UNIVERSIDADE FEDERAL DE PERNAMBUCO
INSTITUTO KEIZO ASAMI
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HIDRADENITE SUPURATIVA HEREDITÁRIA: ANÁLISE GENÉTICA EM FAMÍLIA DO ESTADO DE PERNAMBUCO

Ana Sofia Lima Estevão de Oliveira

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HIDRADENITE SUPURATIVA HEREDITÁRIA: ANÁLISE GENÉTICA EM FAMÍLIA DO ESTADO DE PERNAMBUCO

Defesa de doutoramento apresentada ao Programa de Pós-Graduação em Biologia Aplicada à Saúde, Centro de Biociências, Universidade Federal de Pernambuco, como requisito parcial para a obtenção do título de Doutora em Biologia Aplicada à Saúde.

Área de concentração: Biologia Aplicada à Saúde

Orientador: Prof. Dr. Sergio Crovella

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À minha família.

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RESUMO

A Hidradenite Supurativa (HS) é uma condição de pele que envolve a unidade pilossebácea e é caracterizada por inflamação crônica e pela presença de nódulos e abscessos recorrentes que culminam em secreção purulenta. Em raras ocasiões, a HS pode aparecer concomitantemente com outras doenças como a Doença de Dowling Degos (DDD). A DDD é uma genodermatose autossômica dominante rara, caracterizada por lesões pigmentadas reticuladas e lentamente progressivas que envolvem áreas flexurais da pele. A etiologia de ambas as doenças permanece pouco compreendida. Mutações nos genes queratina 5, potenciador de presenilina (PSENEN), proteína O-fucosiltransferase 1 e proteína O-glucosiltransferase 1 foram associados a DDD, enquanto mutações na nicastrina (*NCSTN*) foram relatadas em indivíduos com HS. PSENEN juntamente com NCSTN, presenilinas (PSEN) e defeito da faringe anterior 1 (APH1) compõe o complexo γ-secretase, que é amplamente distribuído em células e tecidos humanos e está envolvido em processos de clivagem e hidrólise de várias proteínas. Curiosamente, mutações no *PSENEN* já foram associadas à ocorrência simultânea destas doenças, enquanto o papel da *NCSTN* permanece desconhecido. Visando aumentar o entendimento sobre a etiologia desta relação sindrômica, estudamos uma família de quatro gerações com sete membros afetados por HS e DDD. Os pacientes têm um fenótipo de HS grave caracterizado por nódulos inflamatórios, granuloma piogênico, fístulas e comedões simples e duplos espalhados por diferentes áreas do corpo, e um fenótipo de DDD caracterizado por pigmentações reticuladas nas axilas e região inguinal, bem como cicatrizes crateriformes / cribriformes no dorso, dorso nasal e filtro labial. O sequenciamento de exoma total (WES) em dois indivíduos afetados e um não afetado da família identificou uma nova mutação sem sentido no éxon 2 do gene da *NCSTN*, c.T131A:p.L44X. A segregação dessa mutação em todos os membros afetados foi confirmada pelo sequenciamento de Sanger. Para estudar o papel dessa mutação na patogênese da HS e DDD, isolamos as células da bainha radicular externa (ORS) dos folículos pilosos dos pacientes, seguido por análises de expressão gênica e proteica. Como resultado, mostramos que o códon de parada prematuro leva ao decaimento de mRNA pela degradação de mRNA mediada por mutação sem sentido, causando haploinsuficiência da NCSTN nos indivíduos afetados. Além disso, observamos que esta haploinsuficiência também afeta as outras subunidades do

complexo da γ-secretase (PSEN e PSENEN). Deste modo, nossas descobertas sugerem que a haploinsuficiência da NCSTN pode estar associada a HS e DDD nesses pacientes. No geral, este estudo fornece novas informações sobre a etiologia da HS familiar em pacientes pernambucanos e identifica um novo gene associado a coocorrência de HS e DDD.

Palavras-chave: Acne Inversa; Notch; doença de pele; medicina personalizada.

ABSTRACT

Hidradenitis suppurativa (HS) is a skin condition that involves the pilosebaceous unit and is characterized by chronic inflammation and the presence of recurrent nodules and abscesses that culminate in purulent discharge. On rare occasions, HS can appear concomitantly with other diseases such as Dowling Degos Disease (DDD). DDD is a rare autosomal dominant genodermatosis characterized by reticulate and slowly progressive pigmented lesions in flexural areas of the skin. The etiology of both diseases remains poorly understood. Mutations in the genes keratin 5, presenilin enhancer (*PSENEN*), protein O-fucosyltransferase 1, and protein O-glucosyltransferase 1 have been associated with DDD while mutations in nicastrin (*NCSTN*) have been reported in individuals with HS. PSENEN together with NCSTN, presenilin (PSEN) and anterior pharynx-defective 1 (APH1) compose the γ-secretase complex which is widely distributed in human cells and tissues; the γ-secretase complex is involved in cleavage and hydrolysis of various proteins. Interestingly, mutations in *PSENEN* have already been associated with the simultaneous occurrence of these diseases whilst the role of *NCSTN* remains unknown. Aiming to increase understanding about the etiology of this syndromic relationship, we studied a four-generation family with seven members affected by HS and DDD. Patients have a severe HS phenotype characterized by inflammatory nodules, pyogenic granuloma, fistulas, and single and double comedones scattered over different areas of the body, and a DDD phenotype characterized by reticulated pigmentations in the armpits and inguinal region as well as crateriform/cribriform scars on the back, nasal dorsum, and labial philtrum. Whole exome sequencing (WES) in two affected and one unaffected individual of the family identified a new nonsense mutation in exon 2 of the *NCSTN*, c.T131A:p.L44X. The segregation of this mutation in all affected members was confirmed by Sanger sequencing. To study the role of this mutation in the pathogenesis of HS and DDD, we isolated outer root sheath (ORS) cells from patient hair follicles, followed by gene and protein expression analyses. As a result, we show that this premature stop codon leads to mRNA decay by nonsense-mediated mRNA decay, causing NCSTN haploinsufficiency in affected individuals. Furthermore, we observed that this haploinsufficiency also affects the other subunits of the γ-secretase complex (PSEN and PSENEN). Thus, our findings suggest that NCSTN haploinsufficiency may be associated with HS and DDD in these patients. Overall, this study provides new

information on the etiology of familial HS in patients from Pernambuco and identifies a new gene associated with the co-occurrence of HS and DDD.

Keywords: Acne Inversa; Notch; skin disease; personalized medicine.

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Dissertação

LISTA DE ABREVIATURAS E SIGLAS

2D	Duas dimensões
3D	Três dimensões
3T3-J2	Fibroblastos embrionários de camundongos
ADN/DNA	Ácido desoxirribonucleico / Deoxyribonucleic acid
AI	*Acne Inversa*
AMPs	Peptídeos antimicrobianos / Antimicrobial peptides
APH-1	Faringe anterior defectiva 1 / *Anterior Pharynx-defective 1*
ARN/RNA	Ácido ribonucleico / Ribonucleic Acid
BA3R	Anticorpo monoclonal de controle de carga de ß actina / *beta Actin Loading Control Monoclonal Antibody*
BSC	Cabine de segurança biológica / *Biological Safety Cabinet*
BWA	*Burrows-Wheeler Aligner*
cADN/cDNA	DNA complementar / Complementary DNA
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CCL	Ligante CC de quimiocina / *Chemokine (C-C motif) Ligand*
CCL20	Ligante CC de quimiocina 20 / *C-C Motif Chemokine Ligand 20*
CEP	Comitê de Ética em Pesquisa
CXCL	Ligantes C-X-C de quimiocina / *Chemokine (C-X-C motif) Ligand*
CXCL1	Ligante C-X-C de quimiocina 1 / *Chemokine (C-X-C motif) Ligand 1*
CXCL6	Ligante C-X-C de quimiocina 6 / *Chemokine (C-X-C motif) Ligand 6*
CXCL8	Ligante C-X-C de quimiocina 8 / *Chemokine (C-X-C motif) Ligand 8*
Cx26	Conexina 26
DAMP	Padrões Moleculares Associados a Danos / Damage-Associated Molecular Pattern
DDD	Doença de Dowling Degos
DEFB	Beta defensina / Defensin beta
DEFB4	Beta defensina 4
DEFB103	Beta defensina 103
DLQI	Índice de Qualidade de Vida em Dermatologia / Dermatology Life Quality Index
DMEM	*Dulbecco's Modified Eagle Medium*
EDTA	Ácido etilenodiamino tetra-acético / *Ethylenediaminetetraacetic acid*

ESC	Células tronco epidérmicas / *Epidermal Stem Cells*
EUA	Estados Unidos da América
EVA	Escala Visual Analógica
EXOI	Exonuclease I
FastAp	Fosfatase alcalina termossensível
FGFR2	Fator de crescimento do receptor de fibroblastos 2 / *Fibroblast Growth Factor Receptor 2*
FMF	Febre Mediterrânea Familiar
GJB2	Proteína β-2 da *Gap Junction / Gap Junction Beta-2 protein*
GQ	Qualidade de genotipagem / *Genotyping Quality*
GRCh.38	Genoma Humano versão 38
HBD2	Beta-defensina humana / *Human Beta Defensin 2*
HC-UFPE	Hospital das Clínicas da Universidade Federal de Pernambuco
HS	Hidradenite Supurativa
HSS	Pontuação de Sartorius / *Hidradenitis Suppurativa Score*
IFE	Epiderme interfolicular / *Interfollicular Epidermis*
IFN-γ	Interferon gama
IGF-1	Fator de crescimento semelhante à insulina tipo 1 / *Insulin-like Growth Factor 1*
IHS4	Sistema Internacional de Pontuação de Gravidade da Hidradenite Supurativa
IL-1ß	Interleucina 1 beta
IL1RN	Antagonista do receptor de IL-1
IL-6	Interleucina 6
IL-12	Interleucina 12
IL-12Rβ1	Subunidade β-1 do receptor da interleucina-12
IL-17	Interleucina 17
IL-21	Interleucina 21
IL-23	Interleucina 23
IMRB	Instituto Mondor de Pesquisa Biomédica / Institut Mondor de Recherche Biomédicale
Indel	Inserção/Deleção
IVL	Involucrina
KRT1	Queratina 1 / *Keratin 1*

KRT5 Queratina 5 / *Keratin 5*

KRT6 Queratina 6 / *Keratin 6*

KRT10 Queratina 10 / *Keratin 10*

KRT16 Queratina 16 / *Keratin 16*

LL-37 Catelicidina

LPIN2 Lipina 2

LPM Laboratório de Patologia Molecular

LOR Loricrina

M1 Macrófagos do tipo 1

MEC Matriz extracelular

MEFV Gene da febre mediterrânea / *Mediterranean Fever*

MMPs Metaloproteinase de matriz / *Matrix metalloproteinase*

MMP1 Metaloproteinase de matriz 1

MMP3 Metaloproteinase de matriz 3

MMP10 Metaloproteinase de matriz 10

MQ Qualidade de mapeamento / *Mapping Quality*

mRNA RNA mensageiro

mTORC1 Complexo 1 do alvo da rapamicina em mamíferos / *Mechanistic Target of Rapamycin Complex 1)*

MYD88 Gene 88 de diferenciação primária de resposta mieloide / *Myeloid Differentiation primary response 88*

NAP Núcleo de Apoio à Pesquisa

NCBI Centro Nacional de Informações sobre Biotecnologia / *National Center for Biotechnology Information*

NCSTN Nicastrina

NF-κB Fator nuclear potencializador de cadeias leves kappa de células B / *Nuclear factor kappa-light-chain-enhancer of activated B cells*

NLRC4 Proteína NLR contendo o domínio CARD 4

NLRP3 Proteína NLR contendo o domínio de pirina 3

NMD Degradação do RNA mediado por mutação sem sentido / *Nonsense-mediated mRNA decay*

NRS Escala Numérica de Classificação / *Numeric Rating Scale*

OCRL1 Inositol polifosfato-5-fosfatase 1

ORS Bainha radicular externa / *Outer Root Sheath*

PAMP Padrões Moleculares Associados a Patógenos / *Pathogen-Associated Molecular Pattern*

PASH Pioderma gangrenoso, Acne e Hidradenite Supurativa

PAPASH Artrite Piogênica + PASH

PDSE Programa de Doutorado Sanduíche no Exterior

PEN2 Presenilina 2

PI (4,5) P2 Fosfatidilinositol 4,5-bifosfato / *Phosphatidylinositol 4,5-bisphosphate*

PPARγ Receptor ativado por proliferadores de peroxissoma gama/ *Peroxisome Proliferator-Activated Receptor gamma*

POFUT1 O-fucosiltransferase 1

POGLUT1 O-glicosiltransferase 1

PPL Periplacina

PSEN Presenilina

PSENEN Presenilina 2

PSTPIP Proteína 1 que interage com a prolina-serina-treonina-fosfatase / *Proline-Serine-Threonine Phosphatase Interacting Protein 1*

PTC Códon com terminação prematura / *Premature Termination Codons*

qPCR PCR em tempo real

RCP/PCR Reação em Cadeia da Polimerase / *Polymerase Chain Reaction*

RRP Receptores de Reconhecimento de Padrões

SAPHO Sinovite, Acne, Pustulose, Hiperostose e Osteíte

SNP Polimorfismo de nucleotídeo único / *Single Nucleotide Polymorphisms*

SUS Sistema Único de Saúde

TBE Tampão de migração *Tris-borate-EDTA*

TCLE Termo de Consentimento Livre e Esclarecido

TGM Transglutaminase

Th Células T auxiliares / *T helper cells*

Th1 Células T auxiliares 1

Th17 Células T auxiliares 17

TLR Receptor do tipo *toll* / *Toll Like Receptor*

TNF-α Fator de necrose tumoral alfa / *Tumor Necrosis Factor alpha*

Tregs Células T reguladoras

UCSC Universidade da Califórnia em Santa Cruz / *University of California Santa Cruz*

UFPE	Universidade Federal de Pernambuco
UPEC	Université Paris-Est Créteil
US	Ultrassonografia de alta frequência
WDR1	Proteína 1 contendo repetição WD / *WD Repeat Domain 1*
WES	Sequenciamento do exoma completo / *Whole Exome Sequencing*

LISTA DE SÍMBOLOS

g	Grama
ng	Nanograma
µg	Microgrma
ml	Mililitro
µl	Microlitro
mmol	Milimol
µmol	Micromol
pmol	Picomol
nm	Nanômetros
pb	Pares de bases
r.p.m.	Rotações por minuto
V	Volts
α	Alfa
ß	Beta
γ	Gama
κ	Kappa
≈	Aproximadamente
∞	Tempo indeterminado
ΔΔCT	Expressão relativa da expressão gênica

SUMÁRIO

1 INTRODUÇÃO

A Hidradenite Supurativa (HS), também conhecida como *Acne Inversa* (AI), é uma condição de pele que envolve a unidade pilossebácea e é caracterizada por inflamação crônica e pela presença de nódulos e abscessos recorrentes que culminam em secreção purulenta (KATOULIS *et al*., 2017). Tipicamente, lesões de HS são encontradas em áreas do corpo contendo glândulas apócrinas, como axilas, mamas, virilha e nádegas (RALF PAUS *et al.*, 2008). Com a progressão da doença, essas lesões podem evoluir para túneis dérmicos com secreção fétida e cicatrizes desfigurantes, afetando gravemente a qualidade de vida dos pacientes (JEMEC, 2012; MATUSIAK, 2020). Em raras ocasiões, a HS pode aparecer concomitantemente a outras doenças como a Doença de Dowling Degos (DDD) (AGUT-BUSQUET *et al.*, 2019). A DDD é uma genodermatose autossômica dominante rara, caracterizada por lesões pigmentadas reticuladas e lentamente progressivas que envolvem áreas flexurais da pele (PAVLOVSKY *et al.*, 2018).

A etiologia da HS ainda é pouco conhecida (DUFOUR; EMTESTAM; JEMEC, 2014). No entanto, acredita-se que diversos fatores possam estar envolvidos no seu aparecimento, como predisposição genética, disfunção hormonal, disfunção imunológica, desequilíbrio no desenvolvimento epidérmico e desequilíbrio metabólico (MARTORELL *et al.,* 2015). Quando alterados, esses fatores podem contribuir com uma hiperqueratinização folicular, gerando uma obstrução do folículo piloso, com uma subsequente ruptura. Essa ruptura folicular ativa o sistema imunológico, que responde aumentando a expressão de citocinas/quimocinas pró-inflamatórias, bem como de vários outros mediadores pró-inflamatórios (LAFFERT *et al*., 2011; LAFFERT *et al*., 2009; ZOUBOULIS *et al.,* 2020).

Assim como a etiologia da HS, sua prevalência também é incerta; estudos relatam uma prevalência populacional que varia de 0,033% a 4% ao redor do mundo (BETTOLI *et al.,* 2019; COSMATOS *et al.,* 2012; GARG *et al.,* 2017; JEMEC; HEIDENHEIM; NIELSEN, 1996; KIMBALL *et al.,* 2013; REVUZ *et al.,* 2008). Esses dados variam não só entre diferentes países, como também em uma mesma população restrita a uma mesma área geográfica (CARTRON; DRISCOLL, 2019; JEMEC; HEIDENHEIM; NIELSEN, 1996; REVUZ *et al.,* 2008; SHALOM; COHEN, 2019). Acredita-se que essa variação pode ser consequência da falta de um padrão diagnóstico entre os diferentes centros de saúde, bem como da falta de conhecimento

sobre a doença entre os profissionais da área, que não dermatologistas (CHIRICOZZI; MICALI; VERALDI, 2019; KIRBY *et al*., 2015). No Brasil, a prevalência da doença permanece desconhecida (IANHEZ; SCHMITT; MIOT, 2018).

Em geral, pacientes com crises inflamatórias derivadas da HS buscam atendimento em unidades de atenção primária em saúde, como unidades básica de saúde, onde são atendidos por médicos generalistas (KHALSA; LIU; KIRBY, 2015; KIRBY *et al.,* 2015). Como a doença é pouco conhecida fora da área dermatológica, esses pacientes são diagnosticados e tratados incorretamente (KIRBY et al., 2015). Em muitos casos, o diagnóstico correto é realizado anos após o surgimento da doença, e depois de um grande número de consultas e tratamentos equivocados (DEKEULENEER *et al.,* 2019; GARG *et al*., 2018; ROUSTAN, 2019; SAUNTE *et al.,* 2015), o que pode agravar o quadro clínico do paciente (LOPES *et al.,* 2019a; ROUSTAN, 2019).

Além dos danos ao paciente, esta imprecisão ao tratar e diagnosticar a HS também pode trazer gastos diretos e indiretos ao país (KIRBY *et al.,* 2015). Gastos diretos são todos aqueles gastos investidos pelo governo, no caso do Brasil, através do Sistema Único de Saúde (SUS), para cobrir as tentativas frustradas de diversas consultas em postos de saúde e de diversos medicamentos prescritos que serão ineficazes para a doença (MARVEL *et al.*, 2019). Em contrapartida, os gastos indiretos representam os dias que os pacientes precisaram se ausentar da jornada de trabalho por causa da condição cutânea (ZOUBOULIS, 2019).

Deste modo, através deste trabalho, buscamos melhorar o entendimento sobre a etiologia da HS, estudando o perfil genético de pacientes com HS familiar, com possível padrão de herança autossômica dominante, oriundos da Região Metropolitana do Recife, Pernambuco. Uma vez que dados sobre a HS a nível nacional são escassos, é crucial que estudos que explorem a patogênese da doença e que utilizem a população brasileira sejam desenvolvidos.

