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1 **The inversa type of recessive dystrophic epidermolysis bullosa is caused by specific**
2 **arginine and glycine substitutions in type VII collagen**

3

4 Short title: *COL7A1* missense mutations in RDEB-I

5

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1 **ABSTRACT**

2

3 **Background** The inversa type of recessive dystrophic epidermolysis bullosa (RDEB-I) is a
4 rare variant of dystrophic epidermolysis bullosa, characterized by blistering in the body
5 flexures, trunk, and mucosae. The cause of this specific distribution is unknown. So far, 20
6 *COL7A1* genotypes have been described in RDEB-I and genotype-phenotype correlations
7 have not been studied extensively. The aim of the study was to gain more insight into the
8 pathophysiology of this intriguing RDEB-I phenotype.

9 **Methods** We identified 20 Dutch and British RDEB-I patients and full genotypes in 18 of
10 them. We reviewed the literature on RDEB-I genotypes and conducted an extensive
11 genotype-phenotype correlation study for RDEB-I.

12 **Results** All 20 patients had generalized blistering at birth and during early infancy. In most
13 patients, the age of transition from generalized to inversa distribution was before the age of 4
14 years. We noted a spectrum of disease severity ranging from the mildest ‘mucosal only’
15 phenotype to the severest phenotype with limited acral involvement. The 29 genotypes of our
16 RDEB-I patients and those reported in the literature revealed that RDEB-I is associated with
17 specific recessive arginine and glycine substitutions in the triple-helix domain of type VII
18 collagen.

19 **Discussion and conclusion** Why these specific arginine and glycine substitutions cause the
20 inversa distribution remains unknown. We could not identify clear differences in location and
21 nature of substituting amino acids between these mutations and missense mutations causing
22 other RDEB phenotypes. We hypothesize that the higher skin temperature in the affected
23 areas plays an important role in the pathophysiology of RDEB-I.

24

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1 Keywords: recessive dystrophic epidermolysis bullosa, inversa type; genotype-phenotype

2 correlations; type VII collagen; *COL7A1*

3

1 **INTRODUCTION**

2

3 The inversa type of recessive dystrophic epidermolysis bullosa (RDEB-I; OMIM #226600) is
4 one of the rare variants of dystrophic epidermolysis bullosa.[1] RDEB-I was first described by
5 Prof. T. Gedde-Dahl Jr in 1971 and is characterized by generalized blistering from birth and
6 during early infancy.[2] Later in infancy or childhood, the predilection sites for blistering
7 change and the body flexures, axis and mucous membranes become the predominant sites of
8 blistering.[2, 3] Neither the cause of the unusual distribution of blistering in RDEB-I, nor the
9 cause of the “transition” from a generalized to inversa distribution is known. The specific age
10 at which this transition in blistering predilection sites takes place is also not known, but it
11 probably varies between patients. As with the main subtypes of RDEB, there is a large
12 clinical variation between inversa patients.

13 Immunohistochemically, type VII collagen (Col7) is either slightly reduced or
14 normally present in the skin of RDEB-I patients. Anchoring fibrils, which are composed of
15 large numbers of Col7 dimers, can be either reduced or normal in number, and can have a
16 hypoplastic or normal ultrastructural appearance.[4-6] Transient retention of Col7 in basal
17 keratinocytes can be a typical immunohistochemical feature in the early stages.[7]

18 Similar to the other subtypes of DEB, RDEB-I is caused by mutations in the COL7A1
19 gene encoding type VII collagen.[1] However, while genotype-phenotype correlations for the
20 major subtypes of RDEB have been extensively studied in several populations,[1, 8-16] there
21 is little data on genotype-phenotype correlations for RDEB-I. Our study covers the largest
22 RDEB-I cohort reported to date, comprising 20 RDEB-I patients from the Netherlands and the
23 UK. Here we describe this cohort and the genotypes identified in 18 of them, and we discuss
24 the genotype-phenotype correlations identified in these patients and those previously reported
25 in the literature.

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PATIENTS AND METHODS

Patients

A diagnosis of DEB was established in a set of 20 patients on the basis of clinical findings, family history, histopathology, immunofluorescence antigen mapping, electron microscopy, and *COL7A1* mutation analysis. The diagnosis of RDEB-I was based on the typical clinical findings.[1, 3] All patients were evaluated by the same professionals in the University Medical Center Groningen, the Netherlands (MFJ), Great Ormond Street Hospital for Children or St. Thomas' Hospital, London, UK (JEM, AEM, JAM). Informed consent was given for all skin biopsies and venapunctures, and all studies were approved by the local medical ethical committees (IRB) and conducted in accordance with the guidelines stated in the Declaration of Helsinki.

