these children is approximately 27%[20], with a high frequency of spontaneous, asymptomatic nocturnal hypoglycaemia[89].

Monitoring of levels of urinary ketones is usually effective in pre-empting hypoglycaemia related to fasting, activity or illness. This measurement can be used to determine the 'safe fasting time' for a child, which will change with age. Night time hypoglycaemia can be prevented by adding either high molecular weight glucose polymer (for infants under 10 months) or uncooked corn starch (for older infants and children particularly at risk) to the last evening feed. Dental hygiene is important as complex carbohydrates can promote cavities[90]. Severe, non-fasting and non-ketotic hypoglycaemia should always be identified and investigated further.

For episodes of preoperative fasting or febrile illness, intravenous glucose (10% dextrose) might be required.

Children with SRS might need longer periods of gut rest than children with SGA but not SRS before oral or enteral feeding because of their gut dysmotility and intrinsic feeding defects. Before discharge, it is advisable to achieve an absence of ketonuria following at least 12 h of feeding, without intravenous support. When hypoglycaemia remains a problem, early GH therapy should be considered[91,92] (see following section on GH therapy).

6 Recommendations

6.1 Monitoring for ketonuria at home is useful to determine which children need intervention for impending hypoglycaemia. (A++)*

6.2 Develop a plan with the child's local paediatrician and emergency room for rapid admission and intravenous dextrose treatment when the child is ill. (A++)

6.3 Admit children with SRS to hospital early in the course of an illness associated with ketonuria or hypoglycaemia and do not discharge them until they are metabolically stable and can be adequately fed. (A++)

6.4 Glucagon is not recommended to correct hypoglycaemia, because of poor glycogen stores and limited ability for gluconeogenesis. (A+++)

6.5 Provide parents with an emergency guidance plan for illnesses. (A+++)

6.6 Teach parents how to recognize signs of hypoglycaemia, measure ketones, determine the 'safe fasting time' for their child, prevent hypoglycaemia using complex carbohydrates and avoid fasting outside a controlled environment. (A+++)

6.7 In severe cases of fasting hypoglycaemia, where other causes have been excluded and if other alternatives are ineffective, consider:

- Early start of GH therapy to support glucose sources (increase in muscle mass and gluconeogenesis) (A++)
- Placement of a gastrostomy tube or jejunostomy tube. (A++)

*Children with a history of hypoglycaemia who do not have an appropriate ketone response will require formal fasting studies.

Surgery and anaesthesia

Any surgery should be carefully planned due to the increased risk of fasting hypoglycaemia in patients with SRS[93]. As a result of their diminished weight-for-height ratio, low BMI and large head, young patients with SRS are at risk of hypothermia in a cool operating room[94]. Many children with SRS also have abnormal tooth distribution and a small mandible, which affects airway visualization and intubation[95]. Finally, young children with SRS who are malnourished might not heal well following surgery[96].

7 Recommendations

7.1 Review issues related to SRS with the anaesthetist and surgeon in advance. (A+++)

7.2 Consider admission the night before surgery for early administration of intravenous dextrose before surgery to avoid ketonuria and hypoglycaemia. (A++)

7.3 Schedule first on the surgical list where possible. (A++)

7.4 Monitor blood glucose and administer intravenous dextrose during and after surgery. Do not discharge until ketonuria is absent and the child can sustain themselves on oral or enteral feeding. (A++)

7.5 Follow the intraoperative temperature maintenance protocol appropriate for the patient's size, not age. (A+++)

7.6 Delay elective surgery until the child is adequately nourished. (B+)

7.7 Be aware of the high risk of malnutrition after surgery and follow appropriate guidelines. (A+)

Growth hormone treatment

Data on adult height in untreated patients with SRS are limited; however, SRS is associated with a significant reduction in adult height (around −3 SDS; Supplementary information S4 (table)) (REF. 3). SRS is an indication for growth-promoting GH treatment under the SGA registered licence. It is worth noting that SRS was the only syndrome to be included in the clinical trials of GH in short children born SGA that led to the US FDA and the European Medicines Agency (EMA) SGA indications for GH therapy in 2001 and 2003, respectively[97–101]. The results of these clinical trials, therefore, validate the use of GH for patients with SRS.

Overall, clinical trials of GH treatment in patients with SGA (in which patients with SRS were included) demonstrated a satisfactory growth response and an increase in predicted adult height of 7–11 cm at pharmacological doses of GH[97–100,102]. However, the response in patients with SRS was not investigated until a Dutch longitudinal study analysed the response to GH in 62 children with a clinical diagnosis of SRS using the NH-CSS compared with 227 short, non-syndromic children born SGA. Overall, the study showed a similar response to GH in patients with SRS compared with non-SRS children born SGA (mean total height gains of 1.30 SDS and 1.26 SDS, respectively); however, the final adult height attained in patients with SRS was lower (mean adult height −2.17 SDS versus −1.65 SDS for non-SRS children born SGA)[87]. Although the mean height at the start of GH treatment in patients with SRS was statistically significantly lower than in those without SRS, it was shown that patients with all SRS subtypes benefited from GH treatment, with a trend towards increased height gain in patients with upd(7)mat or clinical SRS. In addition, some interim[100,102] and long-term[103,104] studies have focused on the response to GH specifically in patients with SRS, albeit without a control group of non-SRS short children born SGA. Strong predictors of the short-term and long-term responses to GH were age and height SDS at the start of GH treatment (both inversely related)[103–105]. However, the study by Rakover *et al.*[105] of 33 patients with SRS lacked data on adult height. Mean total height gain ranged from +1.2 to +1.4 SDS for GH doses of 35–70 μg/kg per day, which is similar to that achieved in

patients with non-syndromic SGA[97,103,104]. In 2007, an SGA consensus statement advocated early treatment with GH for children born SGA, including those with SRS, who had severe growth retardation (height SDS ≤2.5; age 2–4 years; dose 35–70 μg/kg per day)[106].

Additional potential benefits of GH treatment are increases in appetite, lean body mass and muscle power, which can result in improved mobility[86,107]. In patients with Prader–Willi syndrome, another imprinting disorder, GH treatment started in infancy results in increased lean body mass and motor development, as well as decreased fat mass[108,109]; consequently, GH treatment is now recommended from infancy in this condition. Children with SRS who are <2 years old typically present with low muscle mass and hypotonia, similarly to patients with Prader–Willi syndrome[15], and could also benefit from early GH treatment. Further studies are necessary to investigate this option in patients with SRS.

Classic GH deficiency is neither a common nor a relevant cause of short stature in SRS, nor is it predictive of the response to GH treatment in children born SGA[103,105,110]. Furthermore, given the risk of hypoglycaemia associated with fasting required for GH testing, testing children with SRS might carry added risks.

For most children with SRS, an increase in height velocity of ≥3 cm per year is the lower limit of an effective response range[106]. The growth response depends on the patient's age, GH dose, height deficit, rate of weight gain and confounding problems such as intercurrent illness and scoliosis.

Levels of insulin-like growth factor 1 (IGF1) in response to GH treatment in patients with SRS are difficult to interpret. Children with 11p15 LOM have significantly higher IGF1 levels than children with upd(7)mat and other children born SGA, which suggests an element of IGF1 resistance in patients with 11p15 LOM[73,111]. Basal serum levels of IGF1 in the upper quartile of the normal age-related range or higher can be expected in children with SRS, especially those with 11p15 LOM[73]. In children with 11p15 LOM, serum levels of insulin-like growth factor-binding protein 3 (IGFBP3) are also elevated[111]. IGF1 levels might rise significantly above the reference range in children with SRS on standard doses of GH[87,111]. Further studies are needed to understand how best to use IGF1 and IGFBP3 serum levels to monitor GH doses in children with SRS and IGF1 resistance.

Comprehensive reviews on the use of GH in children born SGA have concluded that GH treatment seems to be safe and effective[112]. Adverse effects due to GH treatment are no more frequent in children with SRS than in those with non-syndromic SGA[87,113] and no specific precautions are advised.

8 Recommendations

8.1 Defer GH treatment until caloric deficits are addressed. (A++)

8.2 Avoid GH stimulation testing. (A++)

8.3 Goals of GH treatment are to improve body composition (especially lean body mass), psychomotor development and appetite, to reduce the risk of hypoglycaemia, and to optimise linear growth. (A++)

8.4 Treat with GH as soon as possible; starting at age 2–4 years is adequate for the majority of patients; however, due consideration should be given to the exceptions listed below*. (A++)

8.5 Start GH at a dose of approximately 35 μg/kg per day. Use the lowest dose that results in catch-up growth. (A+++)

8.6 Terminate GH therapy when height velocity is <2 cm per year over a 6-month period and bone age is >14 years (female patients) or >17 years (male patients). (A++)

8.7 If response to GH is poor, re-evaluate the underlying diagnosis, GH dose, IGF1 response, adherence to therapy and other confounding systemic problems. (A+++)

8.8 Monitor circulating levels of IGF1 and IGFBP3 at least yearly during GH treatment. (A++)

*GH treatment does not have a specific indication for SRS and is prescribed under the SGA indication (height SDS −2.5; age >2–4 years; dose 35–70 μg/kg per day)106. Exemptions from the current SGA licensed indication used in some centres include starting GH therapy below the age of 2 years in case of: severe fasting hypoglycaemia; severe malnutrition, despite nutritional support, which will lead to gastrostomy if no improvement is seen; and severe muscular hypotonia.

Bone age advancement and puberty

The published literature on the natural history of bone age progression in patients with SRS is limited. Early bone age delay is followed by rapid advancement typically at around 8–9 years of age[3,81,113] but sometimes much younger, especially in nonvolitionally overfed children. Onset of puberty is usually within the normal range (8–13 years in girls and 9–14 years in boys)[114] but at the younger end of the spectrum[3,73,87,115]. Adrenarche can be early and aggressive in comparison with children born with non-SRS SGA, particularly in those with 11p15 LOM[116].

Our experience is that in patients with SRS and early adrenarche, the onset of central puberty might be earlier and the tempo faster than expected. In the past few decades, population studies analysing the timing of normal puberty observed a mean age of puberty onset of 9.7–10.0 years in girls[114]. As a group, girls with SRS seem to start central puberty at a mean age of 9.1 years (I. Netchine, unpublished work). This early puberty further accelerates bone age maturation, which leads to an attenuated pubertal growth spurt and compromised adult height. Children with upd(7)mat are likely to progress to central puberty at an even younger age than patients with SRS and 11p15 LOM (mean starting age 8.5 years in girls and 9.5 years in boys) (I. Netchine, unpublished work). A rapid increase in BMI might also exacerbate the tendency to early adrenarche and central puberty[117–119].