1.1 JUSTIFICATIVA

Um bom entendimento sobre a patogênese da condição cutânea é imprescindível para o desenvolvimento de tratamentos eficientes. Mutações nos genes do complexo da gama (γ)-secretase foram previamente atribuídas à HS familiar e à HS na forma sindrômica (DUCHATELET *et al*., 2020; MARZANO *et al*., 2022; PINK *et al.,* 2012). No entanto, essas mutações ocorrem em apenas uma minoria dos

pacientes com HS e, em determinados casos, elas não são suficientes para o desenvolvimento da doença (ZOUBOULIS *et al.,* 2020). Isto sugere que uma série de alterações genéticas possam estar envolvidas na patogênese da HS, ainda que desconhecidas. Assim, pesquisas de ciência básica são necessárias para o entendimento da doença, para o desenvolvimento de uma medicina personalizada mais assertiva e, consequentemente, para o devido manejo de pacientes com HS e uso dos recursos financeiros do SUS.

2 OBJETIVOS

2.1 Objetivo Geral

Identificar genes associados com a Hidradenite Supurativa (HS) hereditária, com possível padrão de herança autossômica dominante, em famílias da Região Metropolitana do Recife, Pernambuco.

2.2 Objetivos Específicos

- Identificar e recrutar famílias com HS hereditária em Pernambuco;
- Sequenciar exoma total (WES) de pacientes membros destas famílias e identificar possíveis variantes genéticas associadas à HS hereditária;
- Confirmar a presença das variantes encontradas nos demais membros familiares através de sequenciamento Sanger;
- Avaliar as vias de sinalização impactadas pelas variantes identificadas e correlacioná-las com a patogênese da HS;
- Avaliar o impacto das mutações a níveis de RNA e de proteína;
- Avaliar a existência de mecanismo de degradação mediada por mutação sem sentido.

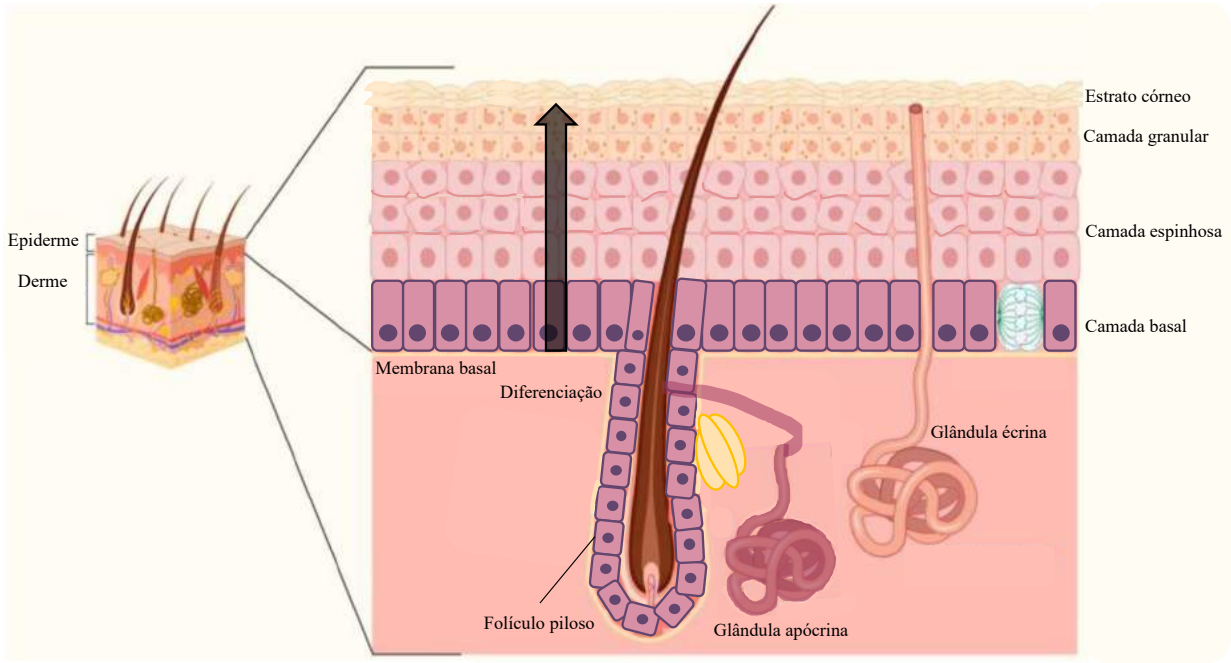
3 FUNDAMENTAÇÃO TEÓRICA

3.1 Histologia da pele

A pele, composta pela epiderme e pela derme, é o órgão mais extenso do corpo humano (GILABERTE *et al.*, 2016). A epiderme é separada da derme por uma membrana basal, a matriz extracelular (MEC), rica em colágeno tipo IV e laminina (WATT, 2014). A epiderme, a camada superior, é constituída por um epitélio escamoso estratificado composto principalmente por queratinócitos e células dendríticas (GRATTON *et al.*, 2020). Já a derme, a camada inferior, é composta por tecido conjuntivo contendo redes vasculares e nervosas, fibroblastos residentes, mastócitos e macrófagos, colágeno amorfo e apêndices epidérmicos (WATT, 2014).

A epiderme é um tecido em regeneração constante com células continuamente proliferando e sofrendo diferenciação terminal (GILABERTE *et al.*, 2016). Ela engloba um epitélio de múltiplas camadas, a epiderme interfolicular (IFE, do inglês, *Interfollicular Epidermis*), e apêndices epidérmicos associados (folículos pilosos, glândulas sebáceas e sudoríparas) (FUCHS; HORSLEY, 2008). A IFE é composta principalmente por queratinócitos em diferenciação progressiva, organizados em camadas específicas que, da mais profunda à mais superficial, são caracterizados pela camada basal, camada espinhosa, camada granular e estrato córneo (**Figura 1**) (WATT, 2014). Nas etapas finais da diferenciação, os queratinócitos são substituídos por células mortas, planas e anucleadas, os corneócitos, que formam o estrato córneo que funciona como o principal elemento da barreira cutânea (GILABERTE *et al.*, 2016).

Figura 1 – Representação esquemática da pele. A camada superior, a epiderme, é definida como um epitélio escamoso estratificado que compreende a epiderme interfolicular (IFE) e vários anexos da pele (folículos pilosos, glândulas sebáceas e glândulas sudoríparas). A camada inferior, a derme, é separada da epiderme pela presença da membrana basal, e está envolvida na proteção e sustentação da pele e das camadas mais profundas, no tato e na termorregulação.



Fonte: A autora (2022).

As células-tronco epidérmicas (ESC, do inglês, *Epidermal Stem Cells*) são células mitoticamente ativas que residem na camada basal. A cada ciclo de divisão celular, essas células se autorrenovam ou sofrem diferenciação terminal estritamente orientada e regulada por sinais do microambiente local, gerando a IFE, folículos pilosos e glândulas sebáceas (WATT, 2014). Durante a diferenciação celular, essas células migram progressivamente para o estrato córneo e adquirem características próprias da camada, como a expressão de queratinas epidérmicas especializadas como queratina (KRT, do inglês *Keratin*) 1, KRT5 e KRT10, fatores de transcrição incluindo o fator nuclear potencializador de cadeias leves kappa de células B (NF-κB) e receptor ativado por proliferadores de peroxissoma gama (PPARγ), involucrina (IVL), transglutaminase (TGM) 1, periplacina (PPL) e loricrina (LOR) (LIN *et al.,* 2011; MEHREL *et al.,* 1990; NICKOLOFF *et al.,* 2002). À medida que a estratificação ocorre, são formados apêndices epidérmicos, como folículos pilosos e glândulas sebáceas ou sudoríparas associadas (FUCHS, 1990; FUCHS, HORSLEY, 2008). Uma vez no estrato córneo, os queratinócitos cessam suas atividades metabólicas.

Diferentemente da epiderme, a derme é organizada em duas camadas: a camada mais fina e próxima da epiderme, a camada papilar, e a camada mais

profunda e espessa, a camada reticular (GILABERTE *et al.*, 2016). Uma maior densidade de fibroblastos caracteriza a derme papilar, enquanto a derme reticular é caracterizada por uma abundância de colágeno fibrilar (GRATTON *et al.*, 2020). Logo após a derme, encontra-se a camada mais profunda, a hipoderme, caracterizada por uma espessa camada de adipócitos brancos (WATT, 2014).

3.2 Cenário atual da hidradenite supurativa: epidemiologia e diagnóstico

3.2.1 Epidemiologia

Hidradenite Supurativa (HS), também conhecida como Acne Inversa e historicamente como doença de *Verneuil*, é uma doença inflamatória da pele, crônica e recorrente, que tipicamente envolve o folículo piloso encontrado em regiões do corpo que possuem glândulas apócrinas (MARTORELL *et al.,* 2015). A HS foi descrita pela primeira vez em 1839, e estudada em 1954 pelo cirurgião francês Verneuil, responsável pelo nome alternativo da enfermidade. Em 1989, com um melhor entendimento da histologia da doença, o termo Acne Inversa foi proposto como uma alternativa mais adequada (CHEN; PLEWIG, 2017). Centenas de anos após o primeiro registro de caso, a etiologia e patogênese da HS continuam pouco compreendidas (OLIVEIRA *et al.,* 2022).

Anteriormente, a HS era caracterizada como uma doença órfã por ser tratada por variadas especialidades médicas e não pertencer a nenhuma delas (JEMEC; KIMBALL, 2015; SUNG; KIMBALL, 2013). Essa fragmentação a tornou uma doença subdiagnosticada, gerando, consequentemente, um atraso de mais ou menos 6 anos no diagnóstico, tornando a determinação da verdadeira prevalência da doença desafiadora (SAUNTE *et al.,* 2015). Dependendo da área geográfica, a prevalência da doença pode variar de 0,033% a 4,10% (COSMATOS *et al.,* 2013; GARG *et al*., 2017; JEMEC; HEIDENHEIM; NIELSEN, 1996; REVUZ *et al.,* 2008). Esses valores não diferem apenas entre diferentes países, dados de prevalência variados também são observados em um mesmo país e/ou cidade (CARTRON; DRISCOLL, 2019; JEMEC; HEIDENHEIM; NIELSEN, 1996; REVUZ *et al.,* 2008; SHALOM; COHEN, 2019). Diante desta situação, estudos levantam a possibilidade da HS ser uma doença negligenciada (DUFOUR; EMTESTAM; JEMEC, 2014).

No Brasil, existem poucos estudos epidemiológicos que caracterizam os pacientes de acordo com os aspectos clínicos e demográficos (TAVORA *et al.*, 2019).

A extensão territorial do país e a dispersão de pacientes entre múltiplas especialidades médicas (ginecologia, cirurgia plástica, proctologia e dermatologia) dificultam o estudo de grandes séries de casos (ANDRADE *et al.*, 2017). Deste modo, a prevalência exata da HS na população geral e local é, atualmente, na melhor das hipóteses, uma estimativa (JEMEC; KIMBALL, 2015). Até o momento, apenas 3 estudos avaliam a prevalência da doença na população brasileira (ANDRADE *et al*., 2017; IANHEZ; SCHMITT; MIOT, 2018; SCHMITT *et al.,* 2012) . Mesmo assim, Ianhez, Schmitt e Miot, através de um estudo populacional com 17 mil habitantes, definiu a prevalência da HS no Brasil como 0,41% (IANHEZ; SCHMITT; MIOT, 2018). No entanto, esse estudo possui uma grande limitação: todos os dados foram obtidos através de autorrelato dos pacientes por telefone, sem um exame clínico confirmatório. Assim, devido a sua baixa especificidade, a real incidência da HS no Brasil permanece desconhecida.

Apesar dos dados sobre a prevalência da doença serem inconclusivos, é possível observar um perfil semelhante entre os pacientes de HS (CANOUI-POITRINE *et al.*, 2009). Artigos de diferentes países demonstram uma maior prevalência da doença entre mulheres (ANDRADE *et al.,* 2017; GARG *et al.,* 2017; KATOULIS *et al.,* 2017; MILLER; MCANDREW; HAMZAVI, 2016). Salvo, por registros de países asiáticos, onde pode-se observar uma maior prevalência em pacientes do sexo masculino (YANG *et al*., 2018). Sabat *et al.* demonstraram que a doença, frequentemente, tem seu início por volta dos 20 anos, sendo a idade média dos pacientes acometidos entre 30 e 40 anos de idade (SABAT *et al.,* 2020). Curiosamente, em pacientes do sexo feminino com mais de 50 anos, é observado uma queda na prevalência da doença; alguns autores atribuem esse fenômeno ao início da menopausa (REVUZ *et al.,* 2008; VAZQUEZ *et al*., 2013). De fato, o surgimento da HS em pacientes pós-menopausa é um acontecimento raro (FITZSIMMONS; GUILBERT, 1985). O mesmo pode ser observado em pacientes pediátricos (DECKERS *et al.*, 2015; PALMER; KEEFE, 2001). Esses achados demonstram uma possível relação entre a HS e os hormônios sexuais (ABU RACHED *et al.*, 2022). Dados que avaliam a influência desses hormônios na HS sugerem que os andrógenos possam estar envolvidos na patogênese da doença (YU *et al.,* 2021). Corroborando, foi observado um aumento da expressão de genes controlados por andrógenos em pacientes do sexo feminino (ZOUBOULIS *et al*., 2020).

Dados relacionados a etnia são, geralmente, originários das estimativas estadunidenses (GARG *et al*., 2018; GARG *et al.,* 2017). Nesses estudos foi observado que pacientes oriundos da população afro-descendente possuem até 3x mais chances de desenvolver a doença que os caucaseanos (GARG *et al.,* 2017; VAZQUEZ *et al.,* 2013). Dentre outros fatores comumente observados nos pacientes acometidos com HS encontram-se um baixo nível sociocultural e um estilo de vida pouco saudável como tabagismo e obesidade (DECKERS *et al*., 2015). De fato, o índice de fumantes ativos ou ex-fumantes entre os pacientes portadores de HS varia de 40% a 92% entre os estudos (CANOUI-POITRINE *et al.,* 2009; KATOULIS *et al.,* 2017; REVUZ *et al.,* 2008). O índice do excesso de peso/obesidade também é alto (GOLDBURG; STROBER; PAYETTE, 2020; REVUZ *et al.,* 2008). Kohorst e seu grupo, através de uma extensa revisão sistemática, demonstrou que a taxa de obesidade entre pacientes de HS varia de 12% a 88%, e demonstrou que essa variação depende do local da população estudada e sua respectiva cultura (KOHORST; KIMBALL; DAVIS, 2015).

3.2.2 Diagnóstico

O diagnóstico de HS é clínico e se baseia nas diretrizes europeias (S1) de Dessau (VANKEVICČIŪTĖ *et al*., 2019; *ZOUBOULIS et al*., 2015). Ou seja, pacientes diagnosticados com HS devem ter lesões típicas, localizadas nas áreas tradicionais, como áreas que possuem glândulas apócrinas, e serem crônicas e recorrentes. Para a lesão ser considerada recorrente, ela deve ter pelo menos duas recorrências em um período de 6 meses e uma persistência de um período menor ou igual a 3 meses (ZOUBOULIS *et al*., 2015). Ainda assim, o diagnóstico clínico pode ser inconclusivo. Nesses casos, é sugerido o uso da ultrassonografia de alta frequência (US) que ajuda de forma segura e não invasiva a detectar nódulos e tratos sinusais superficiais e profundos que não podem ser apreciados com palpação manual (ELKIN; DAVELUY; AVANAKI, 2020).

Para avaliação da gravidade do fenótipo da doença, vários métodos são utilizados. O método de estadiamento Hurley, proposto em 1989, é o mais utilizado para o estadiamento de gravidade da doença, e recomendado por proporcionar uma classificação rápida (VAN DER ZEE; JEMEC, 2015; ZOUBOULIS *et al.,* 2015). Ele categoriza os pacientes com HS em três estágios crescente de gravidade: Hurley I (leve), definido por formação de abscesso, único ou múltiplo, sem formação de trato

sinusal e de cicatrizes; Hurley II (moderada), definido por abscessos recorrentes com formação de trato sinusal, podendo ser lesões únicas ou múltiplas e amplamente separadas; e Hurley III (severa), definido por envolvimento difuso ou quase difuso, de múltiplos tratos sinusais, com cicatrizes e abscessos interconectados em toda a área (DURAN; BAUMEISTER, 2019; ELKIN; DAVELUY; AVANAKI, 2020). No entanto, o Hurley possui limitações, e a sua aplicação é realizada, muitas vezes, em paralelo a outros métodos de estadiamento. A classificação Hurley se baseia em características estáticas da doença, como cicatrizes e fístulas, inviabilizando a monitoração da eficácia terapêutica (ZOUBOULIS *et al.,* 2015). Além disso, um paciente com grau Hurley II ou III nunca poderá regredir a Hurley I sem cirurgia (VAN DER ZEE; JEMEC, 2015). E, uma vez que o fenótipo de casos moderados e severos variam muito entre pacientes, esse estadiamento se torna uma ferramenta de monitoramento pouco precisa no cenário de ensaios clínicos (SARTORIUS *et al.*, 2009).

Dentre as outras ferramentas mais utilizadas para avaliar a gravidade da HS, encontram-se o Sistema Internacional de Pontuação de Gravidade da Hidradenite Supurativa (IHS4) e o Sartorius ou *Hidradenitis Suppurativa Score* (HSS) (SARTORIUS *et al.,* 2009; ZOUBOULIS *et al.,* 2017). Ambos são um método avaliativo mais detalhado e dinâmico que o Hurley. No caso do IHS4, recomendado pela Sociedade Europeia de HS, considera-se a contagem de lesões, atribuindo pesos diferentes a nódulo, abscesso ou fístula drenante (ZOUBOULIS *et al.,* 2017). O Sartorius, por outro lado, é indicado para medir a eficácia dos tratamentos propostos pelo especialista. Seu cálculo considera o número de regiões corporais envolvidas, número das lesões em cada região, distância entre abscessos e a presença de pele sadia entre as lesões (DURAN; BAUMEISTER, 2019).

A classificação da gravidade da HS também considera alguns dados subjetivos relatados pelos pacientes, como dor e qualidade de vida (WŁODAREK *et al.*, 2020). A dor é um dos problemas mais relatados pelos pacientes com HS e está geralmente ligada aos nódulos inflamatórios (ZOUBOULIS *et al.,* 2015). Os pacientes normalmente a descrevem de várias maneiras, podendo ser classificada pelos especialistas através do uso de uma Escala Visual Analógica (EVA) ou por meio de uma Escala Numérica de Classificação (NRS, do inglês *Numeric Rating Scale*), ambos com intervalo de 0 (nenhuma dor) a 10 pontos (dor insuportável) (ONDERDIJK *et al.,* 2013; SMITH; CHAO; TEITELBAUM, 2010; WOLKENSTEIN *et al.,* 2007). Já o comprometimento da qualidade de vida do paciente é avaliado pelo Índice de

Qualidade de Vida em Dermatologia (DLQI, do inglês *Dermatology Life Quality Index*), um questionário com 10 questões que avalia o quanto a doença afeta a vida do paciente considerando sintomas e sentimentos, atividades diárias, lazer, trabalho/escola, relações pessoais e tratamento (FINLAY; KHAN, 1994; PRIGNANO *et al.*, 2019).

Atraso no diagnóstico, e, consequentemente, um tratamento tardio, são comumente relatados por pacientes com HS: o primeiro contato do paciente com o médico, geralmente, se dá após 2,3 anos da incidência da doença, com o diagnóstico correto ocorrendo, em média, 7 anos depois (CLERC *et al.,* 2019; GARG *et al.,* 2018; SAUNTE *et al.,* 2015). Chicorizzi e colaboradores, demonstraram que um paciente com HS, normalmente, passa em média por 5 médicos e mais de 17 consultas para ser diagnosticado corretamente (CHIRICOZZI; MICALI; VERALDI, 2019). Esse atraso afeta severamente os pacientes, que podem desenvolver um estágio mais avançado da doença com lesões irreversíveis (LOPES *et al.,* 2019b; ROUSTAN, 2019).