Immunofluorescence and electron microscopy studies

For immunofluorescence studies (IF), 4 mm punch biopsies of rubbed perilesional skin and/or unaffected skin from non-predilection sites (i.e. the ventral side of the upper arm) were obtained from patients 1-6 and 16 and snap frozen. Four or five μm thick cryostat sections were prepared and the amount of Col7 was determined using monoclonal antibody LH7:2 as previously described.[14, 17, 18] For electron microscopy, 2 mm punch biopsies were taken from the same locations from the same patients and prepared and analyzed as previously described.

1 **COL7A1 mutation analysis**

2 The *COL7A1* gene was fully analyzed for pathological mutations either by complete
3 sequencing or by pre-screening using either Denaturing Gradient Gel Electrophoresis or
4 Conformation-sensitive Capillary Electrophoresis followed by targeted sequencing as
5 described previously.[14, 19] Genomic DNA was extracted from peripheral leukocytes.

6

7

8 **RESULTS**

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10 **Clinical characteristics**

11 In total, we identified 20 patients with RDEB-I, six from the Netherlands (patients 1-6) and
12 14 from the UK (patients 7-20). Clinical characteristics are listed in supplemental Table S1
13 and Figure 1. Patients 1 and 2 are sister and brother; patient 14 reported two similarly affected
14 sibs, but they are not known in our clinics; no other patients had any siblings affected with
15 DEB. Eighteen patients (90%) presented with generalized blistering and skin erosions at birth
16 or shortly after and during early infancy; in one the onset of blistering was within the first six
17 months and in another it was unknown. Seven (35%) had congenital localized absence of skin
18 (CLAS), mostly on the lower legs/ankles/feet, which remained fragile during life. With age,
19 blistering on the extremities decreased markedly in most patients and became more restricted
20 to the body trunk and folds. The most frequently affected skin areas were the proximal body
21 flexures, i.e. the axillae and groins, and the body axis, i.e. the back, neck and buttocks
22 symmetrically. None of the patients had involvement of the acra as the dominant feature. In
23 fact, even after trauma most patients only occasionally experienced blistering of hands and
24 feet, and none of them developed ‘mitten deformities’ as seen in the ‘severe generalized’
25 RDEB (RDEB-sev gen) subtype. However, four patients (3, 9, 19, 20) had more extensive

1 blistering of the acra and developed mild pseudosyndactyly in one of the interdigital web
2 spaces. In contrast, in four patients (7, 13, 15, 16) skin blistering nearly ceased completely
3 after infancy and they only had mucosal involvement and nail dystrophy.

4 In 6/20 (30%) patients, lesions of the oral mucosa were already present at birth,
5 whereas in the other patients the oral mucosa became affected from infancy onwards (Table
6 S1). When the RDEB-I phenotype had established, all patients had severe mucosal
7 involvement, mostly affecting the oral cavity, oesophagus and anus. In 6/10 (60%) women the
8 genital skin and mucosa were affected, and in one this severely complicated healing after
9 childbirth. Swallowing difficulty or dysphagia was reported in 17/20 (85%) patients. Twelve
10 patients (60%) had proven oesophageal strictures and 10 (50%) needed repeated oesophageal
11 dilatations; patient 5 received more than 150 dilatations before the age of 30. Ankyloglossia
12 and microstomia due to scarring of the oral mucosa were present in 15 (75%) and 13 (65%)
13 patients, respectively, and was noted as early as 6 years of age (patient 15). Blistering of the
14 external auditory canal was seen in 7/20 (35%) patients, which was complicated by narrowing
15 of the canal in three, and subsequent conductive hearing loss after the age of 30 in two (10%).
16 None of the patients were growth retarded, had renal failure or developed squamous cell
17 carcinomas. Patients 10, 12, 15, and 20 had anaemic periods and patient 12 required
18 intermittent blood transfusions. Constipation was an almost invariable feature, although only
19 in part due to anal blistering.