The window for effective GH treatment seems to be shorter in patients with SRS than in non-SRS patients with SGA. In a study comparing a cohort of patients with SRS and a cohort of patients born SGA but without SRS, puberty started significantly

earlier in the former (at 10.2 years versus 11.2 years in girls with SRS and non-SRS SGA, respectively, and at 11.4 years versus 12.0 years in boys with SRS and non-SRS SGA, respectively)[87]. Furthermore, a steeper decline in height SDS from the onset of puberty until adult height was seen in patients with SRS, which contributed to a lower adult height and a larger distance to target height than in non-SRS patients with SGA. However, in 17 patients with SRS in this study, puberty was postponed for 2 years with gonadotropin-releasing hormone analogue (GnRHa) due to a low predicted adult height. The effect of GnRHa on final height has been analysed in a cohort of patients with SGA, including patients with SRS[120,121]. This analysis suggested that the combination of GnRHa, started at the initiation of puberty and continued for at least 2 years, along with GH treatment, improves adult height in patients born SGA with a poor adult height prognosis. A retrospective study of GnRHa treatment specifically in patients with SRS did not detect an effect of GnRHa on adult height, but this therapy was used in only 16 of 37 patients and was not standardized[104]. Further studies are required to specifically look at its effects in patients with SRS.

Aromatase catalyses the rate-limiting step in the conversion of androstenedione to oestrone and testosterone to oestradiol. In patients with adrenarche with advancing bone age, but without central puberty, third-generation aromatase inhibitors (such as anastrozole) might be helpful in preventing rapid bone maturation, but are currently not licensed for growth disorders[122]. An 18-month double-blind clinical trial is currently underway to study the efficacy and tolerance of treatment with anastrozole to slow bone maturation related to pathological adrenarche in patients with SRS and Prader–Willi syndrome[123].

9 Recommendations

9.1 Monitor for signs of premature adrenarche, fairly early and accelerated central puberty, and insulin resistance. (A+++)

9.2 Monitor and anticipate acceleration of bone age especially from mid childhood. (A++)

9.3 Consider personalized treatment with GnRHa for at least 2 years in children with evidence of central puberty (starting no later than age 12 years in girls and age 13 years in boys) to preserve adult height potential. (A++)

Long-term metabolic complications

Individuals born with a low birth weight are at increased risk of adult health problems including coronary heart disease[124–126], hypertension, dyslipidaemia, insulin resistance and obesity (the metabolic syndrome)[127–130]. Studies of children born SGA indicate that those who have rapid or disproportionate catch-up in weight are at particularly high risk[119,131,132].

Insulin resistance in young, pre-pubertal, children with SRS can be atypical and difficult to detect in the fasting state; however, impaired glucose tolerance can be confirmed on formal oral glucose tolerance testing[133,134]. Insulin resistance becomes more classic in the pubertal or post-pubertal age groups with elevation in fasting levels of glucose and insulin, and possibly the development of type 2 diabetes mellitus[135,136].

Overall, GH therapy seems to have positive metabolic effects in children born SGA[137], but specific data on such effects in SRS are lacking. Many studies of long-term GH treatment in children born SGA have shown positive outcomes, including increased lean body mass, reduced fat mass, decreased blood pressure and an improved lipid profile[107,120,137,138], which might last after discontinuation of therapy[138,139].

In a study of 110 children born SGA treated with GH, those with the highest baseline levels of IGF1 were the least insulin sensitive. Gains in height and IGF1 response were positively associated with insulin secretion[140]. In SRS, children with 11p15 LOM seem to be at a higher metabolic risk than children who have upd(7)mat and other children born SGA due to poor muscle mass and raised levels of IGF1 (REFS 15,16,73,87). Further research is, therefore, required on the long-term effects of GH therapy on body composition and metabolic parameters in SRS and its various genotypes.

10 Recommendations

10.1 Avoid excessive or rapid weight gain to prevent increased insulin resistance, which is associated with early and rapidly advancing adrenarche, early central puberty, and, in girls, a future risk of developing polycystic ovary syndrome. (A++)

10.2 Raise awareness among gastroenterologists, dieticians, neonatologists, paediatricians and primary health-care providers of the importance of not overfeeding this group of children. (A+++)

10.3 Advise parents, grandparents and care-givers about the risk of insulin resistance associated with intrauterine growth retardation and overfeeding. (A+++)

10.4 Screen for physical and biochemical indicators of insulin resistance during GH treatment, especially in children with low muscle mass and high baseline levels of IGF1. (A+)

10.5 In patients with clinical signs of insulin resistance, consider formal assessment of insulin sensitivity with a 2-h oral glucose tolerance test including measurement of insulin and C-peptide levels (A++)

10.6 Advocate a healthy diet and lifestyle in older children and young adults with particular emphasis on protein calorie balance and regular exercise to avoid disproportionate weight gain, particularly after discontinuation of GH treatment. (A+++)

Neurocognitive problems

Motor and speech delay are common in children with SRS[4,16–18,20] (TABLE 2). Motor delay might be related to reduced muscle bulk and fairly large head size. Verbal dyspraxia and more global developmental delay or learning difficulties, usually mild, have been described in some children with SRS, particularly those with upd(7)mat[12,15,16,20,141]. Autistic spectrum disorder has also been reported more frequently in this subgroup than in

the other subgroups of SRS[15]. Myoclonus dystonia in patients with upd(7)mat is probably associated with altered expression of the paternally expressed *SGCE* on chromosome 7q21 (REFS 20,40,142,143).

11 Recommendations

11.1 Refer infants and children with SRS for a developmental assessment when necessary to ensure appropriate intervention as early as possible. (A+++)

11.2 In patients with upd(7)mat, check for symptoms of myoclonus dystonia at each clinical appointment and refer early to a paediatric neurologist if required. (A+++)

11.3 Monitor children with upd(7)mat for signs of verbal or oromotor dyspraxia and/or signs of autistic spectrum disorders. (A+++)

11.4 Inform parents about increased risk of speech, oromotor and learning disabilities (especially in those with upd(7)mat). (A+++)

11.5 Follow up school-age children for any learning difficulties, psychosocial challenges and/or cognitive delay, to enable appropriate intervention. (A+++)

Orthopaedic problems

Orthopaedic problems seen in association with SRS include limb or body asymmetry, scoliosis, hip dysplasia and hand and/or foot anomalies (TABLE 2).

Limb asymmetry can affect the arms, legs or both. In seven patients with clinically diagnosed SRS, limb length discrepancy was not significantly affected by GH treatment[144]. Limb lengthening surgery performed to equalize limb lengths in patients with SRS has shown positive results[145].

Scoliosis has been reported in 9–36% of individuals with SRS[20,146,147]. The causal relationship to leg length asymmetry is not clear[146,147]. Associated back pain has been reported inconsistently[5,146]. GH therapy might be associated with worsening of existing scoliosis; however, causality has not been established[148]. A study in a large group of children with Prader–Willi syndrome (an imprinting disorder with clinical features that overlap with those of SRS: growth failure; infant hypotonia; early feeding difficulties; and an increased risk of scoliosis) has clearly shown that GH therapy does not influence onset and progression of scoliosis[149]; however, specific studies are required to determine whether GH therapy modifies the risk of scoliosis in patients with SRS.

12 Recommendations

12.1 Where necessary, refer to a paediatric orthopaedic surgeon for collaborative management of body asymmetry, limb length discrepancy and scoliosis. (A+++)

12.2 Routinely examine all patients with SRS for scoliosis. (A+++)

12.3 Before initiation of GH therapy, refer patients with scoliosis to the orthopaedic team and monitor while receiving GH. (A+++)

12.4 Evaluate leg length asymmetry regularly and consider orthopaedic management if necessary. (A++)

Maxillofacial abnormalities

SRS is characterized by craniofacial disproportion, which results in a triangular-shaped face[95]. Delayed dental eruption, microdontia, absence of secondary teeth and blunted condyles have all been reported in patients with SRS[150–152].

In our experience, the upper jaw arch is frequently narrow and crowded, but crowding might be severe in the lower arch, with displacement of lower incisors into a lingual position. Micrognathia is frequent, with lack of mandibular growth, which results in a small, pointed chin and an overbite. Children with notable facial asymmetry might have a crossbite that impairs normal chewing. Velopharyngeal insufficiency with or without a submucous cleft is quite common in patients with 11p15 LOM SRS[20]. Otitis media is frequent in young children with SRS[7] and seems to be improved by orthodontic treatment[20].

Orthodontic intervention in children with SRS can help normalize oropharangeal function and facial appearance. An experienced craniofacial team, including orthodontists, plastic surgeons and ear, nose and throat surgeons is ideal. Multiple orthodontic techniques have been used successfully[153]. Currently, rapid palatal expansion is the most effective technique to change the shape of the face[154].

Many patients with SRS report excessive daytime fatigue, snoring and/or disrupted sleep. However, data are very limited regarding sleep problems, including sleep disordered breathing, in association with SRS. A retrospective study identified mild sleep disordered breathing in 74% of patients with SRS (not exacerbated with GH therapy)[155]. Further studies are necessary.

13 Recommendations

13.1 Develop a referral relationship with a maxillofacial team or orthodontist who has experience caring for patients with SRS. (A++)

13.2 Refer patients to the maxillofacial team for assessment after eruption of primary dentition when necessary. (A++)

13.3 Encourage early orthodontic intervention and compliance with follow-up. (A+)

13.4 Screen for symptoms of sleep disordered breathing (such as snoring, apnoeas, excessive daytime fatigue, disrupted sleep and agitation). (A++)

13.5 Refer patients with suspected sleep disordered breathing to the appropriate specialist for evaluation of obstructive sleep apnoea. (A++)

Other congenital anomalies

Congenital anomalies have been described in a minority of patients with SRS, particularly those with 11p15 LOM (see Supplementary information S1 (table)). Genital abnormalities, including cryptorchidism and hypospadias, occur frequently in boys[16,20]. Mayer–Rokitansky–Kuster–Hauser syndrome in female patients is characterized by congenital hypoplasia or aplasia of the uterus and upper part of the vagina[16,18,156,157]. Structural renal anomalies[18,20] and congenital heart defects[4,18,20,158] have also been reported.

Table 5 | **Checklist for management of patients with Silver–Russell syndrome**

Management issue	At diagnosis	0–2 years	2–10 years	10–18 years
General				
Document molecular subtype	R	N/A	N/A	N/A
Provide support group information	R	N/A	N/A	N/A
Genetic counselling for parents	R	N/A	N/A	N/A
Feeding and growth				
Exclude feeding difficulties	R	R	C	C
Ensure nutritional repletion	R	R	R	R
Screen for gut dysmotility	R	R	C	C
Screen for oromotor or sensory issues	R	C	C	C
Avoid rapid postnatal and/or childhood weight gain	R	R	R	R
Measure head circumference	R	R	R	R
Measure and monitor linear growth	R	R	R	R
Calculate and monitor BMI	R	R	R	R
Screen for symptoms and/or signs of hypoglycaemia	R	R	C	C
Consider growth hormone treatment	R	C	R	R
Monitor IGF1 or IGFBP3 levels (more than yearly)	R	C	R	R
Monitor clinically (with or without biochemical testing) for insulin resistance	R	N/A	R	R
Adrenarche and puberty				
Monitor clinically for early adrenarche	R	R	R	N/A
Anticipate early bone age advancement	R	N/A	R	R
Consider treatment of early or rapid central puberty	R	N/A	R	C
Other medical issues				
Monitor for symptoms of sleep disordered breathing	R	R	R	R
Orthodontic or dental	R	C	R	R
Ear, nose and throat	R	C	C	C
Neurodevelopment				
Developmental assessment	R	R	C	C
Screen for myoclonus dystonia*	R	R	R	R
Speech and language evaluation	R	R	R	C
School progress	R	N/A	C	C
Monitor for speech, motor and cognitive difficulties	R	C	R	C
Psychosocial evaluation	R	N/A	C	C
Musculoskeletal				
Limb length discrepancy or asymmetry	R	C	C	C
Scoliosis	R	C	C	C
Screen for hip dysplasia	R	R	C	C

*upd(7)mat only. C, consider assessment, depending on the clinical features of the patient; N/A, not applicable; R, recommend assessment (unless N/A to age group).