3.3 Patogênese: diferenciação epitelial, aspectos imunológicos e metabolismo

3.3.1 Diferenciação epitelial

A HS é clinicamente caracterizada por nódulos cutâneos inflamados, dolorosos e profundos, tipicamente encontrados em áreas que possuem glândulas apócrinas como axilas, regiões inguinal e anogenital para ambos os sexos, e mamária para as mulheres (VOSSEN; VAN DER ZEE; PRENS, 2018). Lesões de HS, geralmente, afetam a unidade pilossebácea e surgem após o período da puberdade, estágio associado à formação completa da glândula (NAPOLITANO *et al.*, 2017). Com a progressão da doença, esses nódulos tendem a evoluir para túneis dérmicos com secreção fétida e cicatrizes desfigurantes, afetando gravemente a qualidade de vida dos pacientes (JEMEC, 2012; MATUSIAK, 2020).

O mecanismo de formação desses nódulos está principalmente relacionado a um desequilíbrio epidérmico em indivíduos com predisposição genética (GOLDBURG; STROBER; PAYETTE, 2020; MARTORELL *et al.,* 2015). Em condições saudáveis, queratinócitos, o principal tipo celular da epiderme, expressam citocinas anti-inflamatórias, como a interleucina (IL)-1 beta (ß) e o fator de necrose tumoral alfa (TNF-α, do inglês *Tumor Necrosis Factor alpha*), que auxiliam na homeostase da pele através da regulação da proliferação e da diferenciação epitelial (OLIVEIRA *et al*.,

2022). No entanto, na pele de pacientes com HS, acredita-se que esses queratinócitos possuem um perfil inflamatório alterado, afetando a regulação celular nos compartimentos epidérmicos (JIANG *et al.,* 2020). Esse desequilíbrio acarreta num aumento da proliferação de queratinócitos e, consequentemente, numa hiperqueratose considerada responsável pela oclusão do folículo piloso (HOTZ *et al.*, 2016). Essa oclusão leva ao acúmulo intrafolicular da secreção liberada pela glândula apócrina, gerando uma hiperplasia do epitélio do folículo, seguida pelo rompimento e liberação do conteúdo folicular, incluindo queratina e bactérias, para a derme circundante (VOSSEN; VAN DER ZEE; PRENS, 2018). Como consequência do derramamento desses detritos celulares na derme, surge uma intensa resposta inflamatória (VAN DER ZEE *et al.,* 2012).

Em paralelo, modificações na expressão de genes associados a estrutura da pele adicionam um outro nível de complexidade à doença (OLIVEIRA *et al.,* 2022). Por exemplo, a expressão de metaloproteinases de matriz (MMPs, do inglês *Matrix Metalloproteinase*) foi observada alterada em pacientes com HS (WOLK; JOIN-LAMBERT; SABAT, 2020). As mesmas são induzidas por citocinas pró-inflamatórias e atuam na absorção e remodelação da matriz extracelular (LUKES *et al.,* 1999). Neste contexto, um aumento da expressão de citocinas pro-inflamatórias na pele interfere na produção das MMPs, que respondem afinando a membrana basal circundante da unidade do folículo piloso, deixando-o mais vulnerável ao rompimento (WOLK; JOIN-LAMBERT; SABAT, 2020). Assim como as MMPs, o aumento da expressão de genes da queratina, como *KRT6* e *KRT16*, também já foi observado em pele de pacientes com HS (OLIVEIRA *et al.,* 2022). Esse aumento sugere um perfil epidérmico hiperproliferativo presente em diversos fenótipos clínicos da HS, desde a oclusão do folículo à desestabilização da matriz extracelular e do envelope cornificado (ZHANG; YIN; ZHANG, 2019). Expressão anormal desses genes, em conjunto com outros genes, como os associados ao colágeno, também está associado a deformação da estrutura da pele que é característica da HS, com fibras de colágeno dispostas de forma desorganizada ou aleatória e afrouxamento das junções adesivas célula-célula na epiderme (LESSARD *et al.*, 2013). Dentre outros fenótipos clínicos de HS que também podem ser impactados por alterações gênicas, encontram-se: formação excessiva de tecido cicatricial fibrótico, perda dos anexos cutâneos ou deficiência em suas funções e perda parcial da capacidade de reparo da pele (KAMP *et al.*, 2011).

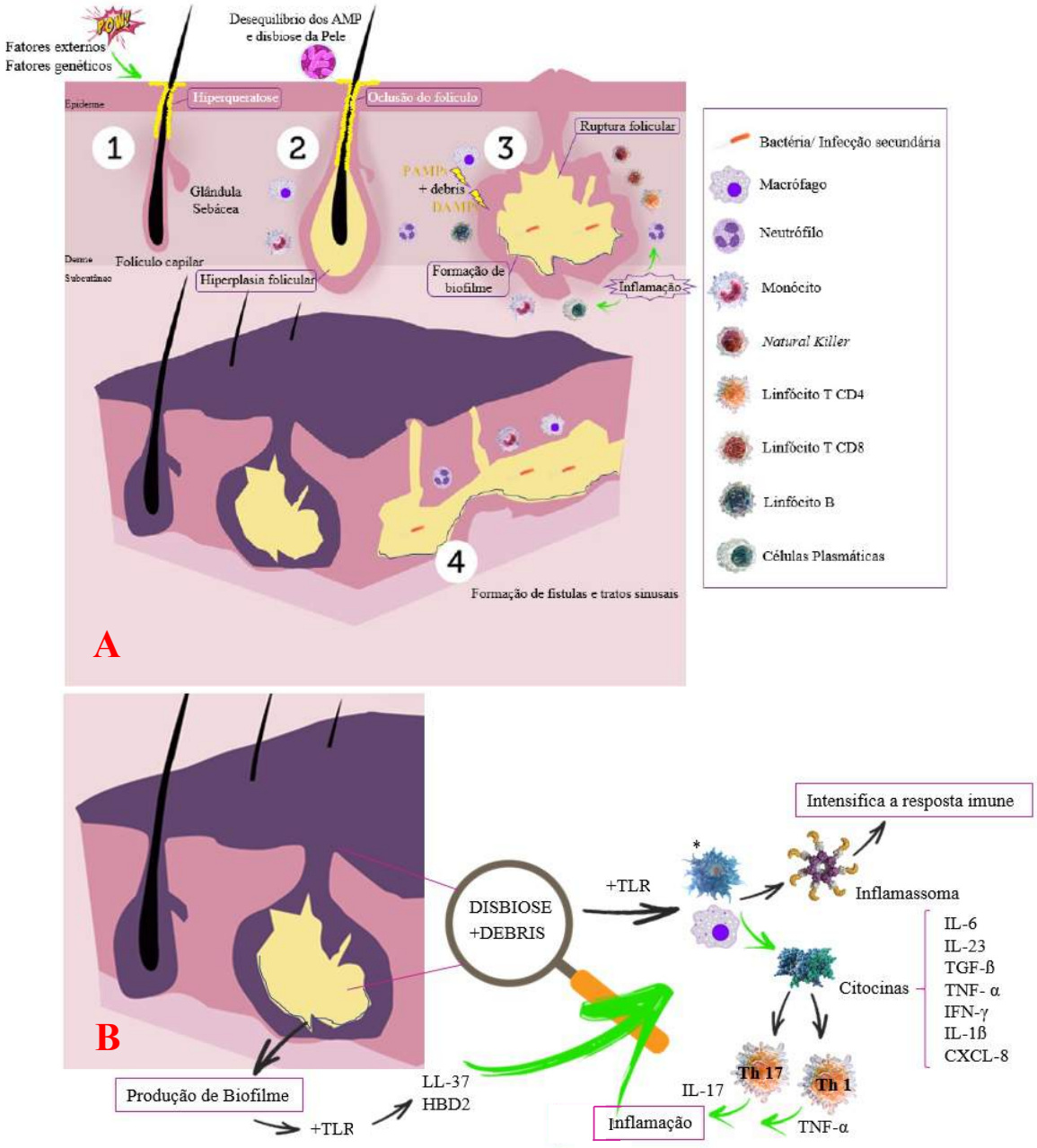
3.3.2 Aspectos Imunológicos

Outro pilar da patogênese da HS é a inflamação crônica autossustentada, desencadeada por uma combinação de fatores genéticos, anatômicos, imunológicos e ambientais (GOLDBURG; STROBER; PAYETTE, 2020). Uma resposta inflamatória inicial pode ocorrer devido a infecções oportunistas que surgem da alteração da microbiota da pele lesionada; a oclusão do folículo piloso cria um ambiente ideal para bactérias produtoras de biofilme responsáveis por essa disbiose (VOSSEN; VAN DER ZEE; PRENS, 2018). De fato, níveis aumentados de peptídeos antimicrobianos (AMPs, do inglês *Antimicrobial Peptides*), como catelicidina (LL-37) e β-defensina humana (HBD2, do inglês *Human Beta Defensin 2*) (EMELIANOV *et al.*, 2012), derivados do desequilíbrio do microbioma da pele, foram observados em pacientes com HS (OLIVEIRA *et al.,* 2022). Adicionalmente, a ruputura do folículo piloso libera debris da unidade pilo-sebácea na derme, gerando uma inflamação (VOSSEN; VAN DER ZEE; PRENS, 2018). Tanto os detritos derramados na derme, quanto a disbiose cutânea, atuam como padrões moleculares associados à patógenos e danos (PAMP/DAMPs, do inglês *Pathogen/Damage-Associated Molecular Pattern*) que ativam os receptores de reconhecimento de padrões (RRP), principalmente os receptores do tipo *toll* (TLR, do inglês *Toll Like Receptor*) e o inflamassoma da família de receptores intracitoplasmáticos do tipo *NOD*, mais especificamente a proteína NLR contendo o domínio de pirina 3 (NLRP3) (HUNGER et *al.,* 2017). Os inflamassomas NLRP3 ativos induzem a expressão de vários mediadores inflamatórios, como IL-1ß e TNF-α, encontrados super expressos nas lesões de pacientes com HS (OLIVEIRA, *et al.,* 2022; WITTE-HÄNDEL *et al.,* 2019). A **Figura 2A** resume o mecanismo inflamatório associado a formação de lesões na pele afetada por HS.

Na pele, o TNF-α induz uma ampla gama de quimiocinas que favorecem a infiltração de células imunes na lesão (INGRAM, 2016). Similarmente, a IL-1ß induz a produção de MMPs, por exemplo MMP1, MMP3 e MMP10, e várias quimiocinas, sendo as que atraem granulócitos neutrofílicos, por exemplo ligantes C-X-C de quimiocina (CXCL, do inglês *Chemokine (C-X-C motif) Ligand*) como CXCL1, CXCL6 e CXCL8, as mais proeminentes (WOLK; JOIN-LAMBERT; SABAT, 2020). Em queratinócitos, os efeitos da IL-1ß são frequentemente amplificados por TNF-α e IL-17, que por estarem também supra regulados nas lesões, mantém constante o ciclo inflamatório (WOLK; JOIN-LAMBERT; SABAT, 2020).

Em paralelo, mediadores típicos de células T, como as citocinas de células T auxiliar (Th, do inglês *T helper*) 17, IL-17A e IL-17F, e a citocina de células Th1, interefon-γ (IFN-γ), foram encontradas super expressas em lesões de HS (OLIVEIRA *et al*., 2022; SCHLAPBACH *et al.,* 2011). Nas lesões de HS, o IFN-γ pode funcionar como o ativador primário dos macrófagos, uma das células imunes mais numerosas nos infiltrados inflamatórios da doença (SHAH; ALHUSAYEN; AMINI-NIK, 2017). Já a IL-17, atua na indução da expressão de proteínas antimicrobianas e quimiocinas selecionadas, como o ligante CC de quimiocina (CCL, do inglês *Chemokine (C-C motif) Ligand*) 20 (CCL20) que atrai para as lesões subpopulações de células Th, células dendríticas, e neutrófilos (HOMEY *et al*., 2000; KAO *et al.,* 2004; WITTE *et al.,* 2014). Na HS, as produções de IL-17 e IFN-γ são mantidas por IL-23, IL-1β, IL-6 e por IL-12 respectivamente, também abundantes na pele de HS (**Figura 2B**) (WOLK et *al*., 2011). De modo geral, essas citocinas atuam na hiperplasia folicular, quimiotaxia de neutrófilos, produção de proteínas antimicrobianas, cicatrização e inflamação (MAAROUF *et al.*, 2018).

Figura 2 – A: Hipótese do mecanismo de formação de lesões da HS. 1- Oclusão folicular que pode ser causada por fatores endógenos em indivíduos portadores de uma predisposição genética, que aumenta o risco de queratinização infundibular e formação de cistos. Fatores exógenos também contribuem para essa oclusão. 2- Dilatação folicular e disbiose do microbioma da pele. 3- Ruptura do folículo dilatado com dispersão do conteúdo folicular na derme, incluindo fibras de queratina e flora comensal, atuando como padrões moleculares associados a patógenos e danos (PAMPs / DAMPs) e desencadeiando uma resposta imune aguda e grave. 4- Inflamação crônica com formação de trato sinusal e fístulas que conectam outros folículos rompidos. B: PAMPs / DAMPs são reconhecidos pelos TLRs de macrófagos e células dendríticas. Este reconhecimento acarreta a ativação de uma cascata imunológica. Células imunes inatas, através da superexpressão de citocinas, ativam o Th1 e Th17, que irão liberar IL-17 e TNF-α, respectivamente, recrutando outras células imunes para o local lesionado. Paralelamente, o inflamassoma intensifica a resposta imune através da ativação de IL-1β pela caspase-1, atraindo mais células imunes para a lesão. Por fim, a produção de biofilme bacteriano intensifica a expressão de LL37 e HBD2, atuando como um *feedback* positivo para a inflamação e a obstrução folicular.



Fonte: A autora (2022).

Esses fatores inflamatórios tendem a formar um ciclo vicioso inflamatório, retroalimentando o recrutamento constante de atores imunológicos. Do ponto de vista clínico, as lesões de HS estabelecidas contêm infiltrados maciços de células imunes compostos numa maior proporção por neutrófilos, macrófagos e as células dendríticas e numa menor proporção por células T, mastócitos, células *natural killer* e células plasmáticas. Comumente, nas lesões iniciais, predominam a formação de abscesso neutrofílico e o fluxo de células imunes inatas, principalmente macrófagos, monócitos e células dendríticas (VOSSEN; VAN DER ZEE; PRENS, 2018). Já nas lesões crônicas, o infiltrado se expande com o aumento da frequência de células imunes adaptativas (VAN DER ZEE *et al.,* 2012).

3.3.3 Metabolismo

Fatores externos/ambientais que afetam o metabolismo também são considerados gatilhos para o desenvolvimento da inflamação e da doença (BOER, 2017). Obesidade e tabagismo, por exemplo, são considerados como fatores predisponentes e desencadeantes da HS em indivíduos geneticamente suscetíveis (MARTORELL *et al.,* 2015). Além disso, pacientes com sobrepeso ou obesos possuem uma maior probablidade de desenvolver fenótipos mais severos da doença e uma pior resposta ao tratamento (REICHERT; FERNANDEZ FAITH; HARFMANN, 2020).

A relação molecular entre o sobrepeso e a HS é hipotetizada de diferentes maneiras. Uma das hipóteses é de que o excesso do tecido adiposo cria um ambiente pró-inflamatório que expressa diversas citocinas capazes de iniciar uma resposta inflamatória (CHOI *et al.*, 2019; POPKO *et al.*, 2010). Células imunes inflamatórias, incluindo macrófagos do tipo M1 e células T, infiltram o tecido adiposo danificado, produzindo citocinas inflamatórias e induzindo um padrão desregulado de mediadores solúveis chamados adipocinas (CURAT *et al.*, 2006). As adipocinas são expressas regularmente por várias células da pele, como queratinócitos, melanócitos, sebócitos e fibroblastos, e sua expressão anormal tem sido associada a várias condições inflamatórias da pele (KOVÁCS *et al.*, 2020). Dentre as adipocinas conhecidas, estão o TNF-α e a IL-1ß, encontrados superregulados em lesões de HS (WOLK; SABAT, 2016). Além da sua função na infiltração tecidual por células imunes, as adipocinas também promovem resistência à insulina, distúrbios do metabolismo da glicose e

lipídios e disfunção vascular (ANDERSSON *et al.,* 2008; GUSTAFSON *et al.,* 2007; GUSTAFSON; SMITH, 2006; YAMAUCHI *et al*., 2001).

Em pacientes obesos com HS, a inflamação pode ser acompanhada de uma desnutrição. As células adiposas expandidas causam estresse oxidativo e alteram as vias de transportes de nutrientes por meio de um esgotamento de nutrientes antioxidantes (PATEL *et al.*, 2017). A deficiência de vitamina D, folato, vitamina A, vitamina B12, vitamina C, vitamina K, vitamina B6, ferro e selênio foram descritas em pacientes obesos com HS (GARCOVICH *et al.,* 2019). Acredita-se que o baixo nível desses nutrientes pode influenciar na desregulação de respostas imunes inatas, na hiperqueratose folicular e na desregulação da homeostase da pele (CHOI *et al.,* 2020; KELLY *et al.,* 2014).

A segunda hipótese sugere que uma alimentação com alto teor glicêmico e rica em proteínas lácteas aumenta a concentração de insulina, de fatores de crescimento semelhante à insulina tipo 1 (IGF-1, do inglês *Insulin-like Growth Factor 1*), e dos aminoácidos de cadeia ramificada de leucina, de isoleucina e de valina no paciente (CHOI *et al.,* 2020). Essas moléculas ativam o complexo 1 do alvo da rapamicina em mamíferos (mTORC1, do inglês *mechanistic Target of Rapamycin Complex 1)* e, consequentemente, acarretam o acúmulo de triacilglicerídeos nas glândulas sebáceas (MELNIK, 2012). Monfrecola e seu grupo observaram uma superexpressão do mTORC1 em lesões de HS em paciente sobrepeso (MONFRECOLA *et al.,* 2016). O mTORC1, quando superativado, aumenta a secreção hormonal de andrógenos e influência na proliferação celular dos folículos sebáceos, causando a oclusão inicial do epitélio folicular (LIM; OON, 2016).

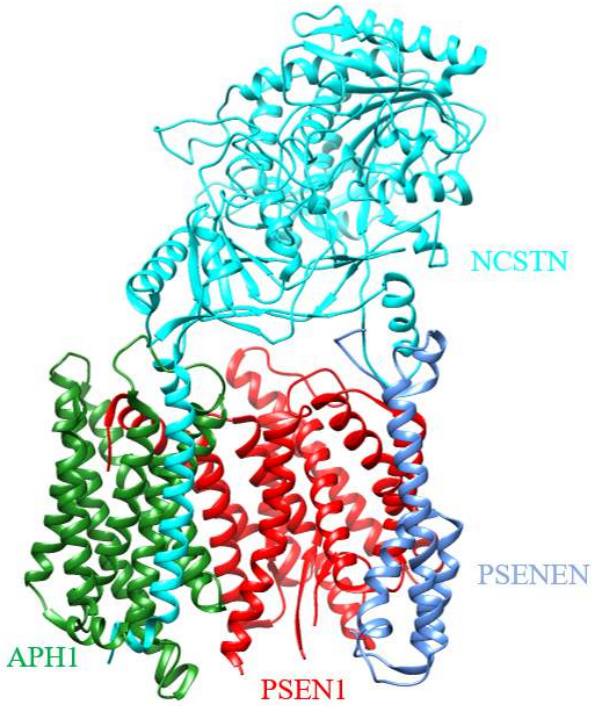
Assim como a obesidade, o tabagismo afeta negativamente pacientes com HS. Em homeostase, célula T reguladoras (Tregs) regulam a tolerância imunológica, suprimindo os linfócitos auto-reativos e os linfócitos reativos a antígenos (CHOI *et al*., 2020). No entanto, pacientes fumantes com HS, tendem a agravar o desequilíbrio da proporção de células Th17 e de células Tregs através do uso da nicotina (MELNIK, B. C. *et al.*, 2018). A fumaça do cigarro, que induz a expressão de IL-8, também aumenta os níveis de expressão de TNFα por queratinócitos, podendo, deste modo, auxiliar no desenvolvimento de fenótipos mais graves da doença (JEONG *et al.*, 2009; MORTAZ *et al.*, 2010). Ademais, a nicotina presente no tabaco superestimula as glândulas sudoríparas a secretar suor com metabólitos nocivos derivados do cigarro, contribuindo para o entupimento dos ductos glandulares (WILTZ *et al.*, 1990).