20

21 **Transition age**

22 The age of transition of blistering from extremities to body axis and flexures was reported in
23 13/20 patients and ranged from one to 18 years. In nine patients, transition was reported
24 between birth and age four years. Patient 6 was the only one in whom the transition age could
25 be objectively determined. He was diagnosed with probable recessive bullous dermolysis of

1 the newborn, since his generalized blisters present at birth disappeared within the first few
2 months, and the immunofluorescence biopsy revealed Col7 retention in basal keratinocytes.
3 However, towards the end of his first year, blistering activity increased on the trunk, axillae,
4 intraorally and especially in the diaper area. Hence, transition age was before the end of the
5 first year.

6

7 **Immunofluorescence and electron microscopy findings**

8 Immunofluorescence studies on healthy skin from non-predilection sites revealed normal
9 Col7 in patients 3, 4, 6, 16, and reduced Col7 in patient 1. Retention of Col7 was noted in
10 basal keratinocytes of patient 3 (aged 33) and patient 6, in whose skin both small and large
11 Col7 positive granules were seen at the age of 1 month. Basal keratinocytes of a peri-lesional
12 skin biopsy of patient 2, obtained a few days after birth, also revealed Col7 retention. Peri-
13 lesional skin from patient 5 revealed reduced Col7. Electron microscopy studies revealed
14 slightly hypoplastic anchoring fibrils in normal (patient 4) or reduced numbers (patients 3, 6,
15 16) at non-predilection sites. Electron microscopy studies of affected skin revealed a reduced
16 number of abnormal or rudimentary anchoring fibrils in patients 1, 2, 3, 4, 5, and 16. Col7-
17 containing vesicles were seen in the rough endoplasmic reticulum in patient 2.

18

19 **Genotypes and genotype-phenotype correlations**

20 Genotypes were available for 18 of our 20 RDEB-I patients. The genotypes of patients 1-5
21 have been reported previously.[14] Table 1 lists the 29 full genotypes associated with RDEB-I
22 identified in this study and the literature.

1 **Table 1. Genotypes of RDEB-inversa patients reported in this study and in the literature.**