14 Recommendations

14.1 Investigate genital abnormalities in boys. (A+++)

14.2 Investigate girls with primary amenorrhoea for Mayer–Rokitansky–Kuster–Hauser syndrome. (A+++)

Adulthood

Very little information exists in the literature regarding the long-term natural history of SRS. The majority of individuals with SRS are not routinely followed up, and the small numbers of adults reported have few medical problems. However, it is well recognized that being SGA at birth with accelerated gain in weight for length, particularly during early life, increases the risk of metabolic problems in adulthood[119,132,159] (see previous discussion). Medical problems reported in adult patients with 11p15 LOM include hypertension, dilated cardiomyopathy, type 2 diabetes mellitus, hypercholesterolaemia, fatty liver infiltration, elevated glucose levels and raised HbA_{1c} levels[135,136,160]; however, these reports might not be representative of the population as a whole.

15 Recommendations

15.1 Consider medical follow-up of adolescents and young adult patients with SRS or develop collaboration with a general or internal medicine team for follow-up. (A+++)

15.2 Avoid losing contact with adult patients with SRS, to facilitate their participation in, and potential benefit from, future clinical research. (A+++)

Genetic counselling

Accurate genetic counselling depends on the underlying molecular cause. 11p15 LOM is associated with a low recurrence risk (with parents of a child with SRS being unlikely to have another affected child). The offspring risk is also low (meaning that individuals with SRS are unlikely to pass the condition on to their children). However, empirical figures are not available. Only three sibships with 11p15 LOM are reported in the literature[13,20], and the underlying mechanism is unknown in all three.

The potential for a familial *trans*-acting gene mutation suggests that the recurrence risk in patients with SRS and MLID could be higher than in other patients with SRS; however, evidence to support this supposition does not yet exist.

Rare familial cases of SRS have been reported with underlying mechanisms including: maternally inherited 11p15 duplication[24,26] (see Supplementary information S2 (table)); maternally inherited *CDKN1C* gain-of-function mutations[60]; and paternally inherited *IGF2* loss-of-function mutations[61]. In these families, the risk of recurrence might be as high as 50%[24,26,60,61]. Investigation for underlying CNVs in patients with 11p15 LOM is, therefore, important. upd(7)mat is associated with a low recurrence and offspring risk (if the karyotype of the patient is normal)[50]. Data are limited regarding the risk of parents of children with clinically diagnosed SRS having another child with SRS; however, the overall risk is probably low. Similarly, the offspring risk for individuals with clinically diagnosed SRS is likely to be low.

Box 2 | **Future research directions for SRS**

Clinical

- Incidence and/or prevalence
- Frequency of associated features (for example, scoliosis, sleep disordered breathing, developmental delay, behavioural issues)
- Frequency and associated phenotype of molecular subtypes, including:
  - 11p15, upd(7)mat
  - MLID
  - 14q32 abnormalities, upd(20)mat, upd(16)mat
- Clinical overlap with other imprinting disorders

Molecular

- Development of testing methodology
- Identification of additional molecular causes in patients with clinically diagnosed SRS
- Prenatal testing: methodology, ethical implications

Management

- Use of cyproheptadine as an appetite stimulant
- Optimal timing of GH use
- Interpretation of IGF1 levels
- Role of aromatase inhibitors to control bone age advancement
- GnRH analogue inhibition of central puberty
- Control of postnatal weight gain
- Limb lengthening

SRS in adulthood

- Natural history, including risk of the metabolic syndrome
- Quality of life indicators
- Reproductive issues (assisted reproductive technology, recurrence risk associated with MLID)

GH, growth hormone; GnRH, gonadotropin-releasing hormone; IGF1, insulin-like growth factor 1; MLID, multi-locus imprinting disturbance; SRS, Silver–Russell syndrome.

16 Recommendation

16.1 Genetic counselling should be performed by a health professional experienced in the field of imprinting disorders. As the recurrence risk associated with CNVs is dependent on their size, location and parental origin, these should be taken into consideration during counselling for the family. (A+++)

Conclusions

Children with SRS and their families face challenges from birth to adulthood. In addition to the problems associated with being born SGA, clinicians treating patients with SRS need to be aware of syndrome-specific management issues. These include substantial feeding difficulties, severe postnatal growth failure with no catch-up, recurrent hypoglycaemia, premature adrenarche, fairly early and rapid puberty, insulin resistance, body asymmetry, orthodontic issues, sleep disordered breathing and the potential for other congenital anomalies.

Presented here are the first international consensus guidelines for the diagnosis and management of SRS, based on published evidence and expert opinion. A summary of all 72 recommendations, including a flow chart for the investigation and diagnosis of SRS, is available as supplementary information online (see Supplementary information S5).

These management recommendations apply to all patients clinically diagnosed with SRS, both with and without a molecularly confirmed diagnosis. However, identification of the underlying molecular subtype can guide treatment with regard to specific risk factors. Management should involve a multi-disciplinary approach and close parental guidance. A practical checklist for use in routine clinical follow up of these patients is proposed in TABLE 5.

As published data specific to SRS are limited, many questions remain (BOX 2). International collaboration and further research is urgently needed to better inform the investigation and management of patients with SRS in the future.

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Author contributions
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Competing interests
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FURTHER INFORMATION
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HUMAN GENETICS

Transcriptional profiling at the *DLK1/MEG3* domain explains clinical overlap between imprinting disorders

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Imprinting disorders (IDs) often affect growth in humans, leading to diseases with overlapping features, regardless of the genomic region affected. IDs related to hypomethylation of the human 14q32.2 region and its *DLK1/MEG3* domain are associated with Temple syndrome (TS14). TS14 is a rare type of growth retardation, the clinical signs of which overlap considerably with those of Silver-Russell syndrome (SRS), another ID related to *IGF2* downregulation at 11p15.5 region. We show that 14q32.2 hypomethylation affects expression, not only for genes at this locus but also for other imprinted genes, and especially lowers *IGF2* levels at 11p15.5. Furthermore, expression of nonimprinted genes is also affected, some of which are also deregulated in SRS patients. These findings highlight the epigenetic regulation of gene expression at the *DLK1/MEG3* domain. Expression profiling of TS14 and SRS patients highlights common signatures, which may account for the clinical overlap observed between TS14 and SRS.

INTRODUCTION

Genomic imprinting is a physiological process defined as the monoallelic expression of a gene according to its parental origin, under the control of a differentially methylated region (DMR), known as the imprinting control region (ICR) (*1*). More than 150 human genes have been shown to be imprinted. Imprinting disorders (IDs), caused by disturbances of imprinted genes, are a group of congenital diseases affecting growth, development, and metabolism in humans, leading to diseases with overlapping features, regardless of the genomic region affected (*2*). Some of this overlap may be explained by the co-regulation of imprinted genes, which belong to an imprinted gene network (IGN) involved in the control of cellular proliferation and differentiation (*3*). Recent studies in mammals have shown how the disturbance of one imprinted gene can affect other maternally expressed genes (MEGs) or paternally expressed genes (PEGs) (*4*–*6*). The overlap between Silver-Russell syndrome (SRS) (*7*) and Temple syndrome (TS14) (*8*) is a particularly demonstrative example of clinical overlap between IDs. Both these syndromes include fetal and postnatal growth retardation, early feeding difficulties, early puberty, and an increase in the risk of metabolic disorders (Fig. 1A) (*8*, *9*). Moreover, TS14 patients also have a number of clinical features in common with another ID, Prader-Willi syndrome (PWS), a differential diagnosis for TS14 (Fig. 1A) (*8*). Most SRS patients carry molecular changes in the 11p15.5 region (Fig. 1B), the most prevalent (~50%) of which is hypomethylation of the DMR *H19/IGF2*:IG-DMR (hereinafter referred to as ICR1), decreasing paternal *IGF2* (a potent fetal growth factor) expression and increasing the maternal expression of *H19* (*10*), encoding a lncRNA. TS14 patients present molecular abnormalities at the paternally methylated imprinted locus on chromosome 14q32.2 (Fig. 1C). The most frequent of these abnormalities is maternal uniparental disomy or upd(14)mat. Paternal deletions of the imprinted *DLK1/MEG3* domain and *DLK1/MEG3*:IG-DMR (hereinafter referred to as IG-DMR) hypomethylation are less frequent (*8*). Last, PWS patients present disturbances of imprinted genes at the *SNRPN* locus and its ICR (IC-*SNRPN*) in the 15q11-q13 region (Fig. 1D), resulting in a loss of the expression of *SNRPN/SNURF* and *IPW*, two PEGs mapping to the minimal deletion interval critical for PWS (*6*). A recent clinical study published in 2017 on a large cohort of 32 TS14 patients with 14q32.2 genetic- and epigenetic-related defects revealed both PWS- and SRS-like phenotypes in 50% of patients (*11*). Moreover, we have recently shown that chromosome 14q32.2 imprinting defects are an alternative molecular diagnosis of SRS (*12*). Evidence is accumulating that these methylation defects in patients with SRS and TS14 are not isolated events, with some patients having multilocus imprinting disturbances (MLIDs) affecting additional imprinted regions (*12*, *13*).