3.4 Genética da Hidradenite Supurativa

Os pacientes com HS frequentemente relatam um histórico familiar, sugerindo um componente genético para esta doença (JFRI *et al*., 2019). Cerca de 30-40% dos pacientes mencionam a existência de pelo menos um outro membro da família com HS (GOLDBURG; STROBER; PAYETTE, 2020). No entanto, a identificação de um locus cromossomal, 1p21.1- 1q25.3, comum entre quatro gerações de uma família, ocorreu apenas em 2006 (GAO *et al.*, 2006). Com a identificação de mutações patogênicas no complexo γ-secretase e alterações da via Notch relacionados com a HS ocorrendo, sucessivamente, em 2010 (WU *et al.,* 2018).

A γ-secretase é um complexo enzimático transmembranar de alto peso molecular, amplamente distribuída em células e tecidos e envolvida em processos de clivagem intramembranar e hidrólise de várias proteínas (LI *et al.,* 2020). Ela é composta por quatro subunidades: Presenilina (PSEN), subunidade catalítica, e três cofatores, Potenciador de Presenilina 2 (PSENEN ou PEN-2), Nicastrina (NCSTN) e faringe anterior defectiva 1 (APH-1, do inglês *Anterior Pharynx-defective 1*), que possui dois homólogos (APH1A e APH1B) (**Figura 3**) (FREW; NAVRAZHINA, 2019). A PSEN é a subunidade ativa do complexo, e as subunidades cofatoras atuam auxiliando a promoção da maturação e estabilização da γ-secretase (LI *et al*., 2020). A figura 3 revela a estrutura do complexo da γ-secretase (YANG *et al.,* 2019), chamando atenção para o ectodomínio extracelular da subunidade da nicastrina, ilustrada na cor azul claro. Sabe-se que o domínio da nicastrina possui homologia com aminopeptidases e pode, deste modo, ligar-se a substratos (SHAH *et al*., 2005). Assim, foi especulado que os substratos se aproximam do sítio ativo da γ-secretase através do lado côncavo do complexo proteico (WOLFE; MIAO, 2022). No entanto, recentemente, foi observado que este sítio ativo é acessado pelo lado convexo da estrutura, sendo a nicastrina (BAI *et al.*, 2015), também, responsável pelo bloqueio de potenciais substratos transmembranares com ectodomínios longos (BOLDUC *et al.,* 2016).

Figura 3 – Estrutura em Crio-microscopia eletrônica (Cryo-EM) da γ-secretase humana com 2.7 Å. As estrutras cofatoras estão destacadas em cores frias: a subunidade na cor azul água corresponde a NCSTN, a verde APH1 e a azul roxa PSENEN. A subunidade catalítica está colorida em cor quente, a subunidade PSEN1 está destacada em vermelho.



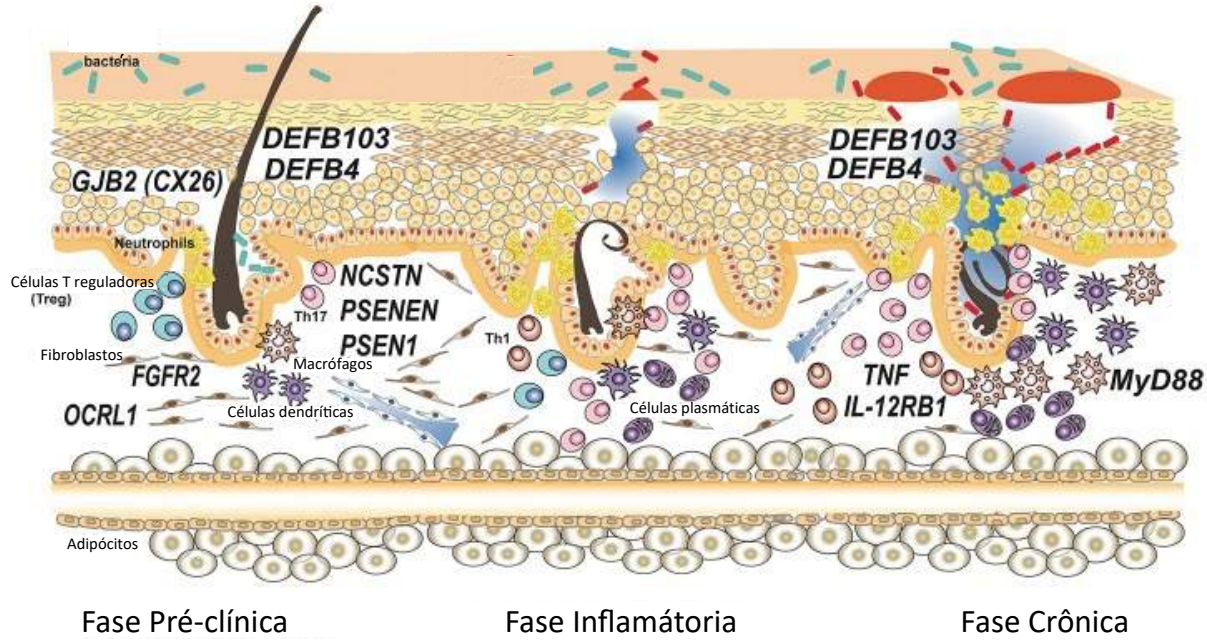
Fonte: A autora (2022). A estrutura foi adquirida no PDB (Protein Data Bank (PDB) ID: 6idf.cif) (YANG, G. *et al.*, 2019) e visualizada no programa Chimera 1.14 (PETTERSEN *et al.*, 2004).

Alguns casos familiares já foram associados a mutações com perda de função em genes que codificam proteínas do complexo γ-secretase (CONSTANTINOU; FRAGOULIS; NIKIPHOROU, 2019). Em famílias com mutações nesse complexo, a HS segue um padrão de herança autossômica dominante com penetrância incompleta, e os membros afetados tendem a ter um fenótipo mais grave (GOLDBURG; STROBER; PAYETTE, 2020). Isso acontece porque a γ-secretase desempenha um papel significativo na transdução do sinal da via Notch (GRATTON *et al.*, 2020), mediando a sua clivagem intramembranar e a subsequente liberação do seu domínio intracelular (NICD) (CONSTANTINOU; FRAGOULIS; NIKIPHOROU, 2019). A via Notch impacta em fenótipos da HS, pois a sua sinalização está envolvida no desenvolvimento, maturação, diferenciação e proliferação de células imunes e queratinóctios, cruciais para a homeostase da pele e seus apêndices (LI *et al.,* 2020; MASSI; PANELOS, 2012). Mesmo assim, essas mutações estão confinadas a uma minoria de indivíduos que desenvolvem a HS e, até o momento, não são suficientes para explicar todos os fenótipos da doença (PINK *et al*., 2012). Deste modo, acredita-

se na existência de genes adicionais e desconhecidos, que não a γ-secretase e o Notch, participantes tanto na HS familiar, quanto na HS esporádica (NOMURA *et al.,* 2014).

Atualmente, outros genes candidatos a influenciar o surgimento da HS estão sendo estudados (**Figura 4)** (TRICARICO *et al.,* 2019). Dentre eles estão: Proteína β-2 da *Gap Junction* (*GJB2*, do *Inglês Gap Junction Beta-2 protein*), responsável pelo contato e comunicação célula-célula e homeostase tecidual, e que codifica a conexina 26 (Cx26), uma das principais conexinas da pele humana e presente em folículos capilares (LUCKE *et al.,* 1999; MONTGOMERY *et al.,* 2004); Fator de crescimento do receptor de fibroblastos 2 (*FGFR2*, do inglês *Fibroblast Growth Factor Receptor 2*), normalmente expresso em queratinócitos, folículos capilares e glândulas sebáceas, e que desempenha um papel essencial na proliferação, diferenciação, migração e apoptose celular (HIGGINS *et al.,* 2017; MELNIK, 2009); Inositol polifosfato-5-fosfatase 1 (*OCRL1*) que forma complexos com componentes de junção, atua na maturação das células epiteliais polarizantes e se liga à clatrina, controlando o transporte membranar e o citoesqueleto de actina, e que quando mutado pode aumentar a susceptibilidade a infecções cutâneas devido ao acúmulo de fosfatidilinositol 4,5-bifosfato (PI (4,5) P2, do inglês *Phosphatidylinositol 4,5-bisphosphate*) na membrana plamática; Fator de Necrose Tumoral (TNF), atuante na regulação da proliferação, diferenciação, apoptose, metabolismo lipídico e (MARZUILLO *et al.*, 2018)coagulação, e relacionado à suscetibilidade à HS e ao curso natural da doença (SAVVA *et al.,* 2013; TRICARICO *et al.,* 2019); Subunidade β-1 do receptor da interleucina-12 (*IL-12Rβ1*), codificante de subunidades dos receptors da IL-21 e IL-23 e importante na resposta immune, podendo agravar o fenótipo clínico da doença (GIATRAKOS *et al.*, 2013); genes da β- defensina (*DEFB*, do inglês *Defensin Beta*) DEFB103 e DEFB4, que codificam os peptídeos antimicrobianos HBD2 e HBD3, respectivamente, e que são importantes na defesa inata epitelial, e que podem ser importante fatores de risco para suscetibilidade a HS (GIAMARELLOS-BOURBOULIS *et al.,* 2016); e Gene 88 de diferenciação primária de resposta (*MYD88*, do inglês *Myeloid Differentiation primary response 88*), codificante de uma proteína adaptadora citosólica que está envolvida na via de sinalização do receptor do tipo *toll* e do receptor IL-1 na resposta imune inata, sendo um fator de risco para o desenvolvimento de HS severa (Hurley III) em pacientes com um genótipo GG na posição rs6853 (AGUT-BUSQUET *et al*., 2018; KFOURY *et al.,* 2014).

Figura 4 – Genes associados à suscetibilidade e progressão da hidradenite supurativa. A suscetibilidade à doença é causada por mutações em genes envolvidos na homeostase dos queratinócitos. A progressão da doença está, muitas vezes, associada a genes que codificam proteínas envolvidas na resposta imune.



Fonte: TRICARICO et al., 2019.

3.5 Mecanismo de degradação de mRNA mediada por mutação sem sentido

Mutação sem sentido é um tipo de mutação pontual que resulta na conversão de um códon em um códon de término, também referido como códon de terminação prematura (PTC, do inglês *Premature Termination Codon*) (KARIJOLICH; YU, 2014). O efeito da mutação sem sentido é, geralmente, deletério, afetando a expressão dos genes afetados e ocasionando no decaimento dos níveis de ácido ribonucleico mensageiro (mARN ou, do inglês, mRNA) e no término prematuro da tradução (MORAIS; ADACHI; YU, 2020). Sucintamente, quando um ribossomo encontra um PTC que está pelo menos 50 nucleotídeos de distância de uma junção éxon-éxon, uma via de vigilância de degradação do RNA mediado por mutação sem sentido (NMD, do inglês *Nonsense-mediated mRNA decay*) é ativada, degradando o mRNA (KUROSAKI; POPP; MAQUAT, 2019). De modo geral, o NMD funciona em pelo menos dois processos celulares distintos: (1) regulação negativa de transcritos anormais que são gerados como consequência de erros na expressão gênica e (2) regulação do nível de transcritos em resposta às necessidades celulares (KUROSAKI; MAQUAT, 2016).

Acredita-se que mutações sem sentido são responsáveis por cerca de 11% dos danos genéticos associados a doenças humanas hereditárias (MORT *et al.*, 2008). Acredita-se também que ao inibir o PTC ou NMD, pode ocorrer a continuação do alongamento do processo de tradução, gerando uma proteína no seu comprimento normal (MORAIS et al., 2017). Se os aminoácidos substituídos não desempenharem uma função essencial num sítio ativo crítico da proteína, talvez a proteína resultante possa ter sua atividade parcial ou normal restaurada (LUECK *et al.*, 2019). Deste modo, as estratégias personalizadas e terapêuticas destinadas a suprimir o mecanismo do NMD podem ter o potencial de fornecer um benefício terapêutico para pacientes em uma ampla gama de doenças genéticas (MORAIS; ADACHI; YU, 2020), dentre elas a HS hereditária.

3.6 Padrões genéticos de comorbidades em pacientes com HS

Em um subconjunto de pacientes, a HS se desenvolve como parte de uma constelação de outras condições inflamatórias, HS sindrômica. As síndromes clássicas da HS incluem as síndromes PASH (pioderma gangrenoso, acne e hidradenite supurativa), PAPASH (artrite piogênica + PASH) e SAPHO (sinovite, acne, pustulose, hiperostose e osteíte) (GARCOVICH *et al.,* 2021). Alguns genes associados a essas síndromes foram descritos: genes do complexo da γ-secretase (PACE; MINTOFF; BORG, 2022), a proteína 1 que interage com a prolina-serina-treonina-fosfatase (*PSTPIP1,* do inglês *Proline-Serine-Threonine Phosphatase Interacting Protein 1),* envolvido na regulação da resposta imune inata (*MARZANO et al.*, 2013), *NLRP3* (PACE; MINTOFF; BORG, 2022), *NLRP12* (MARZANO *et al.,* 2017), antagonista do receptor de IL-1 (*IL1RN*) (MARZANO, *et al.,* 2014), *NOD2* , gene da febre mediterrânea (*MEFV,* do inglês *Mediterranean Fever*), envolvido na codifição da pirina, proteína essencial para a função do inflamassoma (İLGEN *et al.*, 2019), proteína 1 contendo repetição WD (*WDR1,* do inglês *WD Repeat Domain 1)*, envolvido na promoção da renovação do filamento de actina dependente de cofilina (MARZANO, *et al.,* 2022), *OTULIN,* envolvido na regulação da inflamação (MARZANO *et al.,* 2022), *GJB2* (MARZANO *et al.,* 2022), lipina 2 (*LPIN2*), envolvido no desenvolvimento e manutenção do tecido adiposo (MARZANO *et al.,* 2017), e a proteína NLR contendo o domínio CARD 4 (NLRC4), componente chave do inflamassoma (MARZANO *et al.,* 2022).

A HS pode ocorrer também em combinação com outras síndromes hereditárias, por exemplo com a doença de Dowling-Degos (DDD) (NOKDHES *et al.*, 2021). DDD é um raro distúrbio pigmentar reticulado progressivo, geralmente, herdado em um padrão autossômico dominante, apesar da existência de casos esporádicos (STEPHAN; KURBAN; ABBAS, 2021). O principal gene associado à DDD é o gene *KRT5*, um elemento do citoesqueleto dos queratinócitos basais (NOKDHES *et al.*, 2021). No entanto, outros genes já foram documentados: proteína O-fucosiltransferase 1 (POFUT1) (LI *et al.,* 2013), proteína O-glicosiltransferase 1 (POGLUT1) (BASMANAV *et al.*, 2014) e PSENEN (ZHOU *et al.,* 2016), evidenciando o papel da via Notch nessas doenças. Assim como PSENEN, componente da γ-secretase, ambos POGLUT1 e POFUT1 são reguladores da atividade da via Notch através de seus respectivos produtos proteicos (BASMANAV *et al.,* 2014; LI *et al*., 2013). Dentre esses genes, mutações patogênicas em *PSENEN* (PETER *et al.*, 2021), *NCSTN* (GARCOVICH *et al*., 2020) e *POFUT1* (GARCÍA-GIL *et al.*, 2021) já foram identificadas em pacientes com HS associados à DDD.

Além de DDD, vários estudos descreveram a coexistência de um fenótipo de HS em pacientes com variação patogênica no locus *MEFV* associado à febre mediterrânea familiar (FMF) (ABBARA *et al.,* 2017; JFRI *et al.,* 2020; VURAL *et al*., 2017). Este gene codifica a pirina, uma proteína que modula a atividade do complexo da γ-secretase (PACE; MINTOFF; BORG, 2022). Deste modo, acredita-se que HS e FMF são distúrbios autoinflamatórios que podem compartilhar processos fisiopatológicos convergentes.

3.7 Medicina Personalizada: uma nova era

Como discutido neste trabalho, a HS é uma doença de alta complexidade. Como consequência, até o momento, não existe uma cura; o tratamento padrão não leva em consideração a patogênese individual e a variedade fenotípica da doença (INGRAHAM *et al.*, 2022). Deste modo, o objetivo atual do tratamento é prevenir a formação de novas lesões, impedir a agravação das lesões existentes e a formação de cicatrizes e controlar os sintomas (NESBITT; CLEMENTS; DRISCOLL, 2020). Os médicos, normalmente, devem escolher entre as diferentes modalidades de tratamento existentes e, geralmente, precisam combiná-las para obter o controle da doença (SEYED JAFARI; HUNGER; SCHLAPBACH, 2020). Dentre as diferentes

modalidades de tratamento estão o uso de antibióticos e corticóides em terapias tópicas e sistêmicas, as ciruurgias e as fototeraapias (LYONS *et al.*, 2020). Modificações estratégicas no estilo de vida do paciente também podem atuar como terapias adjuvantes e podem mitigar as exacerbações da HS (PRIGNANO *et al.*, 2019).

Diante da necessidade não atendida de um tratamento bem tolerado e eficaz para a HS, a aplicação de uma medicina personalizada para fenótipos específicos de HS tem o potencial de abrir uma nova era promissora de tratamento. Nesse contexto, os biomarcadores da doença ajudariam a definir os fenótipos de um indivíduo a fim de identificar potenciais alvos para novas estratégias terapêuticas (CABANILLAS; BREHLER; NOVAK, 2017). Espera-se que o aumento do conhecimento gerado nos últimos anos sobre as bases moleculares da condição cutânea (OLIVEIRA *et al.,* 2022) contribua para a definição de biomarcadores específicos ou grupo de biomarcadores que categorizem os fenótipos clínicos heterogêneos.

Na tentativa de estudar potenciais alvos terapêuticos para pacientes com HS, algumas técnicas podem ser utilizadas (OLIVEIRA *et al.,* 2022; THEUT RIIS *et al.,* 2021; TRICARICO *et al.,* 2022). Recentemente, células da bainha radicular externa (ORS, do inglês *Outer Root Sheath*) do folículo piloso foram sugeridas como grande atuantes no desenvolvimento da HS (TRICARICO *et al.,* 2022). Protocolos para técnicas não invasivas de coleta de fios de cabelo de pacientes e posterior extração e cultivo de células ORS já foram estabelecidos (LIU *et al.,* 2018; RHEINWALD; GREEN, 1977). Assim, o uso dessas células em cultivo celular de duas e três dimensões (2D e 3D) em paralelo a técnicas de sequenciamento e silenciamento gênico, podem auxiliar na escolha da melhor terapia para determinados indivíduos. Corroborando, Tricarico et al (2022) identificaram variantes genéticas que prejudicam o metabolismo da vitamina D (calciferol) em pacientes com HS, e ao começar uma suplementação da vitamina nos mesmos, foi observado uma melhora clínica da condição da pele, e consequentemente da qualidade de vida (BRANDAO *et al*., 2020; TRICARICO *et al*., 2022).