No.	Mutation 1 ¹			Mutation 2 ¹			Patients	Reference				
	Nucleotide	Amino acid	Exon	Consequence ²	Domain ³	Nucleotide ⁴	Amino acid	Exon ⁵	Consequence ²	Domain ³		
1	c.4039G>T	p.Gly1347Trp	34	Missense	THD	c.8540insC	p.Glu2848X	116	PTC	NC2	Pat 16	This study
2	c.4945C>G	p.Gly1649Arg	53	Missense	THD	NF ⁶					Pat 13	This study
3	c.5282G>C	p.Gly1761Ala	60	Missense	THD	c.5282G>C	p.Gly1761Ala	60	Missense	THD	Pat 6	This study
4	c.5720G>A	p.Gly1907Glu	68	Missense	THD	c.7805G>A	p.Gly2602Glu	105	Missense	THD	Pat 3	[14]
5	c.5720_5721 GA>AT	p.Gly1907Asp	68	Missense	THD	c.4018C>T	p.Arg1340X	34	PTC	THD	2x	[26]
6	c.5720_5721 GA>AT	p.Gly1907Asp	68	Missense	THD	c.5047C>T	p.Arg1687X	54	PTC	THD	Pat 12, 17	This study
7	c.6022C>G	p.Arg2008Gly	73	Missense	THD	c.6022C>G	p.Arg2008Gly	73	Missense	THD	2x	[20, 21]
8	c.6187C>G	p.Arg2063Gly	74	Missense	THD	c.706C>T	p.Arg236X	6	PTC	NC1	1x	[8]
9	c.6205C>T	p.Arg2069Cys	74	Missense	THD	c.425A>G	p.Lys142Arg	3	Splice → PTC	NC1	3x	[20, 24]
10	c.6205C>T	p.Arg2069Cys	74	Missense	THD	c.3551-3T>G		IVS26	Splice → PTC	NC1	Pat 5	[14]
11	c.6205C>T	p.Arg2069Cys	74	Missense	THD	c.3894+1G>A		IVS30	Splice → PTC	THD	1x	[16]
12	c.6205C>T	p.Arg2069Cys	74	Missense	THD	c.3895-1G>A		IVS30	Splice → PTC	THD	1x	[25]
13	c.6205C>T	p.Arg2069Cys	74	Missense	THD	c.4018C>T	p.Arg1340X	34	PTC	THD	1x	[16]
14	c.6205C>T	p.Arg2069Cys	74	Missense	THD	c.4027C>T	p.Arg1343X	34	PTC	THD	1x	[16]
15	c.6205C>T	p.Arg2069Cys	74	Missense	THD	c.5662insG	p.Pro1888AlafsX27	67	PTC	THD	Pat 11	This Study
16	c.6205C>T	p.Arg2069Cys	74	Missense	THD	c.6205C>T	p.Arg2069Cys	74	Missense	THD	3x (pat 4, 8, 18)	This study, [14]
17	c.6262G>A	p.Gly2088Arg	75	Missense	THD	c.676C>T	p.Arg226X	5	PTC	NC1	1x	[26]
18	c.6637G>A	p.Gly2213Arg	83	Missense	THD	c.4918delG	p.Gly1640ValfsX70	52	PTC	THD	Pat 9	This study
19	c.6815G>C	p.Gly2272Ala	86	Missense	THD	c.1363G>T	p.Gln455X	11	PTC	NC1	Pat 15	This study
20	c.7415G>A	p.Gly2472Asp	97	Missense	THD	c.1874delGT	p.Ser625ArgfsX5	14	PTC	NC1	1x	[26]
21	c.7864C>T	p.Arg2622Trp	105	Missense	THD	c.553C>T	p.Arg185X	5	PTC	NC1	1x	[15]
22	c.7864C>T	p.Arg2622Trp	105	Missense	THD	c.2482delCT	p.Ser828CysfsX37	19	PTC	NC1	1x	[9]
23	c.7882C>T	p.Arg2628Trp	106	Missense	THD	c.1332C>A	p.Tyr444X	10	PTC	NC1	1x	[26]
24	c.8065G>A	p.Gly2689Arg	109	Missense	THD	c.1732C>T	p.Arg578X	13	PTC	NC1	Pat 7	This study
25	c.8065G>C	p.Gly2689Arg	109	Missense	THD	c.6527insC	p.Gly2177TrpfsX113	80	PTC	THD	1x	[10]
26	c.8083G>A	p.Gly2695Ser	109	Missense	THD	c.8083G>A	p.Gly2695Ser	109	Missense	THD	Pat 1, 2	[12, 14, 22]
27	c.8156G>C	p.Gly2719Ala	110	Missense	THD	c.2051-1G>C		IVS15	Splice → PTC	NC1	Pat 19	This study
28	c.8323G>A	p.Gly2775Ser	112	Missense	THD	c.425A>G	p.Lys142Arg	3	Splice → PTC	NC1	2x (pat 14)	This study, [23]
29	c.8323G>A	p.Gly2775Ser	112	Missense	THD	c.8323G>A	p.Gly2775Ser	112	Missense	THD	1x	[26]

2 ¹ In **bold** novel *COL7A1* mutations.3 ² PTC, premature termination codon; splice → PTC, splice site affecting mutation resulting in PTC.

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- 1 ³ THD, triple-helix domain; NC1, non-collagenous 1 domain; NC2, non-collagenous 2 domain.
- 2 ⁴ NF, not found; NA, not available.
- 3 ⁵ IVS, intervening sequences (i.e. introns)
- 4 ⁶ Only a single mutation could be identified despite complete sequencing of the other allele.

1 Six genotypes consist of homozygous or compound heterozygous missense mutations within
2 the triple-helix domain (THD) of Col7. Twenty-three genotypes are composed of THD
3 missense mutations combined with mutations generating a premature truncation codon (PTC)
4 or an unidentified second mutation (pat 13). Since PTC mutations are generally considered to
5 result in no functional protein, the functional genotype in these patients is determined by the
6 hemizygous missense mutation.[27] Hence, the functional genotype in RDEB-I is that of a
7 homozygous, compound heterozygous or hemizygous missense mutation within the THD.