Human chromosome 14q32.2 encompasses an imprinted region containing three PEGs (*DLK1*, *RTL1*, and *DIO3*) and a number of MEGs. All the MEGs encode noncoding RNAs (ncRNAs) (*MEG3*/*GTL2*, *MEG8*, *MEG9*, and *RTL1AS*) and several large clusters of microRNAs (miRNAs) and small nucleolar RNAs (snoRNAs) (*14*). The monoallelic parent-specific expression of these genes is controlled by the germline–derived primary intergenic IG-DMR and the postfertilization-derived secondary *MEG3*-DMR, both of which are methylated on the paternal allele and unmethylated on the maternal allele (*15*). PEGs from the 14q32.2 region play a crucial role in cell differentiation and tissue development, whereas the function of the MEGs remains unclear (*16*). It has been shown that the hypermethylation of the IG-DMR results in the reactivation of the normally silenced maternal allele of PEGs and a loss of expression of MEGs (*17*). The effect of ICR1 hypomethylation on the expression of *IGF2/H19* domain genes has been determined for SRS patients (*10*), but the effect

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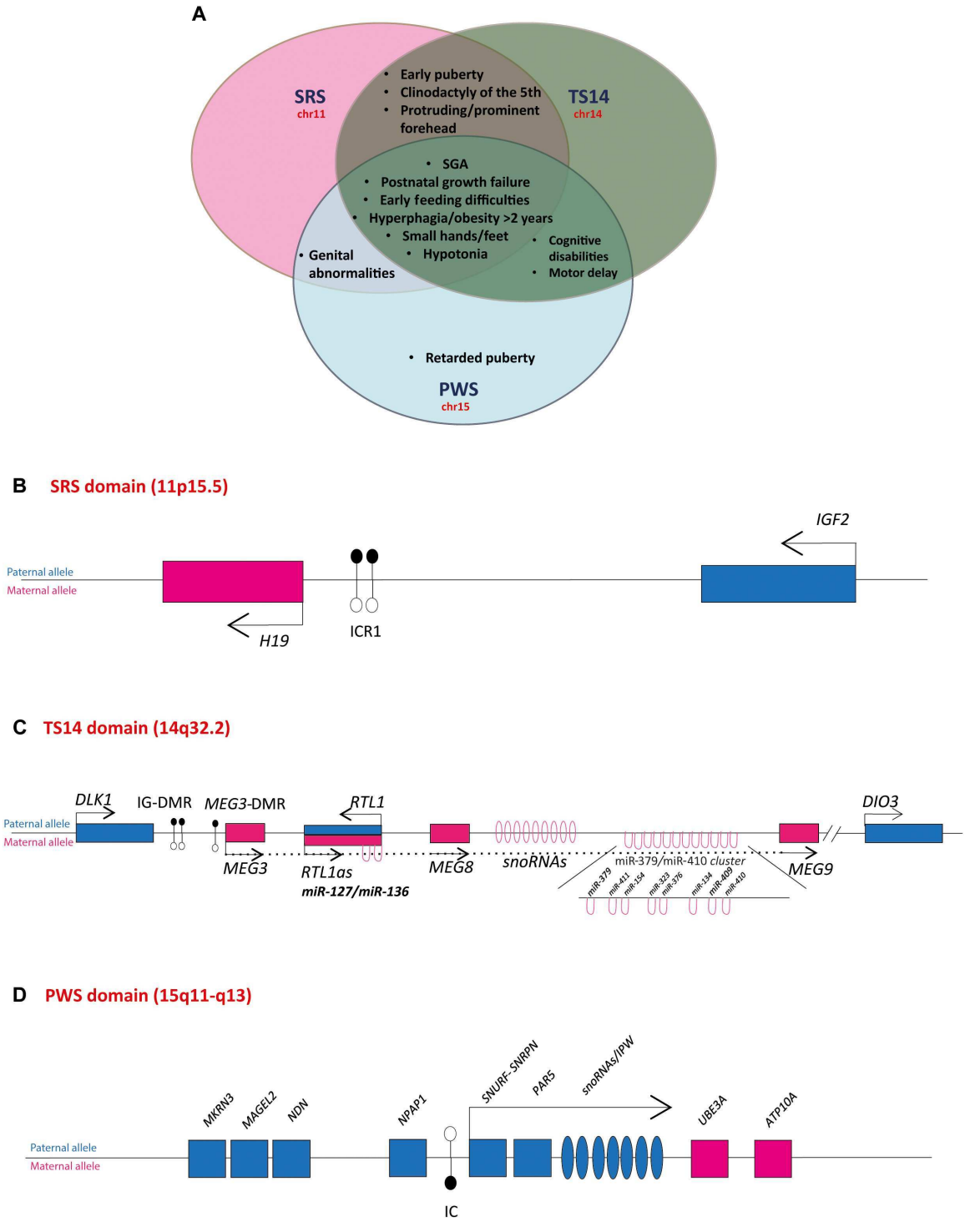

Fig. 1. Overlap of clinical features between SRS, TS14, and PWS and DMRs with parent-specific gene expression from the *DLK1/MEG3*, *IGF2/H19*, and *SNURF/IPW* domains. (**A**) Schematic representation of the overlapping clinical features in SRS, TS14, and PWS patients. Schematic diagram of the regions imprinted in humans (**B**) the *IGF2/H19* domain on 11p15.5 and (**C**) the *DLK1/MEG3* domain of the 14q32.2 region. The relative positions of hairpin-like [pre-microRNA (miRNA)] structures within the miR-379/miR-410 cluster are indicated in the enlargement in the inset and (**D**) the *SNURF/IPW* domain on 15q11-q13. PEGs are shown as blue rectangles, and MEGs are shown as pink rectangles. miRNAs and snoRNAs (small nucleolar RNAs) are depicted as stem loops and ovals, respectively. Arrows indicate the direction of transcription. The DMRs ICR1, IG-DMR, *MEG3*-DMR, and IC, which control monoallelic expression over the domains, are indicated by closed and open lollipops (methylated and unmethylated, respectively). SGA, small for gestational age.

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of IG-DMR hypomethylation has yet to be determined in TS14 patients.

We studied gene expression following the hypomethylation of IG-DMR to characterize the effect of this epigenetic alteration on *DLK1/MEG3* domain gene expression. We performed expression profiling for imprinted and nonimprinted genes in human fibroblasts from TS14 (IG-DMR hypomethylation) and SRS (ICR1 hypomethylation) patients (Fig. 2) to identify possible gene expression signatures common to these two IDs, which present a major clinical overlap.

RESULTS

TS14 patients' molecular diagnostics and collected biological materials

We collected serum from seven TS14 patients with IG-DMR and *MEG3*-DMR hypomethylation (n = 5) or 14q32.2 paternal deletion (n = 2). We also established fibroblast cell cultures for four TS14 patients with IG-DMR hypomethylation, one SRS/TS14 patient with both 11p15.5 ICR1 and IG-DMR hypomethylation, five SRS patients with ICR1 hypomethylation, and five controls (cells were provided by Coriell Cell Repositories). Clinical data and methylation levels for all patients and controls are listed in tables S1 and S2, respectively.

DLK1 is absent from the serum of TS14 patients but present in that of age-matched controls

DLK1 is a single-pass transmembrane protein that can be cleaved by extracellular proteases to release a circulating form (*18*). We assessed the effect of *DLK1/MEG3* domain hypomethylation on *DLK1* expression by first measuring the circulating levels of DLK1 in the serum of healthy children (n = 38, 19 boys and 19 girls) between the ages of 0 and 17 years. We found that serum DLK1 levels decreased considerably after birth, but those patients with paternal deletions or hypomethylation of the *DLK1/MEG3* domain had barely detectable levels of DLK1, regardless of their sex, age, or the molecular defect at 14q32.2 (Fig. 3A).

MEG3, MEG8, and miRNAs are up-regulated because of their expression from the normally silenced paternal allele

We then investigated the levels of transcription of 14q32.2 MEGs in cultured fibroblasts from TS14 and SRS patients (PEGs were not expressed in fibroblasts). We found that the levels of the lncRNA *MEG3* and the ncRNA *MEG8* in all TS14 and SRS/TS14 fibroblasts were at least twice those in fibroblasts from controls (Fig. 3, B and C). SRS fibroblasts had normal *MEG3* levels, but *MEG8* levels were twice those in control fibroblasts (Fig. 3, B and C). In addition to *MEG3* and *MEG8*, we quantified nine miRNAs (encoded by genes distributed throughout the 14q32.2 region), all of which were found

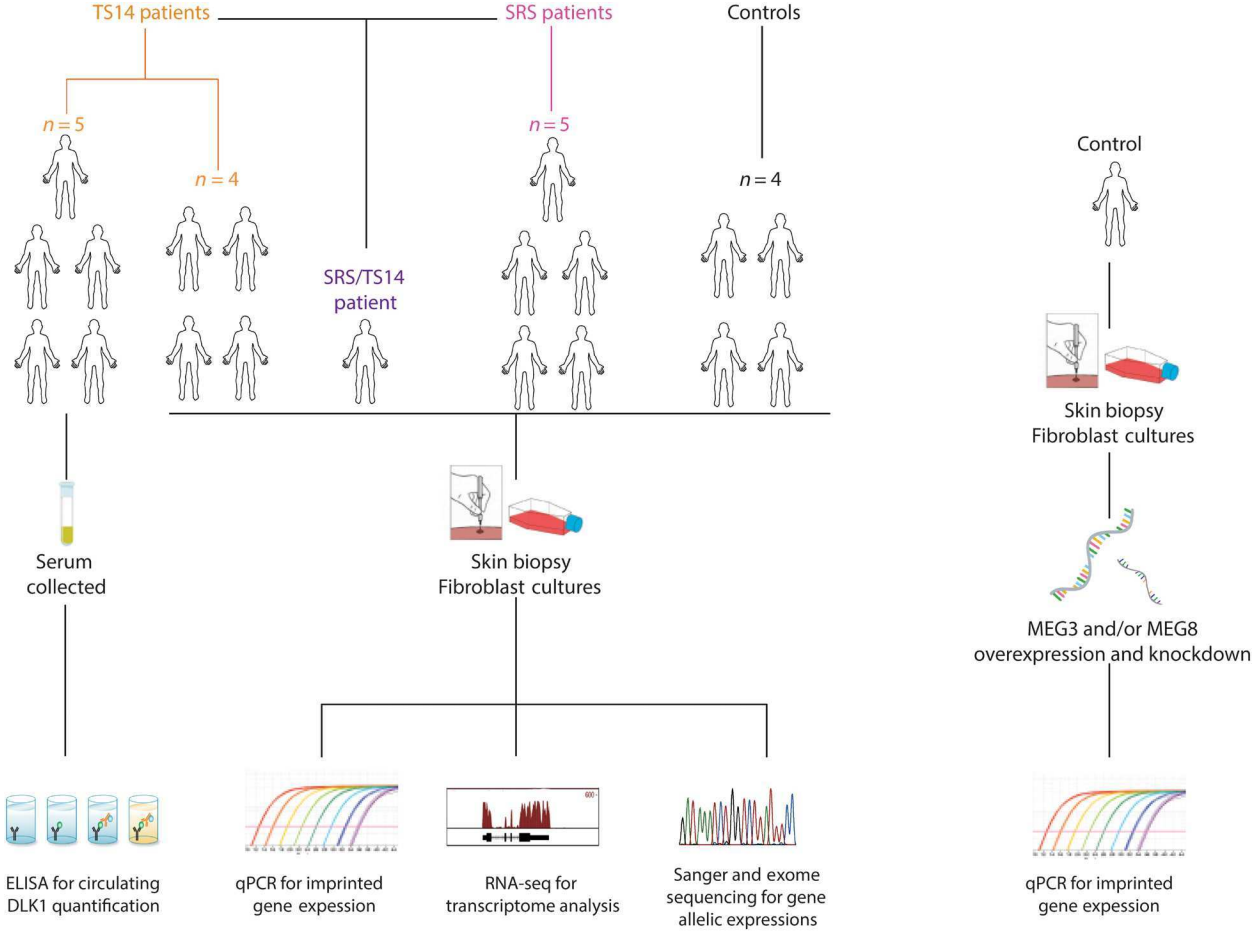

Fig. 2. Schematic presentation of the patients, biological materials, and strategies used in the study. ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative polymerase chain reaction; RNA-seq, RNA sequencing.