3.8 Hipótese

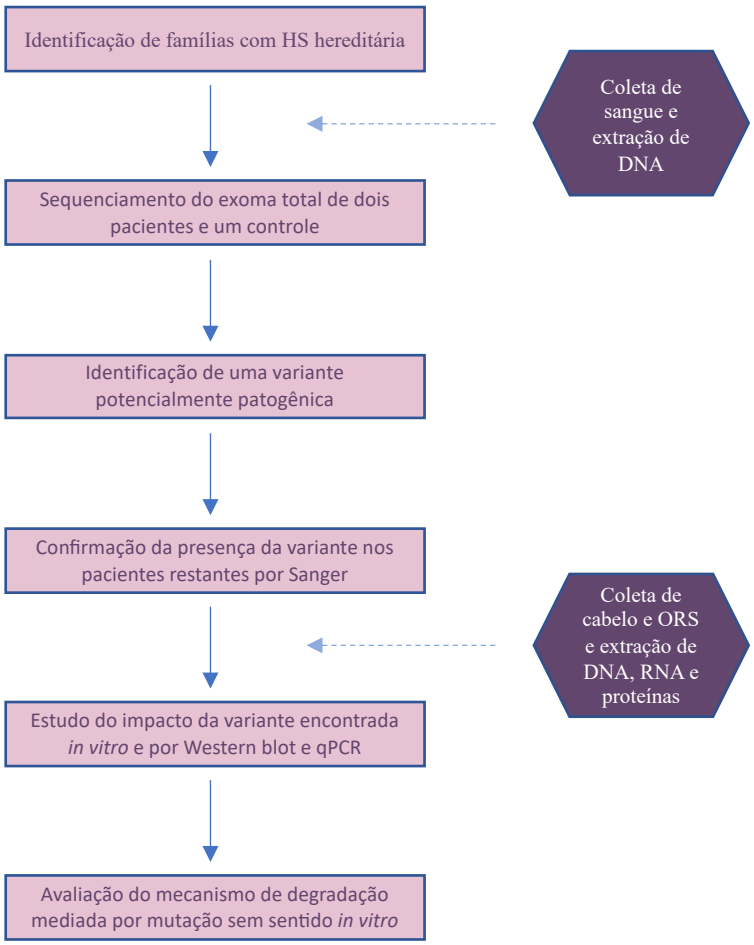
Diante da complexidade fenotípica associada a condição cutânea, a hipótese aqui levantada é a existência de um perfil genético variado associado a HS familiar que possa estar relacionado ao desenvolvimento e progressão da doença.

4 METODOLOGIA

4.1 Desenho do estudo

O estudo trata-se de uma pesquisa observacional e exploratória de casos de pacientes com HS familiar. As etapas metodológicas do estudo estão descritas no esquema a seguir (**Figura 5**).

Figura 5 – Esquema representando as etapas metodológicas utilizadas nesse trabalho.



Fonte: A autora (2022).

4.2 Descrição do local de estudo

A coleta das amostras de sangue e cabelo foi realizada por profissionais da saúde no ambulatório de dermatologia do Hospital das Clínicas da Universidade Federal de Pernambuco (HC-UFPE). O processamento e a análise das amostras sanguíneas ocorreram nas instalações do Instituto Keizo Asami e do Laboratório de

Patologia Molecular (LPM), no Departamento de Patologia, ambos na Universidade Federal de Pernambuco – UFPE.

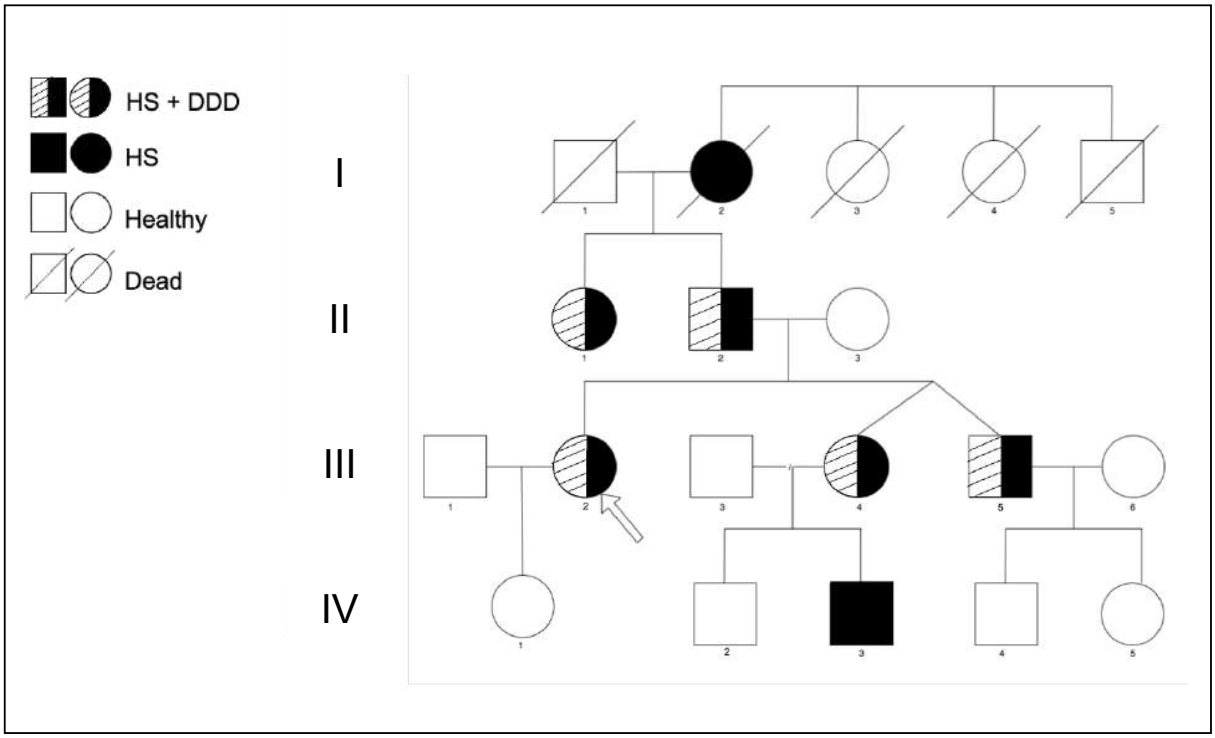
O processamento e a análise das amostras capilares foram realizados na França, através do programa de doutorado sanduíche no exterior (PDSE) financiado pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), nos laboratórios da equipe 15 do Instituto Mondor de Pesquisa Biomédica (IMRB, U955 Inserm – Université Paris Est Créteil, UPEC).

4.3 Grupo Amostral

Após a anuência deste projeto de pesquisa pelo Comitê de Ética em Pesquisa (CEP) e pelo Núcleo de Apoio à Pesquisa (NAP) (HC-UFPE/Ebserh (CAAE: 64777922.0.0000.5208 / Número do Parecer: 5.807.358), os pacientes foram recrutados. Como critérios de inclusão para participação da pesquisa, utilizamos a confirmação da HS por avaliação clínica, independentemente do fenótipo, a possível presença de um padrão de herança autossômica dominante e a aceitação dos pacientes em participar do estudo. Como critério de exclusão, foram aceitos os quesitos de não aceitação pelos indivíduos a ser voluntário no estudo, a ausência da HS e a ausência do padrão de herança autossômica dominante.

Entre o período de abril de 2018 à julho de 2020, 111 pacientes da Região Metropolitana do Recife, diagnosticados com HS, foram recrutados. No entanto, entre os pacientes recrutados, foi identificado apenas um caso de HS familiar com possível padrão de herança autossômica dominante (**Figura 6**). A família em questão foi convidada a participar do projeto de pesquisa e a assinar o termo de consentimento livre e esclarecido (TCLE), previamente estabelecido e aprovado pelo CEP da UFPE e pelo NAP do HC-UFPE. A gravidade da HS, bem como o impacto da doença no cotidiano dos pacientes, foi avaliada pelo estadiamento clínico de Hurley e IHS4 (**Tabela 1**), pelo DLQI e pelo EVA.

Figura 6 – Árvore genealógica da família de 4 gerações afetada por HS hereditária. A família também foi diagnosticada com DDD. Os pacientes femininos estão identificados por um círculo enquanto os pacientes masculinos por um quadrado. A seta refere-se ao probando. Os pacientes com HS e DDD simultaneamente estão representados pela cor preta e por listrado, enquanto os pacientes com apenas HS estão representados pela cor preta. Os pacientes com pontos de interrogação carregam a variante encontrada na NCSTN, mas ainda não desenvolveram a doença ou o diagnóstico é inconclusivo.



Fonte: A autora (2022).

Tabela 1 – Tabela utilizada para a classificação da gravidade dos pacientes. Através da classificação de Hurley, pacientes são agrupados de acordo com os seus respectivos fenótipos clínicos. Hurley I é considerado HS leve, II, moderada e III, grave. De acordo com o IHS4, os pacientes são agrupados de acordo com a pontuação gerada apartir da contagem do número de nódulos, abscessos e fístulas. No cálculo, o número de nódulos é multiplicado por 1, o de abscessos, por 2 e o de fístulas, por 4. A pontuação final indicará qual a gravidade clínica do paciente; pontuação menor ou igual a 3 é considerado HS leve, pontuação entre 4 e 10, HS moderada, e pontuação igual ou maior que 11, severa.

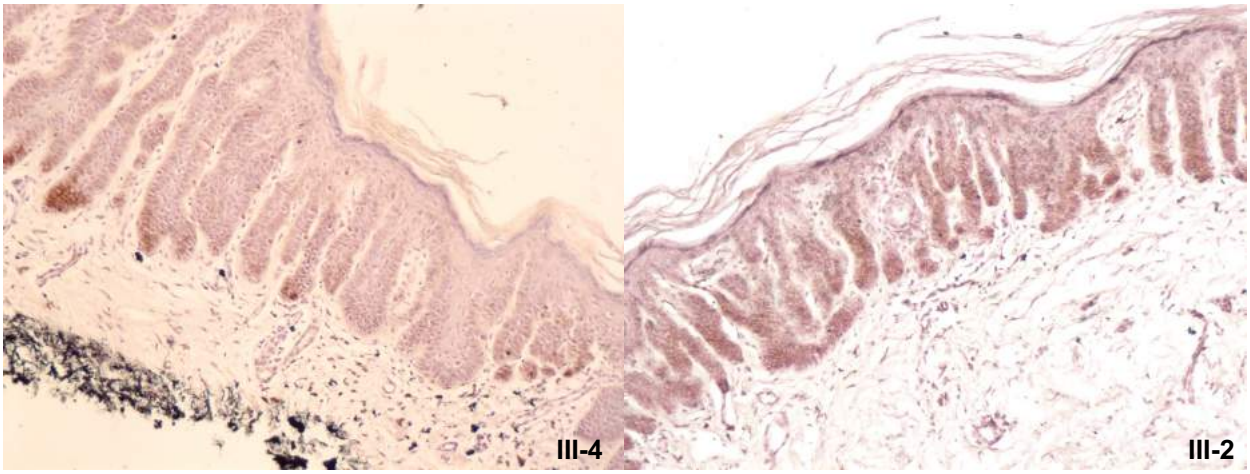
Classificação utilizada para avaliar a severidade da doença	
Estágio Hurley	
I	Abscesso único ou múltiplos, sem túneis ou cicatrizes
II	Abscesso recorrente único ou abscessos múltiplos, separados, com formação de túneis e cicatrizes
III	Múltiplos túneis interconectados e abscessos envolvendo, ao menos, uma área anatômica completa
IHS4	
Número de nódulos (*1)	
Número de abscessos (*2)	
Número de fístulas (*4)	
HS leve, pontuação ≤ 3	
HS moderada, pontuação de 4 a 10	
HS severa, potunação ≥ 11	

Fonte: A autora (2022).

4.4 Análise histológica

As amostras de biópsia de pele dos pacientes foram fixadas em blocos de parafina. Na plataforma de histologia do IMRB, cortes de 5 e 10 mm dessas amostras foram fixadas em lâminas tratadas de microscópio (Fisherbrand™ - Thermo-Fisher Scientific, Massachusetts, Estados Unidos da América (EUA)). Antes de aplicar os corantes hematoxilina e eosina (Abcam, Cambridge, Reino Unido), as lâminas preparadas passaram pelo processo de desparafinação através da submersão das amostras em xilol e subsequente hidratação através de banhos consecutivos em álcool em sequência decrescente de concentração (100%, 95% e 70%). Após a adição dos corantes (**Figura 7**), o processo teve continuidade com a desidratação das amostras através de banhos em série alcoólica crescente (70%, 95%, 100%) e com a montagem da lâmina, que consistiu em cobrir o tecido corado com uma lamínula de vidro.

Figura 7 – Análises histológica de lesões de DDD dos pacientes III-2 e III-4. Coloração efetuada com hematoxilina e eosina, com ampliação de 10 ×.



Fonte: A autora (2022).

4.5 Identificação de variantes patogênicas associadas à HS

4.5.1 Extração e quantificação do ácido desoxirribonucleico (DNA)

A coleta de sangue total foi realizada em tubos Vacutainer® contendo ácido etilenodiamino tetra-acético (EDTA, do inglês *Ethylenediaminetetraacetic acid*) através de uma punção na veia cubital mediana por profissionais da saúde experientes. O DNA total foi extraído do sangue venoso periférico através do kit Wizard® Genomic DNA Purification Kit (Promega, Wisconsin, EUA), seguindo as instruções fornecidas no seu protocolo. Após a extração do DNA, o Nanodrop 2000c (Thermo Scientific, Massachusetts, EUA), foi utilizado para a avaliação da concentração do material genético em ng/µl e da pureza (relação 260/280). A confirmação da qualidade do DNA foi feita através da visualização do DNA em gel de agarose e pelo teste Qubit (Invitrogen™, Oregon, EUA).

4.5.2 Eletroforese

Antes de sequenciar as amostras por Sanger e por sequenciamento do exoma completo (WES, do inglês *Whole Exome Sequencing*), a qualidade do DNA foi analisada em géis de agarose, pela técnica de eletroforese. O gel de agarose, na concentração de 2 porcento (%), foi feito através da mistura de 100 mililitros (ml) de tampão de migração *Tris-borate-EDTA* (TBE) ultrapuro 1X (Invitrogen™, Oregon, EUA), duas gramas (g) de agarose ultrapura (Invitrogen™, Oregon, EUA) e 5 microlitros (µl) de SYBR™ Safe (Invitrogen™, Oregon, EUA). Após a solidificação do

gel, as amostras de DNA, marcadas com azul de bromofenol (Sigma-Aldrich, Missouri, EUA) e acompanhadas pelo GeneRuler 100 bp DNA Ladder, pronto para uso (Thermo-Fisher Scientific, Massachusetts, EUA), migraram no gel a uma voltagem de 110 Volts (V) por 30 minutos no aparelho myGel™ Mini Electrophoresis System (Benchmark Scientific, Nova Jersey, EUA).

4.5.3 Sequenciamento do Exoma Completo (WES)

O WES com 150X da cobertura média esperada foi terceirizado pela empresa Macrogen (Seoul, Coreia do Sul). Nesta etapa, fizemos o sequenciamento de 2 pacientes (III-2, probanda, e III-4) e 1 controle (II-3). As reações do sequenciamento exônico foram realizadas através do sistema Illumina® HiSeq 2500 após a preparação da biblioteca com o kit *SureSelect Human all Exons* V7, na configuração *pair-ended* (150 pares de bases (pb)).

4.5.4 Análise de dados

Após o sequenciamento terceirizado, o resultado foi disponibilizado em uma nuvem virtual da empresa por até 30 dias. Durante esse período, os arquivos gerados foram baixados e armazenados nos computadores do LPM. Antes de começar as análises, a qualidade do sequenciamento, disponibilizada no documento *Raw Data Report,* gerado pela própria empresa, foi avaliada.

Após a averiguação da qualidade do sequenciamento, os adaptadores universais Illumina foram removidos do produto do sequenciamento usando o Trim Galore 0.6.1 (BABRAHAM BIOINFORMATICS). Em seguida, as leituras com o comprimento abaixo de 15 pb e/ou com terminações de baixa qualidade, ou seja, com pontuação Phred33 abaixo de 20, também foram removidas. O controle da qualidade, pré e pós remoção de adaptadores e de sequências de baixa qualidade, foi realizado pelo fastQC (BABRAHAM BIOINFORMATICS).

Os arquivos FASTQ gerados foram alinhados usando o *Software Package Burrows-Wheeler Aligner* (BWA) 13, especificamente a ferramenta *bwa-mem*, tendo como referência o Genoma Humano versão 38 (GRCh.38). Em seguida, a ferramenta Strelka2 (KIM *et al.*, 2018) foi utilizada para realizar a genotipagem. Já as ferramentas do Picard (BROAD INSTITUTE) foram utilizadas para marcar e remover as leituras duplicadas. O GATK v. 4.1.2.0 (BROAD INSTITUTE) realizou a recalibração de bases

e filtragem de qualidade da genotipagem, excluindo as variantes com baixa qualidade de mapeamento e de genotipagem (MQ <40 e GQ <20, respectivamente). Posteriormente, o software ANNOVAR foi utilizado para as anotações de variantes com o auxílio de bancos de dados baseados no genoma de referência GRCh.38 (refGene, cytoBand, wgRna, 1000g 2015 aug_all, gnomad30_genome, exac03, dbscsnv11, dbnsfp35a, clinvar_20200316 e avsnp151).

Através do uso do programa R Software Environment, uma nova estrutura (*framework in-house)* foi desenvolvida para analisar o conteúdo do exoma anotado e, posteriormente, avaliar o seu envolvimento na patogênese da HS. Basicamente, a investigação se concentrou em variantes localizadas nas regiões não intrônicas e não intergênicas.

Por fim, foi avaliado o impacto que as variantes encontradas podem causar na estrutura e na função das proteínas. O possível impacto foi elencado por ordem de relevância com base em 10 algorítimos preditores (sift, polyphen2, lrt, mutationtaster, mutationassessor, fathmm, provean, metasvm, metalr, m_cap). Baseando-se na troca de aminoácidos, esses algorítimos preditores inferem se a variante, seja ela de polimorfismo de nucleotídeo único (SNP, do inglês *Single Nucleotide Polymorphisms*) ou de inserção/deleção (indel), gera consequências estruturais e funcionais na proteína. Cada algorítimo utiliza uma metodologia própria para avaliar se ocorreu ou não algum de tipo de dano protéico. Por exemplo, o SIFT classifica a variante como deletéria baseando-se no grau de conservação da sequência proteica (KUMAR; HENIKOFF, 2009), enquanto o polyPhen2 classifica as variantes como deletérias, possivelmente deletérias ou benignas de acordo com o efeito desta sobre a sequência e estrutura da proteína (ADZHUBEI; JORDAN; SUNYAEV, 2013). Quanto mais preditores demonstrarem um efeito deletério da mutação, mais relevante ela é considerada. Paralelamente, como um outro critério de preditor de dano, foi avaliado a pontuação *phred* do CADD. O CADD gera uma pontuação para a variante de acordo com sua conservação, dados funcionais e análise de outras ferramentas *in silico.* Quanto mais alto for o valor de CADD, mais deletéria a mutação é considerada. Variantes com pontuação ≥ 10 representam variantes entre as 10% mais deletérias, enquanto variantes com pontuação ≥ 20 encontram-se entre as 1% mais deletérias (KIRCHER *et al.*, 2014).