8 To date, 19 missense mutations have been associated with RDEB-I, all of which are
9 either substitutions of an arginine (ASs, n = 5) or a glycine (GSs, n = 14) (Table 1, Fig. 2).
10 The 14 GSs are located at 13 different glycine codons. None of the carrier parents were
11 affected, indicating that the mutations are recessive. The question is whether these recessive
12 ASs or GSs are specific for RDEB-I or whether they can also cause other (R)DEB subtypes.
13 Of the five ASs, the p.Arg2063Gly, p.Arg2622Trp, and p.Arg2628Trp mutations have been
14 exclusively reported in RDEB-I, whereas the p.Arg2008Gly and p.Arg2069Cys mutations
15 were also found in non-inversa RDEB subtypes (Table S2). Nine of the 14 recessive GSs have
16 been exclusively reported in RDEB-I, whereas four GSs were also reported in non-inversa
17 RDEB subtypes and the p.Gly2213Arg mutation was reported as a dominant GS (Table S3).
18 Supplementary tables S2 and S3, and Fig. 2 show detailed information about the ASs and GSs.

19 To check whether ASs in general might cause the RDEB-I phenotype, we studied the
20 phenotypes of patients carrying other ASs. The THD of Col7 contains a total of 94 arginines
21 in Gly-X-Y repeats,[35] of which 12 have been found substituted in DEB (HGMD
22 professional database, www.hgmd.org, and unpublished data (HS), 26 August 2010).
23 Substitutions of 5/12 arginines (42%) are involved in RDEB-I, whereas substitutions of 9/12
24 arginines (75%) are involved in other DEB phenotypes. Hence, it seems unlikely that ASs are
25 in general the cause of RDEB-I.

1

2

3 **DISCUSSION**

4

5 The cause of the typical ‘inverse’ distribution of blistering in RDEB-I is not well understood.

6 We identified 20 Dutch and UK RDEB-I patients and expanded the list of known RDEB-I

7 genotypes from the literature with nine new genotypes, which enabled us to conduct an

8 extensive genotype-phenotype correlation study in RDEB-I. Our results suggest that RDEB-I

9 is caused by a set of specific recessive arginine and glycine substitutions within the THD of

10 Col7.

11

12 **Clinical characteristics**

13 All 20 patients displayed the typical inversa distribution of skin blistering in the proximal

14 body flexures and the body axis. Mucosal involvement, especially of the oral mucosa, was

15 present in all patients. Nearly all patients started with generalized blistering and a third had

16 congenital localized absence of skin. The age of transition from generalized to inversa

17 distribution took place before the age of 4 years in most patients, but ranged from 1 to 18

18 years. Marked clinical variation was noted between patients and the clinical severity of

19 RDEB-I seems to cover a spectrum, as seen in other DEB phenotypes.[1] Four patients had

20 ‘mucosal only’ RDEB-I in which the mucosal involvement dominated, including severe

21 oesophageal stenosis requiring multiple dilatations, but skin blistering was almost absent. A

22 similar phenotype has been described previously,[10, 23, 36] but was not considered to be

23 within the RDEB-I spectrum due to absence of typical blistering in the body flexures. We

24 think that patients with ‘mucosal only’ RDEB form the mildest end of the inversa spectrum

25 (with regard to the skin), because the severe mucosal involvement is not part of the milder

1 forms of RDEB-O.[1, 14] On the other end of the RDEB-I spectrum, four patients had
2 significant involvement of the acra, leading to mild flexion contractures and limited
3 pseudosyndactyly. This end of the RDEB-I spectrum might be difficult to distinguish from
4 RDEB-O or even RDEB-sev gen.[5, 37] However, these RDEB-I patients were not growth
5 retarded and none had developed squamous cell carcinoma.

6 A diagnosis of RDEB-I has important implications for the patient and clinician,
7 especially when skin blistering is relatively mild. Nearly all patients have severe oral and
8 oesophageal blistering resulting in intra-oral scarring, microstomia, ankyloglossia, and
9 oesophageal strictures often requiring repeated dilatations. This can result in decreased food
10 intake, poor dental hygiene and oral infections, which warrant careful follow-up of growth
11 and weight and aggressive dental care. Peri-anal blistering commonly results in constipation
12 that should be asked about carefully and treated if present. In women, genital blistering and
13 scarring can interfere with sexual function, complicate vaginal delivery and recovery
14 afterwards, which may be a reason for elective caesarean section. Moreover, blistering of the
15 external auditory canal may lead to conductive hearing loss, requiring hearing aids after the
16 age of 30. Careful inspection and follow-up of the auditory canals is thus warranted and
17 audiologic evaluation may be indicated at later ages.