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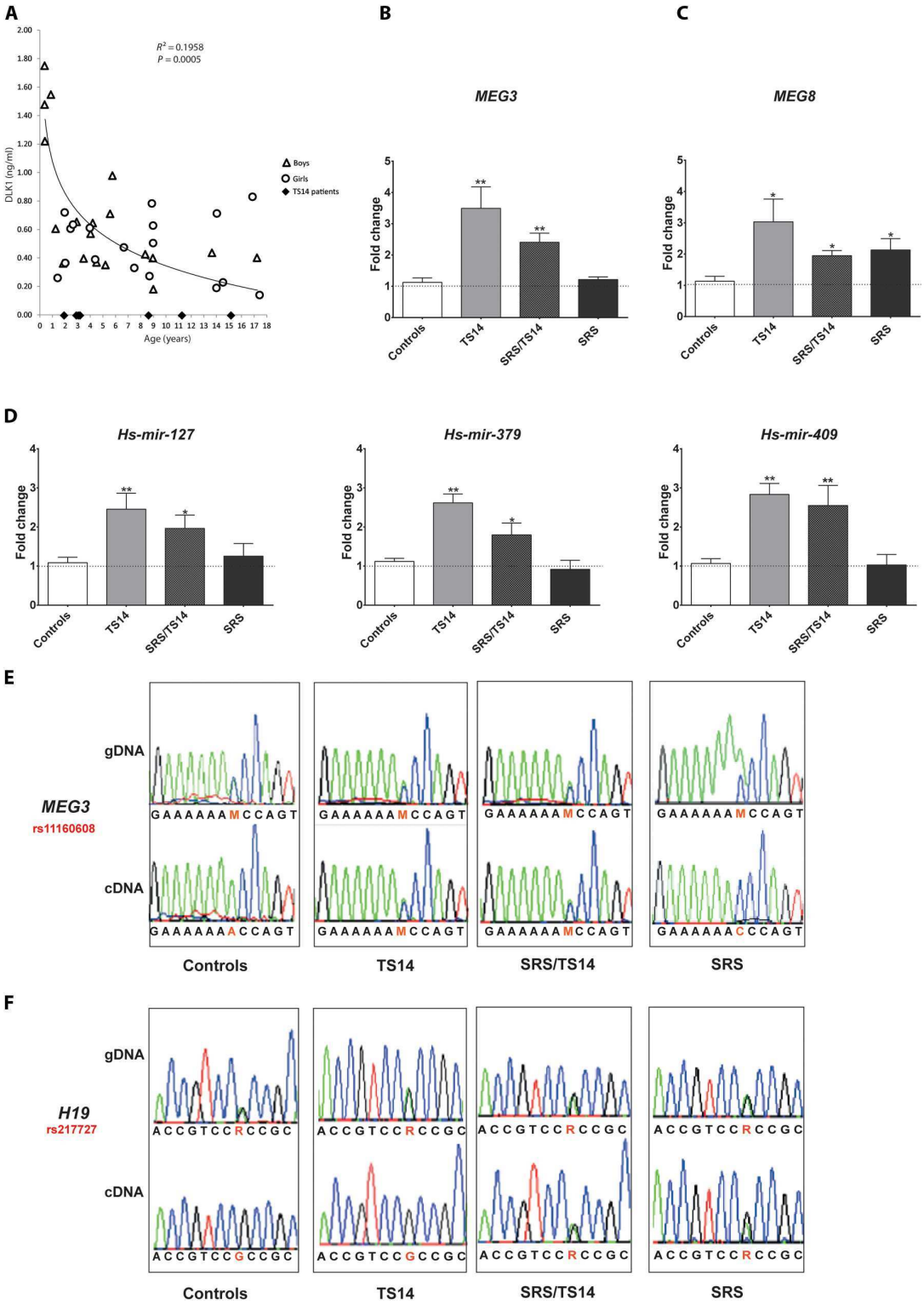

Fig. 3. Expression profiling of 14q32.2 genes from the serum and fibroblasts of TS14 patients. (**A**) DLK1 is absent from the serum of TS14 patients but present in that of age-matched controls. Boys and girls are indicated by open triangles and circles, respectively. TS14 patients are represented by black diamonds. (**B** to **D**) *MEG3*, *MEG8*, and miRNAs are up-regulated in TS14 patient fibroblasts. Relative levels of expression for *MEG3*, *MEG8*, and three miRNAs of the *DLK1/MEG3* domain in skin-derived fibroblast cultures from TS14, SRS/TS14, and SRS patients, compared with control fibroblasts. (**E** and **F**) *MEG3* and *H19* are biallelically expressed upon the hypomethylation of 14q32.2 and 11p15.5, respectively. Electropherogram showing the informative SNPs rs11160608 and rs217727, within the *MEG3* (E) and *H19* (F) coding sequences, respectively, on genomic DNA (gDNA) and cDNA. The data shown are mean values ± SEM for five different passages for the SRS/TS14 patient skin-derived fibroblast cultures, four TS14 patients and five SRS patients, with comparison to five donors as a control. $^*P \leq 0.05$ and $^{**}P \leq 0.01$ versus controls, in Mann-Whitney tests.

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to be up-regulated in fibroblasts from TS14 and SRS/TS14 patients relative to SRS patients and controls (Fig. 3D shows data for three of the nine miRNAs). We assessed the stability and passage independence of the overexpression of these genes by quantifying the mRNAs at five different passages for each patient. We found that, in all patients, all the ncRNAs and miRNAs studied were stably overexpressed in all cultures (fig. S1, A and B). Furthermore, single-nucleotide polymorphism (SNP) genotyping (Fig. 3E) provided the first evidence of biallelic expression of *MEG3* in fibroblasts from patients with TS14, due to reactivation of the normally silenced paternal allele. No SNP assessment was possible for *MEG8* or the miRNAs.

MLIDs at 14q32.2 and 11p15.5 are associated with multi-imprinting relaxation

Fibroblasts from a patient with SRS/TS14 displaying hypomethylation at both 14q32.2 and 11p15.5 loci displayed biallelic expression of *MEG3* from the *DLK1/MEG3* domain. We investigated whether MLID induced multi-imprinting relaxation and biallelic expression at the secondary loci affected by sequencing *H19* genomic DNA (gDNA) and complementary DNA (cDNA) from SRS/TS14 fibroblasts. We found that *H19* was monoallelically expressed in TS14 fibroblasts, whereas it was biallelically expressed in SRS and SRS/TS14 fibroblasts (Fig. 3F).

11p15.5 and 15q11-q13 PEGs are down-regulated by 14q32.2 hypomethylation

We then studied the expression of a large number of imprinted genes from loci involved in growth control in TS14, SRS, and control fibroblasts. For most of the PEGs, expression was barely detectable (*PEG3*, *PEG10*, and *GNAS-XLa*), whereas MEGs were highly variable (*H19*) or comparable to controls (*UBE3A* and *GRB10*) in fibroblast cultures (fig. S1C). We found that the 11p15.5 (*IGF2*) and 15q11-q13 (*SNURF* and *IPW*) PEGs were strongly and stably expressed in cultures of fibroblasts from controls and patients. All TS14 fibroblasts displayed low levels of *IGF2* expression, as did SRS and SRS/TS14 fibroblasts. Furthermore, both TS14 and SRS fibroblasts displayed low levels of *SNURF* expression, whereas only TS14 fibroblasts had low levels of *IPW* expression (Fig. 4). Similar to the ncRNAs and miRNAs, *IGF2*, *SNURF*, and *IPW* were all stably down-regulated in all fibroblast cultures, at various passages, for all patients (fig. S1D).

MEG3 and MEG8 overexpression and knockdown in control fibroblasts are associated with deregulation of the expression of 11p15.5 and 15q11-q13 PEGs

To study whether the clinical overlap could be explained by the cross-regulation between the three imprinted regions, we investigated the possible role of 14q32.2 ncRNAs *MEG3* and *MEG8* in the changes in *IGF2*, *SNURF*, and *IPW* expression. For this purpose, we carried out overexpression and knockdown of MEG3 and/or MEG8 in a control primary fibroblast line.

Expression constructs were available only for *MEG3*. For *MEG8*, we inserted the full-length *MEG8* cDNA obtained from fibroblasts into an expression vector. *MEG8* cDNA amplification revealed the presence of two different transcripts: a 497-base pair (bp) transcript corresponding to NR_024149.2 and a previously unreported 639-bp transcript (GenBank KX237564; fig. S2). This new variant has an additional exon, located between exons 1 and 2 of the NR_024149.2 transcript, flanked by donor and acceptor sites for splicing.

We found that the concomitant overexpression of *MEG3* and *MEG8* in control fibroblast cultures was associated with a moderate but significant down-regulation of *IGF2* expression (Fig. 5A). As for the 15q11-q13 PEGs, we found that overexpressing *MEG8* (regardless of the variant used for transfection) in control fibroblasts was associated with a down-regulation of *SNURF* expression, whereas *MEG3* overexpression was associated with *IPW* down-regulation (Fig. 5A). To investigate whether the down-regulation of *MEG3* and *MEG8* could also lead to deregulation of theses PEGs, we knocked down both ncRNA expression with *siRNA* in the same cell line. We found that *IGF2* expression increased following *MEG3* and/or *MEG8* knockdown, whereas *IPW* and *SNURF* were up-regulated by the knockdown of *MEG3* and *MEG8*, respectively (Fig. 5B). These changes in the expression of *IGF2*, *SNURF*, and *IPW* were observed in all transfected cells, despite the low transfection efficiency due to the use of primary fibroblast cultures as the host cells for transfection assays.

Principal components analysis of overall gene expression in TS14 and SRS fibroblasts

We investigated the dynamic changes in global gene expression levels and searched for possible gene expression signatures common to these two IDs by performing RNA sequencing (RNA-seq)–based expression profiling on TS14, SRS, and control fibroblasts [Gene Expression Omnibus (GEO) accession number GSE109408]. We first analyzed and compared the overall gene expression patterns of TS14 and SRS fibroblasts. For this purpose, we used principal components analysis (PCA), a statistical technique that summarizes large datasets, reducing the number of dimensions while illustrating relationships between samples based on the covariance of the data considered (*19*). PC1, PC2, and PC3 accounted for ~50% of the total variance of the original data (Fig. 6A). When all samples (TS14, SRS, and controls) were plotted in a two-dimensional space defined by PC1 and PC2 (accounting for ~35% of all variation), we were able to distinguish two distinct groups, the first clustering all the TS14 and SRS patients and the second encompassing all the controls (Fig. 6B). However, when the samples were plotted in a three-dimensional space defined by PC1, PC2, and PC3, we were able to separate the TS14 + SRS group into two separate groups, one corresponding to TS14 and the other to SRS (Fig. 6C). This analysis provides the first evidence for overlapping but distinguishable gene expression profiles in TS14 and SRS patients.