4.5.6 Desenho de primers

Foram desenhados pares de *primers* para a reação em cadeia da polimerase (RCP, ou do inglês PCR) e para sequenciamento de Sanger. Primeiramente, as sequências de DNA e de DNA complementar (cDNA) dos genes estudados (GRCh38) foram identificadas na plataforma do Centro Nacional de Informações sobre Biotecnologia (NCBI, do inglês *National Center for Biotechnology Information*) (NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION), utilizando a busca de "gene". Em seguida, os códigos identificadores dessas sequências foram adicionados na plataforma digital do programa *Benchling* (BENCHLING). No programa, foram desenhadas as melhores opções de pares de *primers* para o estudo. De modo geral, buscou-se desenhar *primers* de senso e anti-senso que tinham um tamanho de aproximadamente (≈) 20 nucleotídeos, que se ligavam a éxons diferentes, que tinham uma amplificação ≈ 200 pb, que possuíam uma razão guanina-citosina entre 40-60 % e uma temperatura de fusão ≈ 60 ˚ C. Após a seleção dos pares desejados, foi verificada a especificidade dos mesmos num segundo programa, o PCR *In-Silico* da Universidade da Califórnia em Santa Cruz (UCSC, do inglês *University of California Santa Cruz*) (UNIVERSITY OF CALIFORNIA SANTA CRUZ). Pares de primers específicos para os alvos desejados foram selecionados (**Tabela 2**).

Tabela 2 – Primers usados no estudo.

	Gene	Primer	Sequência (5' to 3')
Sanger			
	NCSTN	Senso	ACGATAAGTGTGTGCCCAAG
		Anti-senso	GGTTGGGGTTGTATGAGATGC
qPCR			
	OAZ1	Senso	ACTTATTCTACTCCGATGATC
		Anti-senso	GAGAATCCTCGTCTTGTC
	NCSTN	Senso	GCGTCCTACTAGCAGGTTTGT
		Anti-senso	AAGGACACTGCAAGACCAGC
	PSEN1	Senso	CGGGGAAGCGTATACCTAATC
		Anti-senso	ACGTACAGTATTGCTCAGGTGG
	PSEN2	Senso	CGGCAGAGCAGGCATTTCC
		Anti-senso	TCACACAGCTCCCTCACATC
	PSENEN	Senso	GCGTGGTTGTTCGTGATCCTT
		Anti-senso	AGCAGAGAACGTGGGACATGA

Nota: qPCR, reação em cadeia de polimerase quantitativa; NCSTN, nicastrina; OAZ1, ornitina descarboxilase antizima 1; PSEN1, presenilina 1; PSEN2, presenilina 2; PSENEN, intensificador de presenilina.

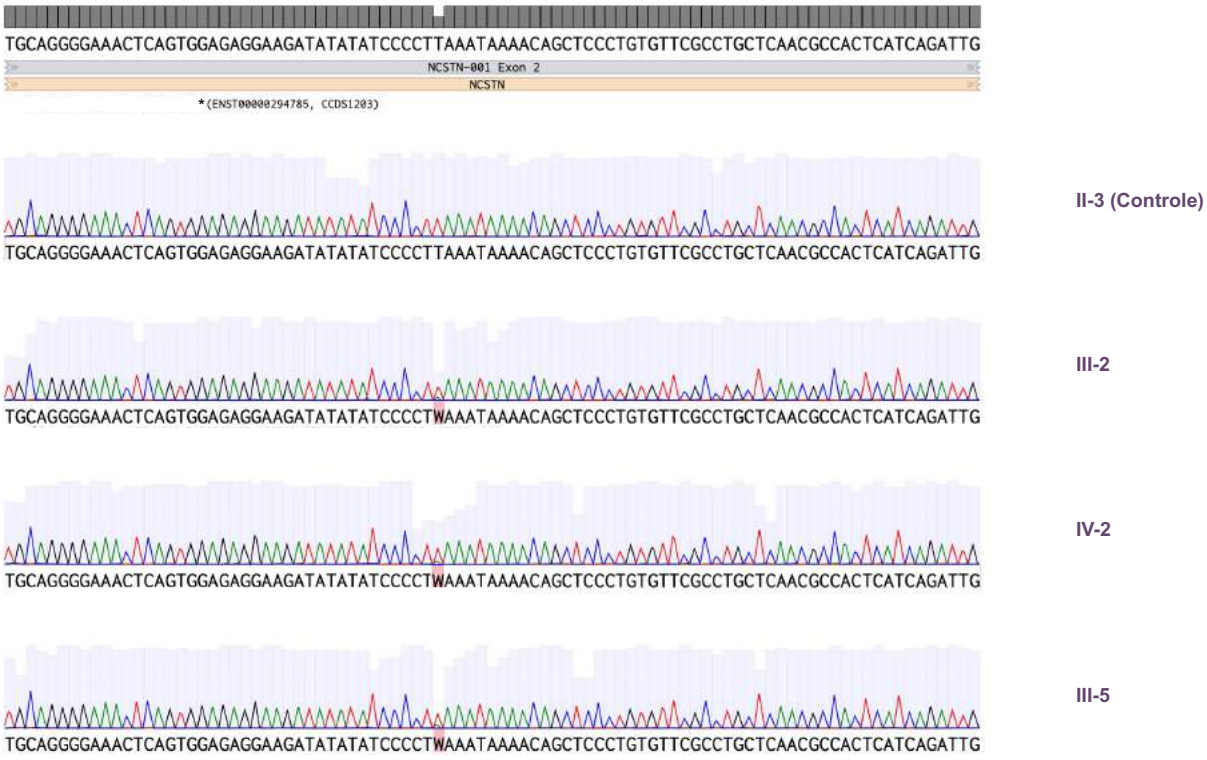
Fonte: A autora (2022).

4.5.7 Sequenciamento por Sanger

O sequenciamento por Sanger foi realizado pela plataforma genômica do IMRB. As sequências de DNA de 11 indivíduos da família (II-1, II-2, II-3 (controle), III-2, III-4, III-5, IV-1, IV-2, IV-3, IV-4 e IV-5) foram amplificadas pelas técnicas de PCR utilizando os primers desenhados e os reagentes do MyTaq™ DNA Polymerase (Bioline - Meridian Bioscience Inc, Ohio, EUA) em conformidade com o protocolo do produto. Após a PCR, os produtos foram purificados através da adição dos reagentes exonuclease I (EXOI) 20U/µl e fosfatase alcalina termossensível FastAP (ambos Thermo-Fisher Scientific, Massachusetts, EUA), seguindo os passos descritos por Werle e seu grupo (WERLE *et al.*, 1994). Brevemente, cinco µl de cada produto de PCR foi misturado com 0,1 µl de EXOI, 0,5 µl de FastAP e 4,4 µl de água livre de nucleases e incubados no termociclador rápido de 96 poços Veriti™ (Thermo-Fisher Scientific, Massachusetts, EUA) por 15 minutos à 37 ˚ C, 15 minutos à 80 ˚ C e 4 ˚ C por tempo indeterminado (∞). Em seguida, as amostras foram preparadas para o

sequenciamento através do kit de sequenciamento de ciclos BigDye terminator V3.1 (Thermo-Fisher Scientific, Massachusetts, EUA), de acordo com as instruções disponibilizadas pela empresa, juntamente com 3,2 picomoles (pmol)/ µl de cada par de *primer* previamente utilizado. A amostra de cada paciente foi separada em dois microtubos de 0,5 ml (BRAND®, Dinamarca). Um microtubo continha os reagentes, o produto de PCR e o *primer* de senso, enquanto o outro microtubo foi constituído pelas mesmas substâncias, porém com o *primer* anti-senso. Todas as amostras foram, em seguida, incubadas no termociclador por um minuto à 95 ˚ C, 25 ciclos de dez segundos à 96 ˚ C, cinco segundos à 50 ˚ C e quatro minutos à 60 ˚ C, finalizando por 4 ˚ C ∞. Por fim, a purificação das reações sequenciadas foi realizada com os reagentes do kit de purificação BigDye XTerminator™ (Thermo-Fisher Scientific, Massachusetts, EUA). As amostras de sequenciamento foram, então, enviadas para a plataforma genômica do IMRB, onde o Sanger foi executado no analisador genético *SeqStudio* (Thermo-Fisher Scientific, Massachusetts, EUA) (**Figura 8**).

Figura 8 – Representação do sequenciamento de Sanger de 4 dos 11 indivíduos, mostrando a ausência da mutação no controle e a presença da mutação nos restantes. * Código da sequência do gene utilizada no alinhamento. A sequência referência do gene foi obtida no site do NCBI (NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION, [s.d.]).



Fonte: A autora (2022).

4.6 Avaliação do impacto da variante a nível de RNA e proteína

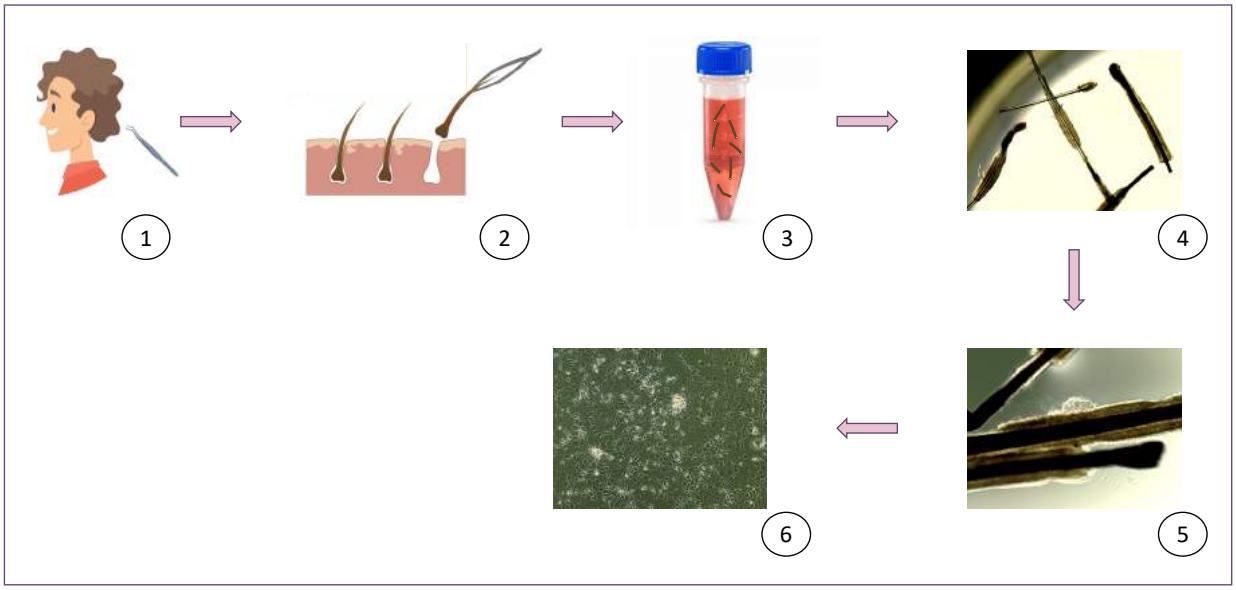
4.6.1 Extração de células da bainha radicular externa (ORS) do folículo piloso

As células ORS de 4 pacientes – controle II-3 e casos III-2, III-5 e IV-2 – foram extraídas da raiz do folículo piloso na fase anágena. A fase anágena é a fase da produção do fio de cabelo, onde as células da matriz do folículo estão se proliferando intensamente (SCHNEIDER; SCHMIDT-ULLRICH; PAUS, 2009). A coleta dos fios e o isolamento das células foram feitos baseados nas instruções publicadas por Aasen e Belmonte (2010) (AASEN; BELMONTE, 2010) uma higienização do local visado com álcool 70 %, seguido pela remoção de pelo menos 10 fios de cabelo da cabeça de cada paciente, com auxílio de uma pinça esterilizada. Os fios de cabelo que possuíam a raiz intacta, foram cortados com tesouras estéreis para obter um tamanho de aproximadamente 5 milímetros (mm) de comprimento. Logo após, esses fios foram armazenados em tubos de 1,5 ml contendo meio de cultivo celular *Dulbecco's Modified Eagle Medium* (DMEM) baixo cálcio (Gibco - Thermo Fisher Scientific, Massachusetts, EUA), com adição de antibiótico e/ou antifúngico, gentamicina e/ou anfotericina 1X (Gibco – Thermo Fisher Scientific, Massachusetts, EUA).

No laboratório, os fios de cabelo de cada paciente, submersos em meios de cultivo, foram transferidos para placas de Petri (Sigma-Aldrich, Missouri, EUA), dentro da cabine de segurança biológica (BSC, do inglês *Biological Safety Cabinet*) de classe II. Na placa de petri, ainda submersos pelo meio de cultivo celular, as raízes foram cortadas por bisturis estéreis e transferidas para placas de 24 poços (Thermo Fisher Scientific, Massachusetts, EUA), contendo 300 µl de *tryple* (Gibco – Thermo Fisher Scientific, Massachusetts, EUA). Os fios de cabelo do mesmo paciente eram agrupados no mesmo poço. Através do uso do *tryple*, foi feito uma digestão enzimática para a dissociação das células, que migraram para o reagente após uma incubação de 40 minutos em estufas de CO_2 com temperatura controlada à 37˚C. Durante esse tempo, para obter uma dissociação celular mais eficiente, foi efetuada pelo menos duas homogeneizações intensas, com o auxílio da pipeta P1000, nos poços contendo as raízes capilares. Quando as células se soltaram por completo da bainha da raiz, a reação enzimática foi interrompida com a adição de 300 µl de meio de cultura. Para finalizar, os 600 µl de reagentes contendo as células foram transferidos para placas de cultivo celular de 6 poços (Thermo Fisher Scientific, Massachusetts, EUA), previamente revestidas por fibroblastos embrionários de camundongos (3T3-J2)

inativados (Kerafast, Boston, EUA), e contendo 3,5 ml de meio para ORS produzido de acordo com os protocolos do laboratório. O processo da extração de células ORS encontra-se resumido na **Figura 9**.

Figura 9 – Esquema representando a extração de células ORS. 1-3: Coleta de fios na fase anágena, com posterior armazenamento em meio de cultura DMEM baixo cálcio, com adição de antibiótico e antifúngico. 4-5: Fios de cabelo são incubados com *tryple* para que ocorra a dissociação enzimática das células ORS aderentes a raiz do cabelo. 6: Após a dissociação, as células que se soltaram do cabelo e se encontram no meio são transferidas para placas revestidas por 3T3-J2 irradiados, onde serão cultivadas pelas próximas semanas.



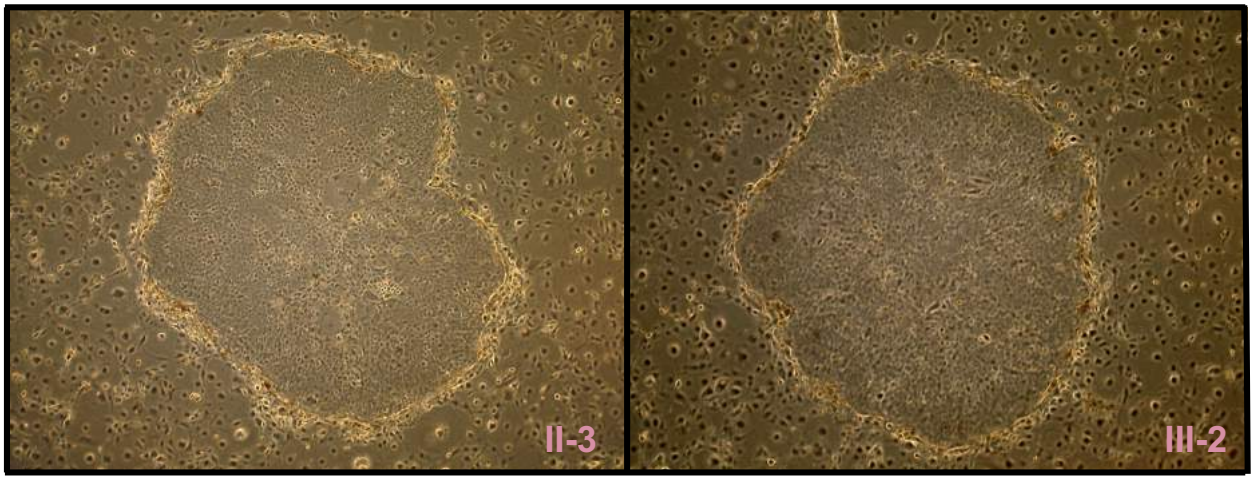
Fonte: A autora (2022).

4.6.2 Cultivo celular

As células ORS foram inicialmente cultivadas em placas de 6 poços, com trocas de meio de cultivo celular a cada 3 dias. Na presença de grandes colônias de queratinócitos primários (**Figura 10**), essas células foram transferidas para frascos 75cm^2 tratados e com filtro (Falcon® - Corning, Nova York, EUA), revestidos com fibroblastos 3T3-J2 irradiados. A irradiação dos fibroblastos é necessária para controlar a proliferação dos mesmos e, deste modo, impedir a competição entre as células que coabitam pelos nutrientes do meio celular. Assim, as células 3T3-J2 irradiadas atuam como uma camada de sustentação metabólicamente ativa para os queratinócitos primários. Para uma boa interação entre 3T3-J2 e ORS, é importante que os fibroblastos sejam utilizados antes da décima passagem e que estejam na confluência de 70%. Nestas condições, as células passaram por uma expansão clonal, onde se adaptaram aos frascos e obtiveram um ritmo de proliferação celular acelerado. Ao aparecerem novas colônias de queratinócitos primários, essas células

foram transferidas para frascos 75cm^2, sem o revestimento por células 3T3-J2, e cultivadas em meio celular CnT-07 Meio de Proliferação Epitelial Certificado (CELLnTEC, Bern, Suíça).

Figura 10 – Colônias de células ORS dos pacientes II-3 (controle) e III-2 (caso).



Fonte: A autora (2022).

4.6.3 Extração e quantificação de ácido ribonucleico (RNA)

A extração de RNA foi realizada diretamente nas placas de cultivo celular das ORS extraídas dos pacientes. O RNA foi extraído utilizando o kit PureLink™ RNA Mini Kit, Cátalogo N. 12183020 (Thermo-Fisher Scientific, Massachusetts, EUA), seguindo as instruções descritas no seu protocolo. Assim como o DNA, as quantificações de RNA foram realizadas com o Nanodrop 2000c, Thermo Scientific®.

4.6.4 Expressão gênica

Após a extração e quantificação das amostras de RNA, foi realizada a conversão do material genético para cDNA. O RNA foi convertido em cDNA utilizando o kit de transcrição reversa de cDNA de alta capacidade (Thermo-Fisher Scientific, Massachusetts, EUA), de acordo com as instruções do protocolo do produto. Em seguida, a expressão gênica dos genes estudados foi avaliada através da técnica de PCR em tempo real (qPCR) utilizando o kit Takyon™ ROX SYBR 2X MasterMix dTTP blue (Eurofins Scientific, Nantes, França), também em concordância com o seu protocolo. O nível de expressão gênica foi avaliado para os seguintes genes: *NCSTN, PSEN1, PSEN2* e *PSENEN.* Por fim, a expressão relativa de cada gene estudado ($\Delta\Delta$CT) foi calculada utilizando o gene *OAZ1* como gene de referência.

4.6.5 Extração e quantificação proteica

Assim como na etapa anterior, a extração de proteínas também foi realizada nas células ORS isolada dos pacientes. Proteínas e RNA de cada paciente foram extraídos simultaneamente nas mesmas placas de cultivo celular, porém de poços diferentes. As proteínas foram extraídas utilizando o reagente RIPA (Thermo-Fisher Scientific, Massachusetts, EUA), com adição do Coquetel de Inibidores de Proteases Halt™ (100X) (Thermo-Fisher Scientific, Massachusetts, EUA), na concentração final de 1X. Para isolar as proteínas dos restos celulares, estas amostras foram centrifugadas na centrífuga 5804 R (Eppendorf, Hamburgo, Alemanha), à 12.000 rotações por minuto (r.p.m.), por 20 minutos, à 4˚C. Após a centrifugação, o sobrenadante, contendo as proteínas, foi transferido para recipientes esterilizados de 1,5 ml (Merck KGaA, Darmstadt, Alemanha), devidamente identificados.