18

19 **Genotype-phenotype correlations**

20 The 29 functional genotypes of our 18 RDEB-I patients and those reported in the literature
21 consist of homozygous, compound heterozygous or hemizygous (i.e. in conjunction with a
22 PTC) recessive ASs or GSs within the THD of Col7. To date, five recessive ASs and 14
23 recessive GSs have been associated with RDEB-I. The collagenous THD domain is composed
24 of many consecutive Gly-X-Y triplets that form a triple-helix.[35] These Gly-X-Y repeat
25 sequences are separated into 20 collagenous subdomains (COL1-20) by 19 interruptions or

1 imperfections (IM1-19) that provide flexibility to the protein. The largest imperfection, the
2 hinge (IM10), is composed of 39 amino acids and divides the THD into amino (COL1-10)
3 and carboxyl (COL11-20) regions. The glycine residues are located at the centre of the triple-
4 helix and are essential for stable triple-helix formation, whereas the amino acids at the X and
5 Y positions are exposed on the helix surface and are less essential for triple-helix stability.[38,
6 39] Substitutions of glycine residues therefore have major disruptive effects on triple-helix
7 folding and/or stability, whereas substitutions of X and Y residues not necessarily exert
8 pathogenic effects. Therefore, pathogenicity of the GSs identified in RDEB-I was not
9 surprising. Pathogenicity of the RDEB-I ASs was also likely, as they are located at highly
10 conserved positions within Col7.

11 Two RDEB-I ASs and five GSs have also been found in patients with phenotypes
12 other than RDEB-I. This observation suggests that some arginine and glycine codons have the
13 potential to result in RDEB-I when mutated, but other as yet unidentified factors determine
14 the resultant clinical phenotype, which can be RDEB-I or sometimes other. The puzzling
15 question remains as to why some specific arginine and glycine codons possess this potential
16 to induce RDEB-I when mutated, since ASs and GSs in general cause phenotypes other than
17 RDEB-I.[28] We therefore looked more closely into the position of the arginine and glycine
18 codons in the THD and into the nature of the substituting amino acids.

19

20 The arginine substitutions

21 The five arginine codons are located in collagenous subdomains in the carboxyl region of the
22 THD, of which three in the first two subdomains after the hinge region (Fig. 2, Table S2). The
23 position of the arginine within the Gly-X-Y triplet does not differ from arginines involved in
24 other phenotypes: the five RDEB-I arginines reside in the Y position as do 7 of 9 arginines
25 involved in other DEB subtypes.

1 Our findings suggest that for each of the five arginines there is only a specific
2 substitution that can lead to RDEB-I (Tables S2). The arginine at codon 2063 was found
3 substituted by glycine in RDEB-I, whereas substituted by tryptophan in other RDEB
4 phenotypes; the arginine at codon 2622 was found substituted by tryptophan in RDEB-I,
5 whereas substituted by glutamine in RDEB-HS. The p.Arg2008Gly mutation was found
6 homozygously in RDEB-I, whereas substitution of this arginine by cysteine or histidine was
7 reported in other RDEB subtypes. Of note, the p.Arg2008Gly mutation was also found
8 homozygously in a single RDEB-HS patient, emphasizing that the *COL7A1* mutation is not
9 the only factor determining the inversa phenotype. On the other hand, the p.Arg2069Cys
10 mutation was not only found in 12 inversa patients, but also in four with RDEB-O. However,
11 it cannot be excluded that these patients will ultimately develop the RDEB-I phenotype, as it
12 can take years for the inversa phenotype to become evident. Therefore, the p.Arg2069Cys is
13 the most frequently observed RDEB-I mutation and can be considered specific for RDEB-I.

14 The specific substituting amino acids leading to RDEB-I, however, are different for
15 each arginine codon and from chemically different groups (Table S2). At codons 2008, 2063
16 and 2069, the large, hydrophilic arginine is substituted by the smaller, nonpolar residues,
17 glycine or cysteine, whereas at codons 2622 and 2628 the arginine is substituted by the large,
18 hydrophobic and aromatic tryptophan. The same substituting amino acids have been found to
19 replace other arginines in the THD in non-inversa phenotypes (Fig 2). This emphasizes that
20 there is not a specific type of amino acid that leads to RDEB-I when replacing an arginine, but
21 it is the combination of a specific substituting amino acid at a specific arginine codon position.
22 Interestingly, the arginine codons 2622 and 2628 in COL19 are located close to the cysteine
23 residue at position 2634. This is the only cysteine residue in the THD and might be involved
24 in disulfide binding with the non-collagenous domain of a pairing Col7 molecule.[35]

1 Substituting either arginine 2622 or 2628 could therefore lead to instable Col7 dimers as a
2 result of local conformational changes.