RNA-seq reveals that imprinting defects lead to the genome-wide deregulation of gene expression

We evaluated differential gene expression patterns in TS14 and SRS fibroblasts by grouping our samples based on their imprinting defects: 14q32.2 hypomethylation (four TS14 patients), 11p15.5 hypomethylation (five SRS patients), and controls (four donors). For this analysis, we selected genes with at least a twofold difference in expression between patients and controls and a q value below 0.05. We increased stringency by considering only genes with a mean expression of $\geq$1 FPKM (fragments per kilobase million) in at least one group. Using these criteria, we identified a total of 11,005, 11,117, and 11,043 expressed genes in control, SRS, and TS14 fibroblasts, respectively. Out of them, 552 genes were differentially expressed genes (DEGs), with expression levels differing between control fibroblasts and fibroblasts obtained from patients with TS14 or SRS (Fig. 6, D and E). Similar numbers of genes were up-regulated

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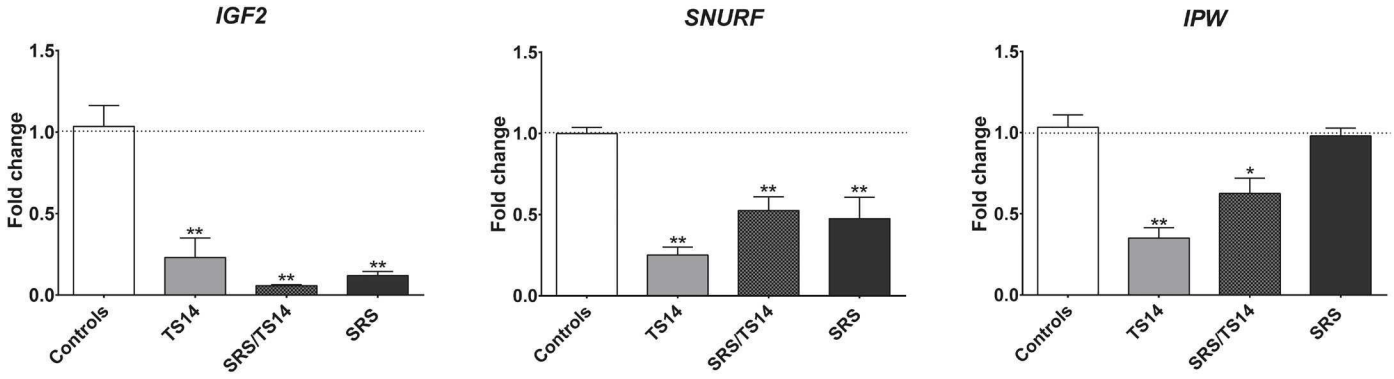

Fig. 4. 11p15.5 and 15q11-q13 PEGs are down-regulated upon 14q32.2 hypomethylation. Relative levels of expression for *IGF2*, *SNURF*, and *IPW* in cultures of skin-derived fibroblasts from TS14, SRS/TS14, and SRS patients compared with control fibroblasts. The data shown are mean values ± SEM for five different passages for the skin-derived fibroblast cultures for the SRS/TS14 patient, four TS14 patients, and five SRS patients, with comparison to five different donors as a control. $*P \leq 0.05$ and $**P \leq 0.01$ versus controls, in Mann-Whitney tests.

(271 genes) and down-regulated (281 genes). We found that 61 of the up-regulated genes were up-regulated in both TS14 and SRS fibroblasts, whereas 62 were up-regulated only in SRS fibroblasts, and 148 were up-regulated only in TS14 fibroblasts. By contrast, 49 of the down-regulated genes were down-regulated in both TS14 and SRS fibroblasts, whereas 53 were down-regulated only in SRS fibroblasts, and 179 were down-regulated only in TS14 fibroblasts (Fig. 6F). Together with the quantitative polymerase chain reaction (qPCR) findings in patients and transfected normal fibroblasts, these results show that imprinting defects induce the deregulation of both imprinted and nonimprinted genes. Moreover, 50% of the DEGs in SRS fibroblasts was also deregulated in TS14 fibroblasts (Fig. 6F). The list of all the DEGs in SRS and TS14 fibroblasts is available in data file S1.

A gene signature common to TS14 and SRS identified by gene set analysis

We then determined whether networks of genes with similar functional annotations were dysregulated in TS14 and SRS patients relative to controls. We analyzed the genes displaying differential expression between these groups using gene ontology pathways from gene set enrichment analysis (GSEA) to annotate genes on the basis of the functional biological processes in which their products are involved. GSEA with a false discovery rate (q value) threshold of $q < 0.05$, to correct for multiple testing, identified one major pathway as down-regulated and two as up-regulated in both TS14 and SRS (Fig. 6G: [1]Go Cell Development, Go Regulation Of Cell Differentiation, Go Positive Regulation Of Developmental Process, Go Tissue Development. [2]Go Cellular Response To Organic Substance, Go Response To Oxygen Containing Compound. [3]Go Immune System Process, Go Response To External Stimulus, Go Cytokine Mediated Signaling Pathway, Go Inflammatory Response.). We found that the level of gene expression was lower (87 genes for TS14 and 41 genes for SRS) in both TS14 and SRS fibroblasts for genes encoding products known to enhance cell or tissue proliferation, growth, and development, whereas genes from the immune system involved in inflammatory, cytokine, and reactive oxygen species reactions were overexpressed. In all cases, TS14 fibroblasts had three to four times more DEGs from these pathways than SRS fibroblasts. Once again, 50% of the DEGs in SRS was also found among the DEGs in TS14 dysregulated pathways.

Allele-specific expression of imprinted genes from TS14 and SRS/TS14 fibroblasts

We previously performed whole-exome sequencing (WES) on DNA extracted from the fibroblasts of two TS14 patients (TS14-1 and TS14-2) (*12*) and the SRS/TS14 patient. We then used a method combining RNA-seq and WES of these patients to accurately assess allele-specific expression (ASE; fig. S3). On average, 3825 genes were found to be expressed biallelically versus 103 expressed monoallelically (3941, 3638, and 3895 versus 105, 122, and 82 from TS14-1, TS14-2, and SRS/TS14 patients, respectively). We first focused on imprinted human genes from the list of genes available from the geneimprint database (www.geneimprint.com). We compared their ASE to the bulk/single-cell fibroblast ASE recently reported for five healthy individuals (table S3) (*20*). Consistent with their known imprinted status, we found that four imprinted genes had monoallelic expression in both patient and control fibroblasts (*OSBPL5*, *NDN*, *ZDBF2*, and *PEG10*). The *H19* gene was found to be monoallelically expressed in patient TS14-1, whereas the *PRIM2* gene, predicted to be imprinted on the basis of previous studies, also displayed monoallelic expression in both TS14 patients. However, we identified 16 other imprinted genes with biallelic expression in our patients' fibroblasts (table S3). Thirteen of these genes were also biallelically expressed in bulk fibroblast cultures from controls, suggesting that these genes may not be imprinted in fibroblasts. For the remaining three genes, *CPA4* was biallelically expressed in all three patients, whereas *ZNF597* and *H19* (consistent with our Sanger sequencing results for cDNA) were biallelically expressed only in the SRS/TS14 patient.

Fibroblasts from the SRS/TS14 patient displaying hypomethylation at both 14q32.2 and 11p15.5 loci displayed biallelic expression of *MEG3* and *H19* from the *DLK1/MEG3* and *IGF2/H19* domains, respectively. We investigated whether the biallelic expression of *CPA4* and *ZNF597* also resulted from MLID by assessing the methylation levels of the two imprinted regions regulating these genes (the *PEG1/MEST* and *ZNF597*-DMRs, respectively). The *PEG1/MEST* locus was hypomethylated in all three patients, whereas *ZNF597* was hypomethylated only in the SRS/TS14 patient, consistent with the biallelic expression profiles of these patients (table S4). These results confirm our previous finding for MLID and multi-imprinting relaxation together with biallelic expression at the affected secondary loci. However, as both *CPA4* and *ZNF597* are expressed from the

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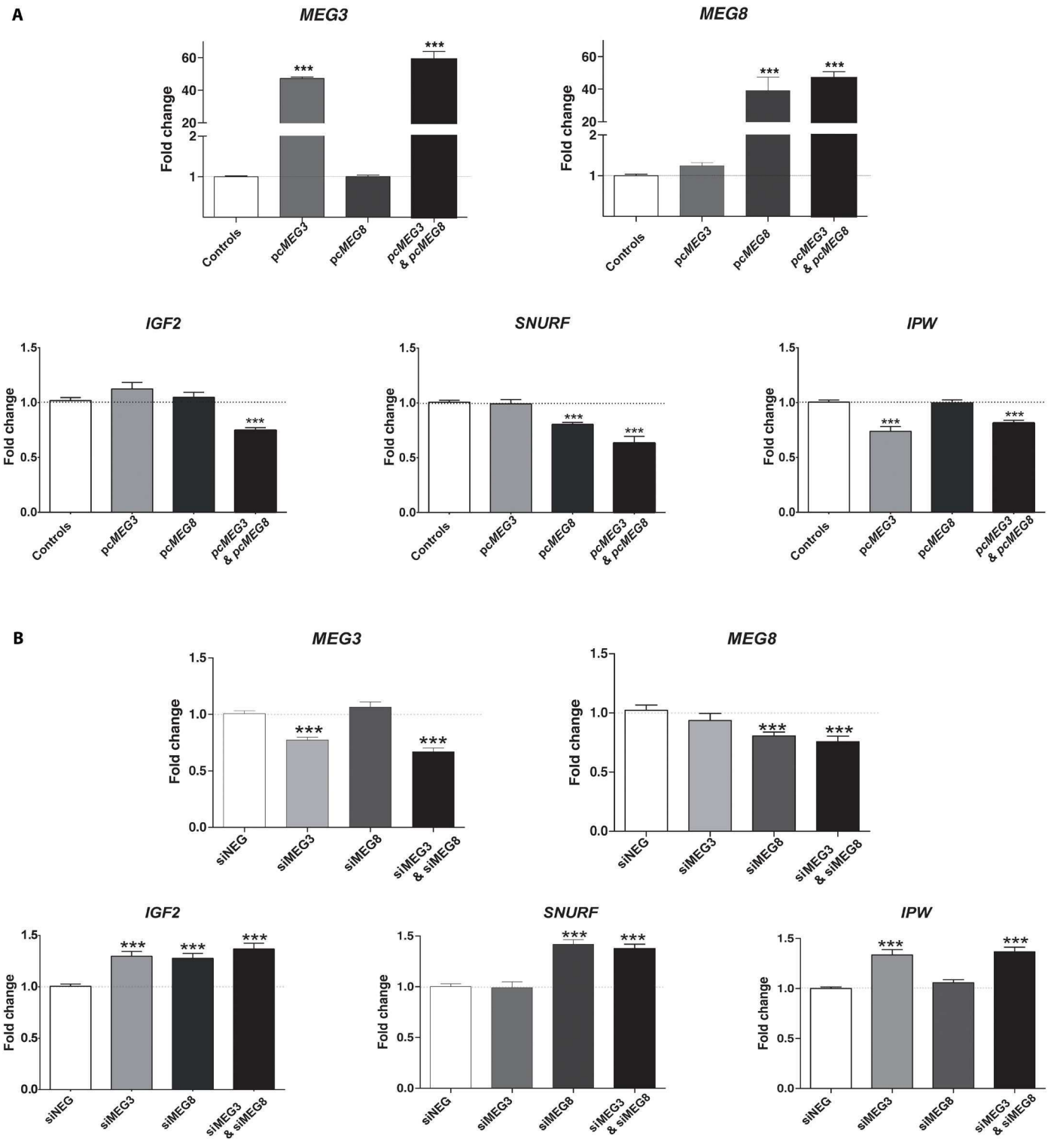