A quantificação proteica foi realizada através da detecção a base do corante *Bradford* (Thermo-Fisher Scientific, Massachusetts, EUA). O ensaio de *Bradford* foi realizado de acordo com as instruções fornecidas no protocolo do produto. As absorbâncias emitidas pela reação entre o corante e a proteína foram lidas no leitor de placas Infinite® 200 (TECAN, Männedorf, Suíça) e comparadas às da curva de albumina de soro bovino (Sigma-Aldrich, Missouri, EUA) nas concentrações de 0, 4, 8, 16, 32, 48 ug/ml. A absorbância 595 nanômetros (nm) aumenta de forma proporcional à quantidade de proteínas na amostra. Para determinar quantitativamente a concentração proteica dessas amostras, uma curva-padrão foi utilizada. Essa curva-padrão, baseada na relação entre a quantidade de albumina presente e a sua absorbância, foi criada no programa Excel, gerando uma equação linear que determinou os valores quantitativos da proteína das amostras estudadas.

4.6.6 Expressão proteica

Após a quantificação proteica, a expressão de determinadas proteínas foi analisada através da técnica de Western Blot. Sucintamente, 15 µg de proteína de cada paciente foram preparadas com agentes redutores NuPAGE™ (10X) (Invitrogen™, Oregon, EUA), tampão de amostra Pierce™ LDS, não redutor (4X) (Invitrogen™, Oregon, EUA), e aquecimento térmico a 75 ˚ C por 15 minutos. Em seguida, essas amostras migraram em géis de 12 poços NuPAGE™ 4-12%, Bis-Tris, 1,0 mm (Invitrogen™, Oregon, EUA), ao lado de marcadores de peso de proteína, pré-

corado, PageRuler™ Plus, 10-250 kDa (Invitrogen™, Oregon, EUA). Ao final da corrida, o gel foi inserido no iBlot PVDF (Invitrogen™, Oregon, EUA), onde as proteínas migraram do gel para a membrana, numa voltagem de 20 V, com uma corrente de 1.5 amperes, por 5 minutos. Ao finalizar a transferência de proteínas, as membranas foram incubadas com anticorpos específicos no iBind (Invitrogen™, Oregon, EUA), por pelo menos duas horas e meia. Dentre os anticorpos utilizados encontram-se: o anticorpo monoclonal de controle de carga de ß actina (BA3R) (Thermo-Fisher Scientific, Massachusetts, EUA), NCSTN (R&D systems, Minnesota, EUA), PSEN1, terminal C (Cell Signaling Techonology, Massachusetts, EUA), PSEN1, terminal N (Cell Signaling Techonology, Massachusetts, EUA), PSEN2 (Cell Signaling Techonology, Massachusetts, EUA) e PSENEN (Cell Signaling Techonology, Massachusetts, EUA).

4.7 Avaliação da hipótese do mecanismo de degradação mediada por mutação sem sentido

A hipótese do mecanismo de degradação mediada por mutação sem sentido foi testada pela incubação das células ORS com medicamentos já disponíveis no mercado e descritos como inibidores de NMD ou PTC, diluídos em meio celular CnT-07-HC. Células ORS do paciente probando (III-2), na confluência de 70%, foram incubadas por 24 horas com Amlexanox (Selleckchem, Houston, EUA), na concentração de 33,5 mmol, ou com gentamicina (Gibco™ - Thermo-Fisher Scientific, Massachusetts, EUA), na concentração de 50 µmol ou de 250 µmol. Como controle para esses 3 tratamentos, foi utilizado células ORS do mesmo paciente, incubadas apenas com meio de cultivo CnT-07-HC.

5 RESULTADOS E DISCUSSÃO

Os resultados deste trabalho encontram-se divididos entre as seções “Apêndices A-C”, seguindo a sugestão da Biblioteca Central da UFPE para trabalhos compostos por artigos já publicados em revistas científicas. Os resultados encontram-se divididos da seguinte maneira:

- Apêndice A – Artigo publicado no periódico *Experimental Dermatology* – Wiley (Fator de impacto – 4,511), relativo ao estudo da mutação encontrada na *NCSTN,* e o impacto dela na patogênese da HS e DDD.
- Apêndice B – Artigo publicado no periódico *Biomolecules* - MDPI (Fator de impacto 2022 – 4,569), relativo a uma meta-análise de transcriptoma de pele afetada pela HS, avaliando a assinatura genética-molecular presente nesses pacientes.
- Apêndice C – Artigo publicado no periódico *International Journal of Molecular Sci*ences - MDPI (Fator de impacto 2020 – 5,523), relativo ao estudo do papel da via Notch em doenças dermatológicas.

6 CONSIDERAÇÕES FINAIS

- Foi recrutado uma família de quatro gerações, com sete pacientes afetados por HS com possível herança de padrão autossômica dominante.
- As análises histológicas confirmaram a presença de DDD concomitante a HS.
- Foi identificado, através do WES, e confirmado por Sanger, um *stop códon* no segundo éxon do gene da *NCSTN* (c.T131A:p.L44X) nos pacientes afetados pela condição e ausente no controle. Acreditamos que esta mutação esteja associada com ambas as doenças.
- O gene da *NCSTN* faz parte da γ-secretase que regula a via Notch, responsável pelas interações entre melanócitos e queratinócitos, regulando a proliferação e diferenciação dessas células, assim como de células imunes.
- Acredita-se que esta mutação na *NCSTN* ocasiona uma desregulação da via Notch, que pode levar à hiperpigmentação observada na DDD e ao desequilíbrio da resposta imunológica e hiperqueratose epidérmica e folicular, com formação de cisto epidérmico, observados na HS.
- Níveis de RNA e de proteína, extraídos de células primárias da bainha radicular externa do folículo piloso dos indivíduos da família, confirmaram a haploinsuficiência da NCSTN nos pacientes afetados.
- Foi observado que os níveis proteicos das outras subunidades da γ-secretase (PSEN1, PSEN2 e PSENEN) também estavam alterados na presença da mutação, sugerindo uma associação com o desenvolvimento de DDD.
- Mostramos que o códon de parada prematuro leva ao decaimento de mRNA mediado por NMD.
- Esses pacientes poderiam se beneficiar com o aumento dos níveis de mRNA de *NCSTN*. Baseado nos nossos resultados preliminares, hipotetizamos que o uso de moléculas que tenham como alvo o mecanismo NMD, como amlexanox e gentamicina, poderiam aumentar os níveis de mRNA da *NCSTN* nesses pacientes. Esta reconstituição poderia, consequentemente, auxiliar na reconstituição do complexo γ-secretase e, consequentemente, melhorar o fenótipo de HS e prevenir ou retardar o DDD.

7 ASPECTOS ÉTICOS

Este projeto faz parte de um projeto intitulado “Análise biomolecular para medicina personalizada em pacientes com Hidradenite Supurativa” e possui aprovação do comitê de ética em pesquisa do Centro de Ciências da Saúde da Universidade Federal de Pernambuco – UFPE/CCS e pelo Núcleo de Apoio à Pesquisa do Hospital das Clínicas da Universidade Federal de Pernambuco – **HC-UFPE**/Ebserh (CAAE: 64777922.0.0000.5208/ Número do Parecer: 5.807.358).

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APÊNDICE A – Artigo 1: A loss-of-function NCSTN mutation associated with familial Dowling Degos disease and hidradenitis suppurativa (Fator de impacto 2023 – 4,511)

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RESEARCH ARTICLE

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A loss-of-function *NCSTN* mutation associated with familial Dowling Degos disease and hidradenitis suppurativa

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Abstract

Dowling Degos disease (DDD) is a rare autosomal dominant genodermatosis characterized by acquired, slowly progressive reticulated pigmented lesions primarily involving flexural skin areas. Mutations in *KRT5*, *POGLUT-1* and *POFUT-1* genes have been associated with DDD, and loss-of-function mutations in *PSENEN*, a subunit of the gamma-secretase complex, were found in patients presenting with DDD or DDD comorbid with hidradenitis suppurativa (HS). A nonsense mutation in *NCSTN*, another subunit of the gamma-secretase, was already described in a patient suffering from HS and DDD but whether *NCSTN* could be considered a novel gene for DDD is still debated. Here, we enrolled a four-generation family with HS and DDD. Through Whole Exome Sequencing (WES) we identified a novel nonsense mutation in the *NCSTN* gene in all the affected family members. To study the impact of this variant, we isolated outer root sheath cells from patients' hair follicles. We showed that this variant leads to a premature stop codon, activates a nonsense-mediated mRNA decay, and causes NCSTN haploinsufficiency in affected individuals. In fact, cells treated with gentamicin, a readthrough agent, had the *NCSTN* levels corrected. Moreover, we observed that this haploinsufficiency also affects other subunits of the gamma-secretase complex, possibly causing DDD. Our findings clearly support *NCSTN* as a novel DDD gene and suggest carefully investigating this co-occurrence in HS patients carrying a mutation in the *NCSTN* gene.

KEYWORDS
Dowling Degos disease, genetic mutation, gentamicin, hidradenitis suppurativa, NCSTN

Ana Sofia Lima Estevao de Oliveira and Roberta Cardoso de Siqueira should be considered joint first author' and Michele Boniotto and Lucas André Cavalcanti Brandão should be considered joint senior authors.

1 | INTRODUCTION

Dowling-Degos disease (DDD) (ORPHA:79145) is a rare autosomal dominant genodermatosis characterized by slowly progressive reticulated pigmented lesions in flexural skin areas.[1] The first gene associated with DDD was *KRT5*.[2] Mutations in the head region of keratin 5 (KRT5) protein are found in ~1/3 of DDD patients, usually presenting an acantholytic variant named Galli-Galli Disease (GGD).[3,4] Loss-of-function (LOF) mutations in the genes encoding the protein O-fucosyltransferase 1 (POFUT1) and the protein O-glucosyltransferase 1 (POGLUT1) have been found in sporadic cases and families suffering from DDD.[5,6]

Hidradenitis suppurativa (HS), also known as Acne Inversa, is a chronic inflammatory skin disease clinically characterized by recurrent painful nodules and abscesses on body areas containing apocrine glands.[7] In severe HS phenotypes, recurrent nodular rupture leads to sinus tract formation, a hallmark that severely impacts patients' quality of life[8]. HS may appear in a sporadic, familial or syndromic form and is associated with a mutation in *NCSTN*, *PSEN-1* or *PSENEN* in 5% of patients.[9]

The first patient suffering from DDD and HS was described in 1990[10] and, ever since, several patients have been reported raising the hypothesis that this co-occurrence is due to a shared pathogenic mechanism rather than a simple coincidence. Interestingly, mutations in *PSENEN* were first associated with HS and only later found in patients suffering from DDD alone or DDD and HS,[11-14] thus confirming the pathogenic link between DDD and HS. Even if the molecular events leading to HS and DDD are not well elucidated, the common hypothesis is that a reduced NOTCH signalling in keratinocytes and melanocytes could be responsible for both diseases. In fact, most of the mutated proteins in HS or DDD are involved in NOTCH Receptors processing or maturation. Presenilin enhancer (PEN-2 -encoded by *PSENEN*) together with nicastrin (NCSTN), presenilins 1 or 2 (PSEN) and anterior pharynx defective 1 A or B (APH1) compose the gamma-secretase complex,[15] a high molecular weight transmembrane enzyme complex that is involved in intramembrane cleavage of the NOTCH Receptors.[16] In addition, POFUT1 and POGLUT1 add O-fucose and O-glucose, respectively, to most of the EGF-like repeats of NOTCH Receptors.[17] Why mutations in certain genes are associated with HS and others with DDD is not known. In this respect, even if mutations in *NCSTN* have already been found in patients suffering from both HS and DDD, *NCSTN* remains not recognized as a gene for DDD.

In this paper, we report the genetic study of a four-generation family from Brazil where HS and DDD co-segregate as an autosomal dominant trait. Whole exome sequencing (WES) performed in two affected and one non-affected family member allowed the identification of a novel nonsense mutation in the *NCSTN* gene that was found in all affected individuals. Functional studies conducted on outer root sheath keratinocytes isolated from plucked hairs showed that the mutation caused haploinsufficiency of NCSTN triggered by nonsense-mediated decay (NMD) and also affected other subunits of the gamma-secretase complex. Interestingly, this haploinsufficiency could be corrected by gentamicin, a read-through stimulating antibiotic.

2 | MATERIALS AND METHODS

2.1 | Patients

In October of 2019, a Brazilian family was recruited in a multidisciplinary ambulatory of HS at Hospital das Clínicas–Federal University of Pernambuco, situated in Recife, Brazil. This study, managed in accordance with the Declaration of Helsinki standards, has been approved by the local and federal ethical committee (CAAE: 03096118.1.0000.5208/Approval number 3.588.204). To be included in the study, written consent from all the patients has been collected after a scrupulous explanation of the research's purpose.

HS clinical diagnosis was given by a dermatologist based on the European guidelines (S1) of Dessau.[18] DDD diagnosis was later confirmed in two patients through a 5 mm biopsy punch and histopathologic analysis stained with haematoxylin and eosin. Biopsies were only performed on patients that went through HS surgery. Although histopathologic analyses were not performed in the remaining patients, similar clinical DDD lesions were observed.

2.2 | Whole exome sequencing analysis

Subsequent to diagnosis, patients' blood samples were collected for DNA isolation. DNA was isolated from peripheral venous blood using the Wizard® Genomic DNA Purification Kit (Promega), following the instructions provided in its protocol. DNA WES was performed through outsourcing sequencing (Macrogen) with a 150× average coverage. WES was performed in two patients diagnosed with HS and DDD (the propositus III-2 and the family member III-4) as well as one healthy individual from the family (II-3). Briefly, the exonic sequencing reactions were done using the Illumina® HiSeq 2500 system with the library SureSelect Human all Exons V7 kit in the pair-ended configuration (150 base pairs). After checking the sequencing quality, Illumina adapters were removed with Trim Galore 0.6.1 and FASTQ files were aligned with Software Package Burrows-Wheeler Aligner (BWA) 13 to the Human Genome version 38 (GRCh.38) as a reference. Finally, variants were annotated using ANNOVAR and visualized using the R software v. 4.2.2.

To assess the segregation between the variant and the disease, Sanger sequencing was performed for all the affected individuals using BigDye terminator v3.1 cycle sequencing kit (ThermoFisher Scientific) according to manufacturer's instructions, and analysed with SeqStudio Genetic Analyser (ThermoFisher Scientific). Primers used are indicated in Table 1.

2.3 | Cells isolation and culture

Outer root sheath (ORS) keratinocytes were isolated from 10 patients' hairs in anagen phase. Cells were isolated by enzymatic digestion using 0.25% trypsin with 0.03% EDTA at 37°C following the protocol by Rochat et al..[19] To obtain a cell suspension, the isolated

TABLE 1 Primers used in the study.

	Gene	Primer	Sequence (5′–3′)	Sequence ID
Sanger sequencing	NCSTN	Forward	ACGATAAGTGTGTGCCCAAG	ENST00000294785
		Reverse	GGTTGGGGTTGTATGAGATGC	
qPCR	OAZ1	Forward	ACTTATTCTACTCCGATGATC	NM_004152.3
		Reverse	GAGAATCCTCGTCTTGTC	
	NCSTN	Forward	GCGTCCTACTAGCAGGTTTGT	NM_015331
		Reverse	AAGGACACTGCAAGACCAGC	

Note: qPCR denotes quantitative real-time PCR; NCSTN, nicastrin; OAZ1, ornithine decarboxylase antizyme 1.

cells were filtered through a 40-mm-pore nylon mesh cell strainer (BD Falcon). Cells were grown and maintained following the protocols by Rheinwald and Green[20] in the presence of feeder cells 3T3-J2 (kindly donated by Dr Y. Barrandon). Cells were further amplified and maintained in DermaCult Keratinocyte Expansion Medium (STEMCELL Technologies).

2.4 | Cells treatment by read-through agents

ORS keratinocytes from patient III-2 (1.5×10^6 cells) were seeded at ~80% confluency and cultured in DermaCult Keratinocyte Expansion Medium (STEMCELL Technologies) for 24 h. Cells were then left untreated or treated with Amlexanox (33.4 mM) or Gentamicin (50 μM) for additional 24 h and lysed for RNA extraction. The experiment was repeated three times.

2.5 | Quantitative PCR

About 2×10^5 cells were seeded and cultured in DermaCult Keratinocyte Expansion Medium (STEMCELL Technologies) for 3 days to reach confluency. Cells were lysed and RNA extracted using the PureLink RNA Mini Kit (ThermoFisher Scientific), following manufacturer's instructions. *NCSTN* expression level was measured by quantitative real-time PCR using the kit Takyon™ ROX SYBR 2X MasterMix dTTP blue (Eurogentec). The *NCSTN* relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method, and *OAZ1* as a housekeeping gene (Table 1).

2.6 | Western blot

About 2×10^5 cells were seeded and cultured in DermaCult Keratinocyte Expansion Medium (STEMCELL Technologies) for 3 days to reach confluency. Cells were then lysed in RIPA buffer (ThermoFisher Scientific) supplemented with the Halt Protease Inhibitor Cocktail (ThermoFisher Scientific). The protein contents in cell lysates were determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), and the concentrations were adjusted with sample buffer (250 mM Tris–HCl, 10% (v/v) SDS, 50% (v/v) Glycerol, 0.5 M β-mercaptoethanol, 0.5% (w/v) Bromophenol blue, pH 6.8) prior to samples boiling 5 min at 95°C. Equal amounts of protein lysates were loaded on 4%–12% Bis-Tris gels (NuPage, Invitrogen) and transferred to 0.2 μm pore-size nitrocellulose membranes (ThermoFisher Scientific). Membranes were blocked 30 min in PBS supplemented with either 0.1% (v/v) Tween-20 and 4% (w/v) non-fat dried milk or 0.1% (v/v) Tween-20 and 5% (w/v) BSA and incubated up to 12 h at 4°C with respective primary antibodies, and 45 min with secondary antibodies diluted in PBS/ 0.1% Tween-20. Membranes were washed 30 min at least twice in PBS/ 0.1% Tween-20, developed using ECL Plus western blotting detection system (GE Healthcare) and visualized by Amersham Hyperfilm ECL (GE Healthcare). All molecular weights (MW) were in kDa. Antibodies used in this study: beta Actin Loading Control Monoclonal Antibody (Thermo Fisher Scientific Cat# MA5-15739, RRID:AB_10979409–1/20000), Invitrogen; Mouse antibody against NCSTN (R and D Systems Cat# MAB53781, RRID:AB_11128467–1/500); Rabbit antibodies against PSEN1-C (Cell Signaling Technology Cat# 5643, RRID:AB_10706356–1/1000), PSEN1-N (Cell Signaling Technology Cat# 87146, RRID:AB_2800100–1/1000); PSEN2 (Cell Signaling Technology Cat# 9979, RRID:AB_10829910–1/1000), PEN2 (Cell Signaling Technology Cat# 8598, RRID:AB_11127393–1/1000), and HRP conjugated secondary antibodies against mouse (Cell Signaling Technology Cat# 7076, RRID:AB_330924–1/2000) and rabbit (Cell Signaling Technology Cat# 7074, RRID:AB_2099233–1/2000). Densitometry values were obtained using the ImageJ software[21] for proteins of interest and β-actin, as a control protein. Data from at least three different experiments are reported as the ratios of protein of interest/β-actin. This ratio was normalized to wild-type values (individual II-3), which was set to the value of 1.

2.7 | Statistical analyses

Statistical analyses were performed using GraphPad Prism (version 9, GraphPad Software) in all experiments that were repeated at least three times. Results were reported as mean ± standard error of the mean (SEM). Statistical analyses between two sets of data were performed by using the two-tailed unpaired Student's *t*-test. Significant

differences between control or treated samples are indicated as ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$. Only $p<0.05$ was considered as statistically significant.