3

4 The glycine substitutions

5 All 13 glycine codons associated with RDEB-I are located in collagenous subdomains of the

6 THD (Fig. 2, Table S3). Four of 13 (31%) glycine codons are located in subdomain COL19.

7 This number is surprisingly high, even though COL19 is the largest subdomain, containing

8 almost 16% of Gly-X-Y repeats. These four GSs, as well as p.Gly2775Ser in COL20 are

9 located close to the carboxyl end of the THD. Additionally, the p.Gly1347Trp mutation in the

10 COL2 subdomain is located near the amino end of the THD and glycine codon 1907 (COL10)

11 is located near the hinge region, the end of the amino region of the THD. It can thus be

12 speculated that GSs located at either end of the amino and carboxyl regions of the THD can

13 cause the relatively mild RDEB-I phenotype. This agrees with the finding that GSs near the

14 ends of the THD cause less severe triple-helix destabilization than central GSs and

15 concordantly lead to less severe disease.[23] Interestingly, the amino acids replacing the

16 glycines are either chemically very different and destabilizing, or chemically quite similar and

17 less destabilizing.[38]

18 Recently, Chiaverini et al. suggested that GSs located near the borders of collagenous

19 subdomains exert different, i.e. milder, pathogenic effects than GSs located in the centre of

20 collagenous subdomains and consequently result in RDEB-I.[26] However, the combined

21 genotypic data of our RDEB-I patients and those reported in the literature show that of the 13

22 glycine codons involved in RDEB-I only 6/13 (46%) are located in the first three or last three

23 Gly-X-Y triplets of their respective collagenous subdomain. The other 7/13 (54%) are located

24 in the central portion of the subdomain (Table S3). Hence, a GSs location near the border of a

1 collagenous subdomain might predispose to RDEB-I, but it is certainly not the only factor,
2 since specific central GSs also have the potential to induce RDEB-I.

3 There were no differences in the location of the glycine codons or the nature of the
4 substituting amino acids between GSs causing the ‘mucosal only’ phenotype and the
5 phenotype with limited acral involvement (Tables 1 and S3). However, the four glycine
6 codons involved in RDEB-I with limited acral involvement have been reported in the RDEB-
7 sev gen or DDEB phenotypes. In contrast, of the glycine codons involved in the RDEB-I
8 ‘mucosal only’ phenotype only glycine codon 1347 was reported in other, milder RDEB
9 phenotypes.[10, 30, 32]

10

11 **Putative temperature related pathophysiology in RDEB-I**

12 Because the lesions in RDEB-I are mainly restricted to body locations where skin
13 temperatures are the highest (Fig. 3),[40, 41] we hypothesize that the pathophysiology in
14 RDEB-I is temperature-dependent. Kern et al. showed by *in vitro* experiments that Col7
15 homotrimers from a ‘mucosal only’ patient (genotype 18, Table 1) dissociated at lower
16 temperatures ($T_m = 33-35^\circ\text{C}$) compared to normal Col7 ($T_m = 39-41^\circ\text{C}$).[10] Moreover,
17 Fritsch et al. showed that Col7 trimers harbouring three different dominant GSs in the THD
18 had even more reduced thermostability ($T_m 20-33^\circ\text{C}$, mono-expression).[39] Furthermore,
19 four GSs in the THD of type XVII collagen also reduced the thermostability of recombinant
20 proteins.[42] This reduced thermostability reflects folding defects of procollagen molecules
21 into Col7 trimers and/or packing defects of Col7 trimers into anchoring fibrils. The lower
22 dissociation temperatures of Col7 homotrimers harbouring the dominant GSs suggest that
23 dominant GSs have more dramatic destabilizing effects on Col7 than recessive GSs. Hence,
24 we speculate that the recessive GSs found in RDEB-I only slightly lower the temperature
25 dissociation thresholds for Col7 homotrimers. The only body regions where skin temperatures