Fig. 5. 11p15.5 and 15q11-q13 PEGs are deregulated upon *MEG3* and/or *MEG8* overexpression or knockdown in experimental models. (**A**) Relative levels of expression for *IGF2*, *SNURF*, and *IPW* in a control primary fibroblast line transfected with an empty, *MEG3*, and/or *MEG8* expression vector. (**B**) Relative levels of expression for *IGF2*, *SNURF*, and *IPW* for a control primary fibroblast line transfected with a negative control, *MEG3*, and/or *MEG8* small interfering RNA (siRNA). The data shown are the mean values ± SEM for four independent transfection assays (with at least four wells transfected per experiment). ***$P \leq 0.001$ versus controls (transfected with an empty vector), in unpaired *t* tests.

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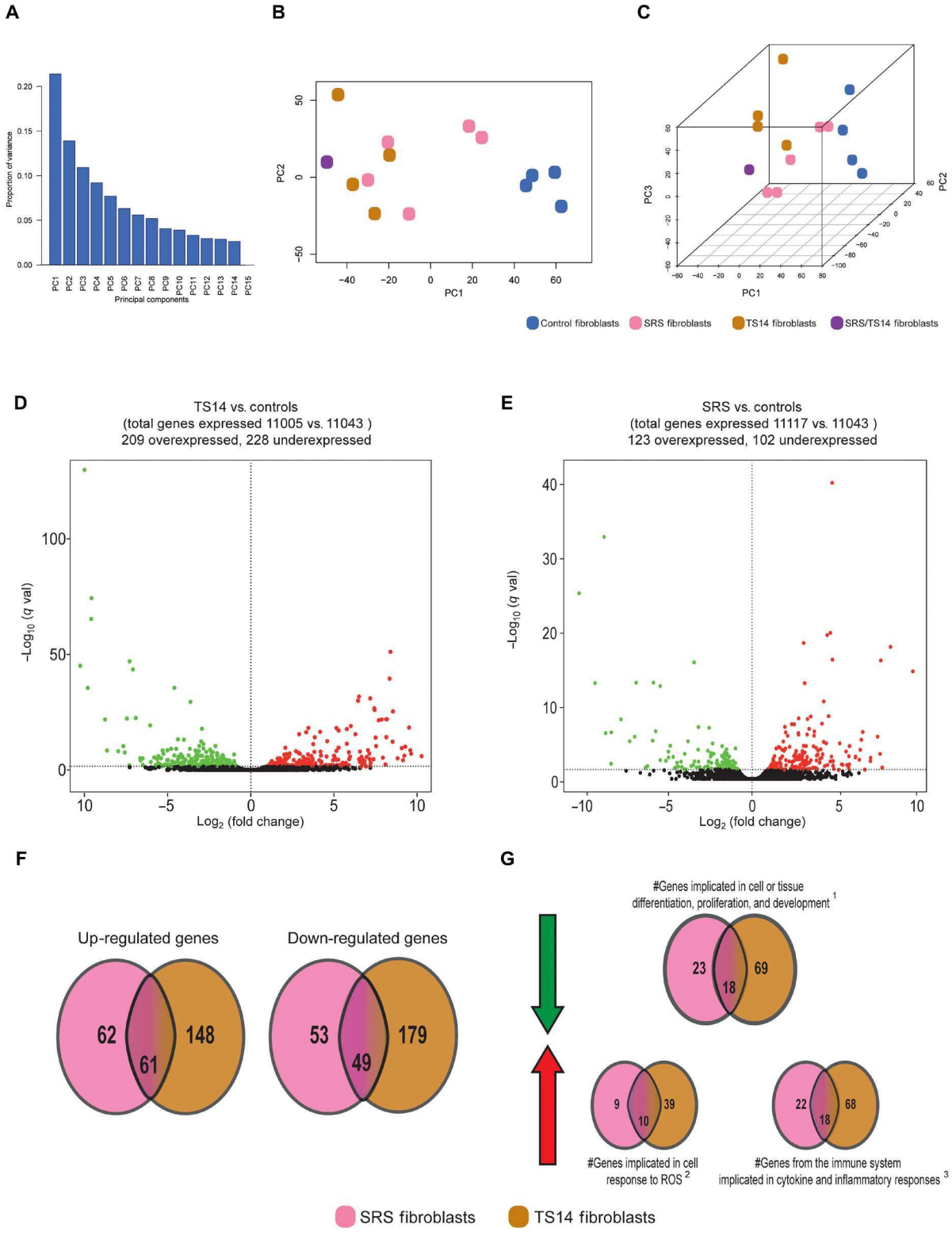

Fig. 6. PCA and DEGs from TS14, SRS, and control fibroblasts. (**A**) Proportion of the variance accounted for by each principal component (PC). (**B**) Two-dimensional and (**C**) three-dimensional projection plots of the first three PCs: PC1, PC2, and PC3. (**D** and **E**) Volcano plots of the expression of all genes (with FPKM of ≥1) from TS14 and SRS, respectively, relative to controls. Green and red dots represent gene displaying statistically significant underexpression and overexpression, respectively [$\log_2(\pm 2)$ fold change, with a q value threshold of 0.05]. (**F**) The number of genes differentially regulated in both TS14 and SRS fibroblasts or in one of these types of fibroblasts only. (**G**) Schematic representation of gene ontology pathways from the GSEA displaying significant up- and down-regulation in patients relative to controls fibroblasts after correction for multiple testing ($q < 0.05$). GSEA reveals a dysregulated pathway signature common to TS14 and SRS.

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methylated maternal allele, their biallelic expression upon hypomethylation of their respective domains is discordant with their parent-of-origin expression. Such discordances have been reported for other imprinted genes but never before for these two genes (*20*). Last, we looked at the remaining genes of unknown imprinting status displaying monoallelic expression (137 genes; table S4) in our patients. We identified seven putative imprinted genes clustering at four different loci across the genome (fig. S4). These variants were not validated by another targeted sequencing technique.

DISCUSSION

IDs often affect growth in humans, leading to diseases with overlapping features, regardless of the genomic region affected (*2*). We characterized the effect of a loss of methylation at the imprinted *DLK1/MEG3* locus on gene expression at the 14q32.2 locus in TS14 patients, and we investigated whether the overlap in clinical phenotype between TS14 and SRS and PWS might be due to similar patterns of gene deregulation between these syndromes.

DLK1 is an important PEG in the 14q32.2 domain. This gene encodes a transmembrane protein that generates both membrane-bound and serum-soluble isoforms through alternative splicing (*16*). We found that TS14 patients with paternal deletions or hypomethylation of the *DLK1/MEG3* domain had no circulating DLK1, whereas serum DLK1 concentration is negatively correlated with age after birth in healthy children [consistent with the rapid decrease in serum Dlk1 concentration observed in newborn mice (*21*)]. Thus, unlike circulating IGF2, which is produced biallelically in the liver after birth, under the control of a specific nonimprinted promoter (*22*), circulating *DLK1* probably continues to be generated by monoallelic expression from the imprinted gene under normal conditions after birth. An analysis of the MEGs from the 14q32.2 domain showed that *MEG3*, *MEG8*, and miRNAs were up-regulated in TS14 fibroblasts. We demonstrated that *MEG3* overexpression resulted from the reactivation of the normally silenced paternal allele. We hypothesized that the overexpression of *MEG8* and miRNA was also due to expression from both parental alleles in TS14 patients. This hypothesis is consistent with the finding of previous studies that *DLK1/MEG3* hypermethylation leads to a reactivation of the expression of *RTL1* and *DLK1* from the maternal allele (*17*). These findings highlight the parent-of-origin–specific effects of DNA methylation at ICRs on gene expression in the *DLK1/MEG3* domain.

We investigated the possible overlap in gene expression patterns between TS14 and SRS and between TS14 and PWS. We found that imprinted genes within the primary affected domains for SRS (*IGF2* at 11p15.5) and PWS (*SNURF* and *IPW* at 15q11-q13) were down-regulated in all TS14 fibroblasts and in SRS/TS14 fibroblasts, despite the normal levels of methylation at their imprinted control regions, that are generally altered in SRS and PWS patients (*6*, *23*). We hypothesized that this deregulation of the IGN might affect gene expression throughout the genome. An unsupervised PCA on the RNA-seq data from control and TS14 and SRS patient fibroblast cultures resulted in the clustering of samples into two groups (controls on the one hand and SRS and TS14 patients on the other hand) when the data were projected onto a two-dimensional space. However, the SRS and TS14 patients could be distinguished when the data were projected onto a three-dimensional space. Gene ontology annotations showed that the genes displaying expression alterations belonged to common pathways. Genes encoding products that promote cell and tissue growth were found to be down-regulated in fibroblasts from both TS14 and SRS patients. These results confirm our hypothesis of an overlapping but distinguishable, genome-wide deregulation of the pattern of expression in TS14 and SRS patients, affecting not only other imprinted genes from the IGN but also a number of nonimprinted genes. We showed that hypomethylation at the *DLK1/MEG3* domain leads to the down-regulation of other imprinted PEGs, such as *IGF2*, *SNURF*, and *IPW*, mostly genes known to enhance growth (*24*), and of 87 other nonimprinted genes implicated in promoting growth. Moreover, TS14 fibroblasts had about twice as many deregulated (1.6× up- and 2.2× down-regulated) genes as SRS fibroblasts, with about 50% of the deregulated genes (61 up- and 49 down-regulated) common to both syndromes, regardless of the imprinting defect. This finding may partly explain the phenotypic overlap and also some of the differences between SRS and TS14, such as the much early onset of puberty and greater early weight gain in TS14 than in SRS patients.

Last, we hypothesized that these changes in expression might reflect, directly or indirectly, the involvement of 14q32.2 ncRNAs *MEG3* and *MEG8* in this process. ncRNAs have been shown to regulate gene expression both in cis and in trans (*25*, *26*), through association with chromatin modifiers (*27*). This raises the possibility of 14q32.2 ncRNAs regulating the expression of these genes from the 11p15.5 and 15q11-q13 regions. We found that overexpressing or knocking down the expression of *MEG3* and *MEG8* in normal fibroblast cultures was associated with deregulation of PEGs from the 11p15.5 and 15q11-q13 regions. We found in our experimental models of normal fibroblasts that (i) overexpressing *MEG3* was associated with decreased level of *IPW* transcripts, (ii) overexpressing *MEG8* was associated with lower levels of *SNURF*, and (iii) concomitant overexpression of *MEG3* and *MEG8* was associated with lower levels of *IGF2* transcripts. These results are in accordance with observations in TS14 and SRS fibroblasts, where (i) *IPW* levels were lower only in TS14 fibroblasts where *MEG3* was overexpressed; (ii) *SNURF* levels were lower in TS14 and SRS patients, both having increased levels of *MEG8*; and (iii) *IGF2* levels were lower in TS14 patients overexpressing *MEG3* and *MEG8* upon *DLK1/MEG3* hypomethylation. These results suggest that *MEG3* and *MEG8* can regulate, in trans, the expression of other imprinted genes. We hypothesize a synergic role of MEG3 and MEG8 to control directly or indirectly the expression of *IGF2*. Overexpressing one of the two is not enough to increase *IGF2* expression, since the other component is missing, while knocking down only one is enough to disrupt the complex and lead to decreased *IGF2* expression. Similar patterns of regulation within the *IGN* have been described before (*3*, *5*), involving *IPW* and *MEG3* in particular (*6*), for which *IPW* was found to repress *MEG3* expression. Together with our results, this strongly suggests that a system of reciprocal control operates between ncRNAs at imprinted loci and that this system might contribute to the clinical overlap between IDs. However, additional functional studies of the role of *MEG3* and *MEG8* are required to decipher the mechanisms by which these ncRNAs could regulate gene expression, particularly for imprinted genes. These mechanisms are probably unrelated to gene imprinting status because all TS14 patients have normal methylation levels at the ICRs corresponding to the deregulated imprinted genes (ICR1 for *IGF2* and *SNRPN*-IC for *SNURF* and *IPW*). Single-cell RNA-seq has been shown to be more powerful than bulk RNA-seq for studies of the expression of imprinted

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genes (*20*) and therefore would allow the generation of a more extensive map of the deregulated IGN upon hypomethylation of the *DLK1/MEG3* and/or *IGF2/H19* domains.

In conclusion, we show that 14q32.2 hypomethylation affects the expression not only of genes within this locus but also of other imprinted and nonimprinted genes, some of which are involved in controlling tissue growth. We also observed changes in gene expression common to TS14 and SRS patients, in particular, a diminished *IGF2* expression, and to TS14 and PWS patients, with diminished *SNURF* and *IPW* expressions (fig. S5). This transcriptomic overlap may account for the clinical overlap between the two syndromes.

MATERIALS AND METHODS

Study approval

Patients were followed up at Armand Trousseau Children's Hospital or were referred by other clinical centers for molecular analysis for suspected TS14 or SRS. A geneticist and/or a pediatric endocrinologist examined each patient, and a detailed clinical form was completed. All patients or their parents and the controls included in this study gave written informed consent for participation, in accordance with French national ethics rules for patients recruited in France (Assistance Publique–Hôpitaux de Paris authorization no. 682) and the rules of Institutional Review Board I00000204 of the Mount Sinai School of Medicine for patients recruited in the United States.

Quantification of serum DLK1 levels

Serum DLK1 levels from healthy children and TS14 patients were quantified in 96-well plates, with the Quantikine Immunoassay Control for Human Pref-1/DLK1/FA1 (R&D Systems, France), based on sandwich ELISA (enzyme-linked immunosorbent assay), according to the manufacturer's instructions.

Skin-derived fibroblast cultures

Skin-derived fibroblasts from patients and controls were cultured to confluence in RPMI 1640 (Gibco, Cergy Pontoise, France) supplemented with 10% fetal calf serum and ampicillin (50 U/ml)/streptomycin (50 μg/ml) at 37°C (number of passages, <10). Cells were then treated with trypsin and centrifuged. The cell pellet obtained was washed twice with 1× phosphate-buffered saline and used for DNA and micro/large-scale RNA extractions.

Nucleic acid extraction and quantification

DNA was extracted from blood leukocytes and from skin-derived fibroblast cultures, by an in-house protocol, after cell lysis by a salting-out procedure, as previously described (*28*, *29*). Total RNA and miRNA were extracted from cultured fibroblasts with the NucleoSpin miRNA Kit for the isolation of small and large RNAs (MACHEREY-NAGEL, France). Both DNA and RNA were quantified with a NanoDrop ND-1000 spectrophotometer (Invitrogen, France).

Reverse transcription and real-time PCR quantification of micro/mRNAs

We synthesized cDNA from micro/mRNAs isolated from fibroblasts and used it for quantitative PCR with the miScript PCR System (Qiagen, France). Expression in the controls was arbitrarily set to 1, and fold changes (FCs) between two groups were calculated as $FC = 2^{-\Delta\Delta Ct}$. Details from the reverse transcription and real-time quantification analysis are provided in Supplementary Methods.

Bisulfite treatment of DNA and methylation analysis

gDNA (1 μg) was treated with sodium bisulfite, with the EZ DNA Methylation Kit (Zymo Research, Orange, CA), according to the manufacturer's instructions. The methylation status of the studied loci was assessed by allele-specific methylated multiplex real-time quantitative PCR (ASMM RTQ-PCR), as previously described (*30*–*36*). Details for the treatment and technique are provided in Supplementary Methods.

gDNA and cDNA sequencing

Two SNPs from the *MEG3* and *H19* gDNA and cDNA were sequenced by standard Sanger sequencing methods, with the ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Kit and the ABI 3100 Genetic Analyzer. The sequencing products were then analyzed with SeqScape v2.6 (Life Technologies, Courtaboeuf, France).

Cell cultures and transfection assays

GM05757 (control human skin–derived fibroblast) cells were cultured in 24-well plates for overexpression and small interfering RNA (siRNA) transfections. Details of culture and transfection conditions and constructs used are detailed in Supplementary Methods.

WES, RNA-seq, and bioinformatics analysis

Library preparations, sequencing, and data analysis were carried out by IntegraGen SA (Evry, France). The sequencing methods and bioinformatics analysis are described in detail in Supplementary Methods.

RT-qPCR statistical analysis

For RT-qPCR, we compared data for pairs of groups in Mann-Whitney tests (expression in fibroblasts from patients versus that in control fibroblasts, with $n = 5$ each) and unpaired t tests (overexpression and siRNA assays with $n > 20$ per condition). We considered P values below 0.05 to indicate statistical significance. The analyses were performed with GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/2/eaau9425/DC1

Supplementary Methods

Fig. S1. Expression of 14q32.2 MEGs and 11p15.5 and 15q11-q13 PEGs in five different passages of cultured fibroblasts from TS14 patients.

Fig. S2. Schematic representation of the subcloned transcripts of MEG8 (*MEG8a* and *MEG8b*).

Fig. S3. Distribution of minor allele frequency according to combined exome and RNA-seq data for fibroblasts from the TS14-1, TS14-2, and SRS/TS14 patients.

Fig. S4. Schematic representation of the four putative imprinted loci identified on the basis of allele-specific gene expression and DMRs.

Fig. S5. Schematic representation of the molecular findings and the hypothesized mechanism from this study.

Table S1. Methylation levels for all patients and controls, as determined by ASMM RT-qPCR.

Table S2. Clinical features for TS14 patients with imprinting defects at the *DLK1/MEG3* domain described here.

Table S3. The allelic status of imprinted genes, as determined by RNA and exome sequencing for TS14 and control fibroblasts.

Table S4. List of all genes with monoallelic expression, as determined by RNA and exome sequencing in TS14 patients.

Table S5. List and sequences of the primers used in this study.

Data file S1. List of all gene FPKMs for each patient and control.

Data file S2. Supervised study of DEGs for each group of patients compared to controls.

References (*37*–*40*)

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Résumé

La croissance fœtale est sous la dépendance de nombreux facteurs environnementaux, génétiques et hormonaux dont les interactions vont en conditionner le bon déroulement. Le système des insulin-like growth factors (IGFs) joue un rôle prépondérant, à l'interface de ces différents facteurs, pour assurer une bonne croissance fœtale. Dans ce travail, nous nous sommes intéressés aux différents acteurs du système des IGFs dans des pathologies de la croissance fœtale. Dans une approche clinique et expérimentale, nous avons décrit les conséquences fonctionnelles d'anomalies génétiques ou épigénétiques intéressant IGF-I, IGF-II et leur récepteur commun IGF1R. Ainsi, nous avons mis au point un test fonctionnel permettant d'apprécier l'activité *in vitro* d'IGF1R chez les patients présentant une restriction de croissance fœtale et postnatale. Nous avons également documenté la biodisponibilité d'IGF-I chez des patients présentant un syndrome de Silver-Russell, qui est une pathologie liée à l'empreinte parentale responsable d'une restriction de croissance à début ante-natal. Enfin, nous avons caractérisé le chevauchement clinique et moléculaire entre les patients présentant un SRS ou un syndrome de Temple (autre pathologie liée à l'empreinte parentale), confirmant le rôle prépondérant du défaut d'expression d'*IGF2* dans ces deux syndromes. Ces résultats confirment un fonctionnement des gènes soumis à empreinte en réseau et le rôle majeur du système des IGFs dans la croissance fœtale, particulièrement altérée en cas de pathologie intéressant ces gènes soumis à empreinte parentale.

Mots clés : croissance fœtale, système des IGFs, empreinte parentale, anomalies multiples de la méthylation, réseau de gènes soumis à empreinte parentale, IGF-I, IGF2, IGF1R.

Abstract

Growth and insulin-like growth factors (IGF): physiopathology insights from imprinting diseases

Fetal growth is dependant of environemental, genetic and hormonal factors which interact to ensure a proper development. Insulin-like growth factors (IGF) system plays a key role in fetal growth by interactions with these differents systems. In this work, we studied the roles of the IGF system in fetal growth restriction diseases. We used both clinical and experimental approaches to enhance knowledge on functional consequences of genetic ou epigenetic defects of IGF system actors. We set-up a functional test to assess IGF1R activity in vitro in patients with restricted fetal and postnatal growth. We also documented the IGF-I bioavailability in patients with Silver-Russell syndrome, which is an imprinting disorder responsible for fetal and postnatal growth restriction. We characterized the clinical and molecular overlap of Silver-Russell and Temple syndrome (another imprinting disease affecting growth and metabolism) and confirmed the central role of IGF2 in the physiopathology of these disorders. These results confirmed the integration of imprinted genes in a large co-regulation network and the major role of IGF system actors in fetal growth which is usually impaired when these imprinted genes are affected.

Keywords: fetal growth, IGF system, imprinting diseases

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