1 are continuously high enough to cross these temperature thresholds are the inversa regions.
2 This leads to increased Col7 destabilization with subsequent blistering only in these body
3 regions and not on the more distal body parts where skin temperatures are generally lower.
4 The invariable involvement of the oral mucosa and oesophageal involvement in most patients
5 supports this hypothesis. Temperature-dependent pathophysiology has been shown for
6 bathing suit ichthyosis (BSI), in which skin distribution is quite similar to RDEB-I.[41]
7 Moreover, also in BSI the skin features are more severe in infancy than later on. In BSI,
8 specific mutations in the transglutaminase-1 gene, *TGMI*, lead to decreased enzyme activity
9 at temperatures above 33°C,[43] explaining the ‘inversa-like’ skin distribution. Interestingly,
10 most BSI causing *TGMI* mutations found to date are arginine substitutions.[44] The ASs
11 found in RDEB-I could thus exert the same effects as the GSs. A temperature-dependent
12 effect in skin disorders has also been noted for mutations in the *TYR* and *CDKN2A* genes.[45,
13 46] Temperature-dependent pathophysiology could also explain the transition from
14 generalized to inversa distribution, as one can assume that skin temperature *in utero* will be
15 higher than postnatally in all body regions. An obvious question is whether patients have
16 more severe blistering during warmer circumstances, but this was denied by all patients.
17 Hence, the temperature hypothesis is an interesting topic for future studies.

18

19 **Conclusion and clinical implications**

20 Our results suggest that RDEB-I is caused by specific recessive arginine and glycine
21 substitutions in the THD of Col7. The p.Arg2069Cys mutation is the most frequently
22 observed RDEB-I mutation. This study provides detailed insights into genotype-phenotype
23 correlations in RDEB-I, which is a first step towards understanding the pathophysiology of
24 the typical inverse distribution in this rare RDEB subtype. The exact mechanisms by which

1 these ASs and GSs cause RDEB-I are not yet understood, but we hypothesize that the
2 pathophysiology of the inversa distribution is temperature-dependent.

3 These findings have important implications for the diagnosis and prognosis in DEB,
4 since the identification of one of the specific ASs or GSs as the only functional allele in a
5 newborn with DEB is predictive of the future development of RDEB-I. However, a phenotype
6 with more severe skin involvement may occasionally develop due to currently unknown
7 genetic, epigenetic or non-genetic modifiers.

8

9

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20

21

22 **COMPETING INTERESTS**

23

24 None.

25

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10

11

12 **PATIENT CONSENT**

13

14 We obtained written consent for publication of all patient images.

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1 **FIGURE LEGENDS**

2

3 **Figure 1. Clinical features of patients with RDEB-inversa.**

4 Typical distribution of skin blistering in RDEB-I patients in the proximal body flexures and
5 body axis with nail dystrophy and involvement of the mucosal membranes. Skin blistering
6 and erosions in (A) the neck of patient 9, (B) the left axilla of patient 17, (C) the lower back in
7 patient 3, and (D) the groins and lower abdomen in patient 6. (E and F) The hands and feet of
8 patient 7 show absent and dystrophic nails, with no completely normal nails. There is no
9 current blistering, but small scars indicate past blistering. (G) Microstomia, ankyloglossia and
10 dental decay of the lower dentition (upper dentition has been repaired) in patient 17. Written
11 consent was obtained for publication of all images.

12

13 **Figure 2. Arginine and glycine substitutions associated with RDEB-I.**

14 Schematic representation of the triple-helix domain of type VII collagen. White boxes are
15 collagenous subdomains COL1-20, black bars represent collagenous imperfections IM1-19,
16 and the hinge region (IM10) is hatched. The glycine substitutions are shown above the triple-
17 helix domain, and the arginine substitutions below. All known arginine and glycine
18 substitutions, as summarized by Dang and Murrell,[28] are shown. Glycine and arginine
19 substitutions associated with RDEB-I are depicted above and below the dashed lines,
20 respectively. Glycine and arginine substitutions exclusively reported in RDEB-I are shown in
21 bold. Novel glycine substitutions are shown in italics.

22

23 **Figure 3. Digital thermographs of a healthy human body.**

24 Photos obtained by digital infrared thermal imaging of (A) front and (B) back of a healthy
25 female human body showing higher body temperatures in the typical inversa body sites, i.e.

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- 1 the proximal flexures, neck, lower back and submammary folds, compared to more distal
- 2 body parts. The colour scales indicate the temperatures that correspond to the colours used in
- 3 the images. Images published with permission from Meditherm Inc, Hilversum, the
- 4 Netherlands.