3 | RESULTS

3.1 | Clinical features

We identified a four-generation family with five affected patients with DDD comorbid with HS (Figure 1). The proband (III-2) was classified with a severe phenotype of HS (Hurley III) characterized by inflammatory nodules, abscess, comedones, interconnected tunnels, scars, sebaceous cysts, follicular hyperkeratosis and folliculitis in several body areas. Apart from her, the other HS/DDD family members have a mild HS phenotype (Hurley I) characterized by several sebaceous cysts ranging in size from small to large, nodules, follicular papules, folliculitis, pyogenic granuloma, scars and single and double comedones in different areas of the body (Figure 2). Some of them also presented inactive pilonidal cyst. In particular, the individual II-2 was also diagnosed with invasive ulcerated squamous cell carcinoma (SCC) located between the inguinal and scrotum regions of the right side. At the time of the examination, patient II-1 did not show active typical HS lesions. Thus, the diagnosis was made based on the scars that were present, and family reports. DDD was characterized by reticulated pigmentations in the armpits and groin, and crateriform/cribriform scars on the back, nasal dorsum and lip philtrum (Figure 2).

As depicted in Figure 1, one family patient (IV-3) may be affected by HS only. In this case, HS may be presented in an HS conglobate-like type that presents itself like conglobate acne, a severe acne that mainly occurs on a patient's back[22]. However, the patient did not meet the criteria for HS diagnosis, which includes the involvement of HS typical areas of the body: axilla, genitofemoral area, perineum and gluteal area.[18] He reported a history of inflammatory lesions located on the face and back since he was 12 years old. Clinical analysis revealed the presence of erythematous papules, pustules, small nodules, atrophic scars, and simple and double-ended open comedones (pseudocomedones) along the entire length of the back, lumbosacral region and face. A linear lesion was also observed in the right lumbar region, below the waistline, which could correspond to a fistula or a scar. Such findings can occur both in the conglobata HS subtype[23] and in acne, and it is also known that these two conditions can coexist.

3.2 | Identification of a novel variant associated with DDD and HS

We performed WES in two patients of the family affected by HS and DDD, III-2 and III-4, as well as in one healthy individual, II-3. We identified a novel *NCSTN* heterozygous single nucleotide variation (SNV) (NM_015331.3:c145T>A) located in exon 2, predicted to create a premature stop codon (NP_056146.1:Leu44Ter[p.L44*]). The segregation of this variant was confirmed by Sanger sequencing in all family members. Note that all affected subjects carried the p.L44X variant (Table 2). It is noteworthy that the three unaffected individuals of the fourth generation who have the SNV, have not yet reached puberty, the period associated with HS development.[24] There was also no sign of DDD in these patients, a progressive disease that is acquired over the years .[1]

In order to evaluate the impact of this SNV, ORS cells were isolated from plucked hairs of subjects II-3 (healthy control), III-2, III-5 and IV-2 (cases). A significant decrease in both *NCSTN* mRNA and protein level was observed by qPCR and western-blot assays in subjects carrying the heterozygous premature stop codon when compared with the unmutated individual (Figure 3). This suggests that the phenotype observed in mutated individuals results from haploinsufficiency of *NCSTN*.

After confirming *NCSTN* haploinsufficiency in three mutated subjects, we questioned if lower levels of *NCSTN* could be a consequence of NMD surveillance. Since the SNV is predicted to generate a premature termination codon (PTC), a strategy to skip NMD is through the inhibition of this mechanism or through the incorporation of a random amino-acid at the PTC position, causing a PTC read-through.[25] A few commercially available molecules that target NMD have been described.[26,27] To answer this question, we treated the III-2 ORS cells with two of these compounds, amlexanox and gentamicin (Figure 3C). As seen in Figure 3C, after treating cells, levels of *NCSTN* mRNA were significantly increased, corroborating that the mutation was activating the NMD mechanism in these cells.

3.3 | Evaluation of gamma-secretase subunits stability

Since pathogenic mutations in genes that were previously associated with DDD namely *KRT5*, *POGLUT1*, *POFUT1* and *PSENEN*, were not found (Table S1), it is safe to assume that the *NCSTN* mutation was associated with both conditions in this family. *NCSTN* belongs to the gamma-secretase complex alongside *PSENEN* and other genes. As mutations in *PSENEN* have already been reported in subjects with both HS and DDD, we wondered whether haploinsufficiency of NCSTN could lead to gamma-secretase complex instability or degradation of its subunits. We thus evaluated mRNA and protein relative expression levels of *PSEN1*, *PSEN2* and *PSENEN* in mutated and non-mutated subjects' ORS cells. There was no statistically significant difference between patients and control regarding the mRNA levels of these genes (not shown). However, protein levels of these gamma-secretase subunits were significantly lower in two out of three mutated individuals when compared with the unmutated one (Figure 4). This suggests that *NCSTN* haploinsufficiency may result in protein degradation of the other subunits of the gamma-secretase complex. Interestingly, the individual IV-2, who carries the mutation but has not developed HS yet, has significantly lower levels of NCSTN mRNA and the

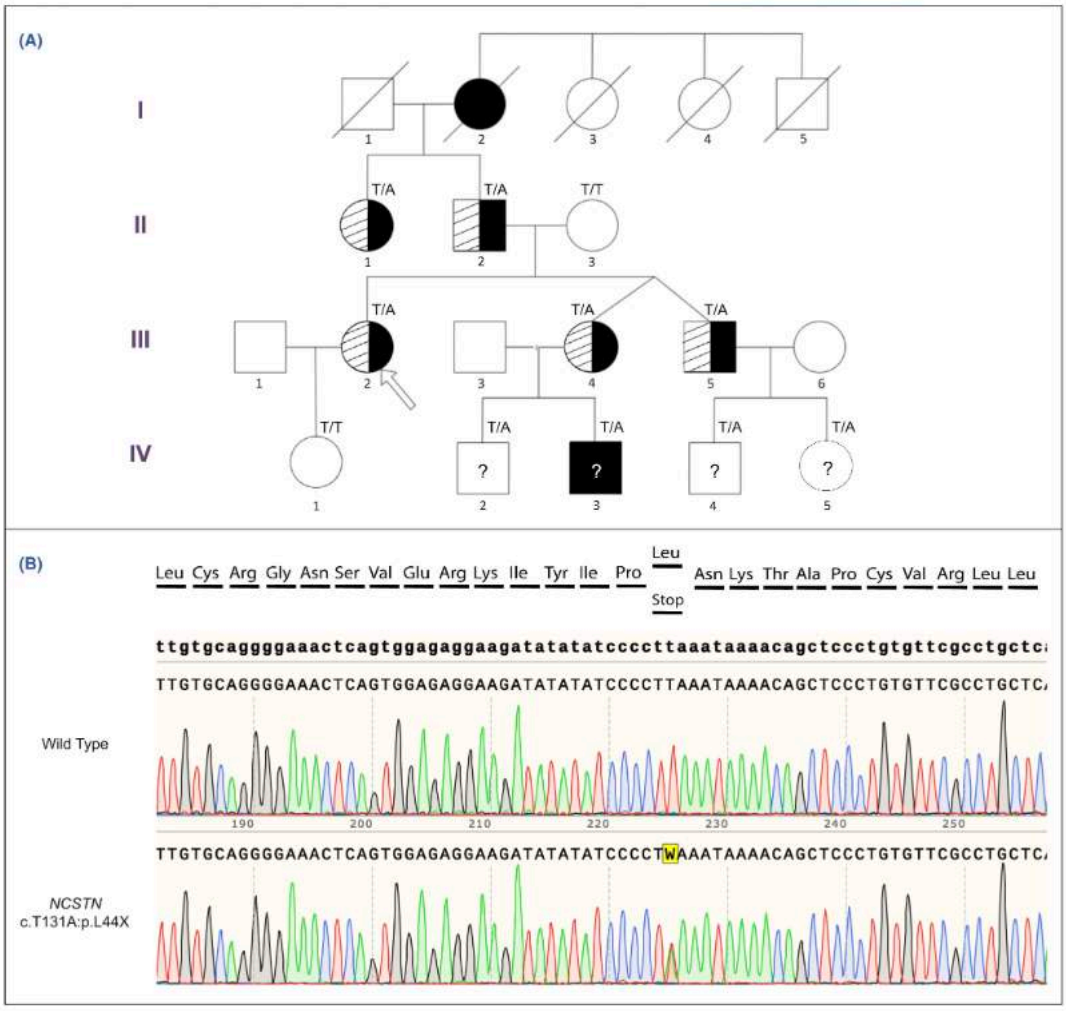

FIGURE 1 (A) Family pedigree of the four-generation Brazilian family affected by HS and DDD. The arrow refers to the proband. Males are shown with squares and females with circles. A diagonal line indicates deceased individuals. HS and DDD are shown with solid and hatched patterns, respectively, whereas unaffected individuals are shown with clear symbols. T/A genotype indicates the presence of the variant at the *NCSTN*, NM_015331.3:c145T>A; NP_056146.1:Leu44Ter[p.L44*]), while T/T indicates the wild type. Patients with question marks carry the mutation but have not yet developed the disease or have an inconclusive diagnosis. (B) Partial sequence of the exon 2 of the *NCSTN* gene. The top electrogram belongs to the control (II-3), whereas the bottom one belongs to the patient III-2, indicating the mutation, NM_015331.3:c145T>A.

immature form of the protein but normal levels of its mature form. In this patient, the protein levels of the other gamma-secretase subunits were not significantly different from the unmutated subject, corroborating the hypothesis that the HS and DDD phenotypes may result from a lower level of the whole gamma-secretase complex. Altogether, our results support the hypothesis that the *NCSTN* mutation is not only responsible for HS onset in these patients; it is also associated with gamma-secretase instability that causes DDD.[28]

4 | DISCUSSION

To date, LOF mutations in *KRT5*, *POGLUT1* and *POFUT1* have been associated with DDD and its acantholytic form GGD.[3,29] LOF mutations in *PSENEN* were first associated with HS[11] and then found in patients affected by DDD or presenting DDD comorbid with HS.[1,14] The opposite holds true for *POFUT1*, whose mutations were first associated with DDD and later described in sporadic patients presenting both HS and DDD.[30,31] Recently, sporadic patients suffering

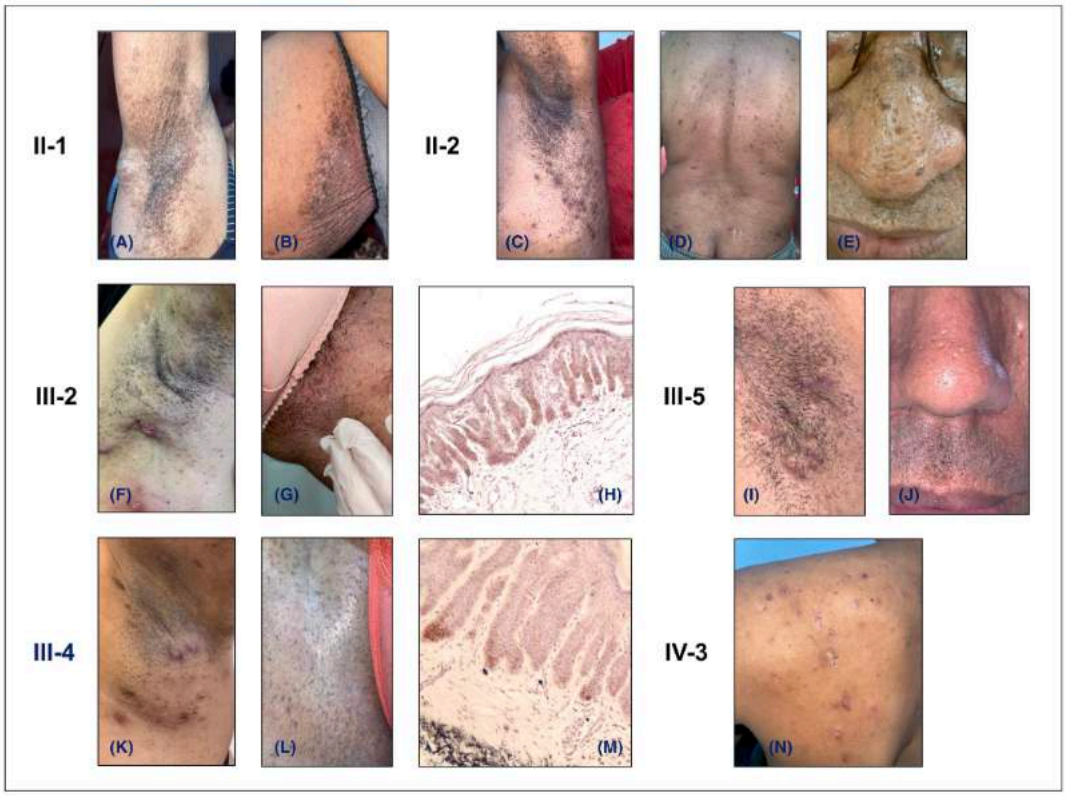

FIGURE 2 (A–K,M,N) Skin images of the affected family members. (A) hyperpigmented macules in a reticulate pattern in the axillary region; (B) hyperpigmented macules in a reticulate pattern in the groin and inner thigh; (C) hyperpigmented macules in a reticulate pattern, comedones, follicular papules and nodules in the axillary region; (D) cribriform scars on the back; (E) cribriform scars on the nasal dorsum and lip philtrum; (F) hyperpigmented macules in a reticulate pattern, nodules and sinus tracts in the axillary region; (G) hyperpigmented macules in a reticulate pattern in the groin and inner thigh; (I) hyperpigmented macules in a reticulate pattern, nodules and atrophic scars in the axillary region; (J) cribriform scars on the nasal dorsum and lip philtrum; (K) nodules in the axillary region; (L) hyperpigmented macules in a reticulate pattern in the groin; (N) nodules and cribriform scars on the back. (H, M) Histologic images of DDD biopsies show filiform acanthosis in an 'antler-like' pattern with hyperpigmentation in the basal layer; haematoxylin and eosin stain, magnification (H) 5× and (m) 10×.

from HS and DDD have been described with damaging mutations in *NCSTN*.[32] Garcovich et al.[28] have also identified a nonsense *NCSTN* mutation associated with familial HS where the older father also suffered from DDD. Even with those results, the NCSTN role in DDD remains debated for the lack of sufficient evidence.

Here, we describe the first family where HS and DDD co-segregated across two generations. All patients carried a nonsense mutation in the *NCSTN* gene (NM_015331.3:c145T>A; NP_056146.1:Leu44Ter[p.L44*]), including five of them with HS and DDD. This demonstrates that *NCSTN* should be considered as a gene associated with DDD comorbid with HS. It remains to be understood whether, in the case of mutations in *NCSTN*, DDD is a subphenotype of HS or the opposite is true.

In this family, affected members suffer from a follicular HS phenotype and developed folliculitis and opened and closed comedones, which are characteristics of HS patients carrying *NCSTN* mutations.[33] Interestingly, out of the five family members with a conclusive diagnosis of HS and DDD, only the proband was diagnosed with a severe HS phenotype. The others have a mild HS phenotype, thus supporting the hypothesis by Garcovich et al. that DDD lesions become clinically evident when HS skin inflammation is kept at bay.[28] As for the onset age, these five individuals suffering from DDD and HS developed HS symptoms around the age of 15, 5 years earlier than the age reported by Xu et al.[33] while DDD lesions probably arose later as they usually have an age of onset between 20 and 50 years.[34] This could be recapitulated by the clinical history of the patient IV-3, who has not been diagnosed with HS but has been suffering from conglobate acne since the age of 12 and still does not show any DDD lesions at the age of 17. Moreover, his clinical history indicates that the *NCSTN* nonsense mutation NM_015331.3:c145T>A causes HS

TABLE 2 Clinical features of the HS and DDD family individuals.

ID	II-1	II-2	II-3	III-2	III-4	III-5	IV-1	IV-2	IV-3	IV-4	IV-5
Sex	F	M	F	F	F	M	F	M	M	M	F
Age	60	65	68	37	36	36	15	11	17	12	10
Age when HS started	NA	17	–	15	15	15	–	–	12	–	–
BMI	NA	37.52	NA	27.13	37.98	33.22	NA	NA	18.7	NA	NA
Smoker	NA	–	NA	–	–	+	NA	NA	–	NA	NA
NCSTN, c.T131A:p.L44X	+	+	–	+	+	+	–	+	+	+	+
DDD	+	+	–	+	+	+	–	–	–	–	–
Face	–	+	–	–	–	+	–	–	–	–	–
Trunk	–	+	–	+	–	–	–	–	–	–	–
Axillae	+	+	–	+	–	+	–	–	–	–	–
Inguinal folds	+	NA	–	+	+	–	–	–	–	–	–
DDD histology	–	–	–	+	+	NA	–	NA	NA	NA	NA
HS	+	+	–	+	+	+	–	–	?	–	–
Axillae	+	+	–	+	+	+	–	–	–	–	–
Inframammary region	NA	–	–	–	+	–	–	–	–	–	–
Inguinal folds	NA	+	–	+	+	–	–	–	–	–	–
Genitals	NA	+	–	–	–	–	–	–	–	–	–
Trunk	NA	+	–	–	–	–	–	–	+	–	–
Sebaceous cyst	NA	+	–	NA	+	–	–	–	+	–	–
Hurley	I	I	–	III	I	I	–	–	Acne conglobata?	–	–
Comorbidities											
Metabolic syndrome	NA	+	NA	–	–	NA	NA	NA	NA	NA	NA
Type 2 diabetes mellitus	+	+	NA	–	–	–	NA	NA	–	NA	NA
Systemic hypertension	+	+	NA	–	–	+	NA	NA	–	NA	NA
Squamous cell carcinoma	–	+	–	–	–	–	NA	NA	–	NA	NA
Pylonidal cyst	NA	+	NA	+	+	+	NA	NA	–	NA	NA
Acne	–	–	NA	–	+	–	NA	NA	+	NA	NA

Note: F denotes female; M, male; NA, information is not available; +, presence of a feature; –, absence of a feature.

even in the absence of predisposing factors such as obesity, present in the adults with HS and DDD of this family, and cigarette smoking. Finally, patient II-2 showed the association of HS, DDD and ulcerated SCC that has been reported in many patients suffering from HS and DDD.[35,36]

Regarding the pathogenic mechanisms, *NCSTN* deficiency has been found to impact keratinocyte differentiation and proliferation, responsible for cyst formation and hyperkeratosis commonly observed in HS patients.[33] Patients with HS and DDD carrying PSENEN mutations were also distinguished by follicular hyperkeratosis,[12] suggesting a potential link between gamma-secretase dysfunction and keratinocyte proliferation. Wild-type mice treated with a gamma-secretase inhibitor corroborate the association between this enzymatic inhibition and epidermal abnormalities.[37] Moreover, since most of the genes mutated in DDD and HS encode for proteins with roles in ligand-dependent NOTCH receptors processing or their post-translational maturation, the common hypothesis is that a decreased NOTCH signalling is the common pathogenic mechanism leading to both HS and DDD. However, NOTCH signalling-deficient mice showed a coat colour dilution and hair greying that resulted from abnormally localized and differentiated melanocyte stem cells and melanoblasts in the hair follicles.[38,39] The association of *NCSTN* with pigmentation defects was studied mainly in zebrafish (ZF). A *Ncstn* LOF mutation induced tyrosinase leakage out of melanosomes causing the necrotic death of melanophores and mis-pigmentation of ZF.[40] Another recent study showed that knock down *Ncstn* larvae had a defect in melanophore migration, shape and number resulting in non-homogenous body pigmentation[41] as already shown for *Psenen* knock down larvae.[12] Noteworthy, in humans, no difference in melanocytes number or tissue distribution were associated with DDD; instead, an atypical biogenesis of melanosomes in melanocytes and their subcellular distribution or retention in basal keratinocytes are key features

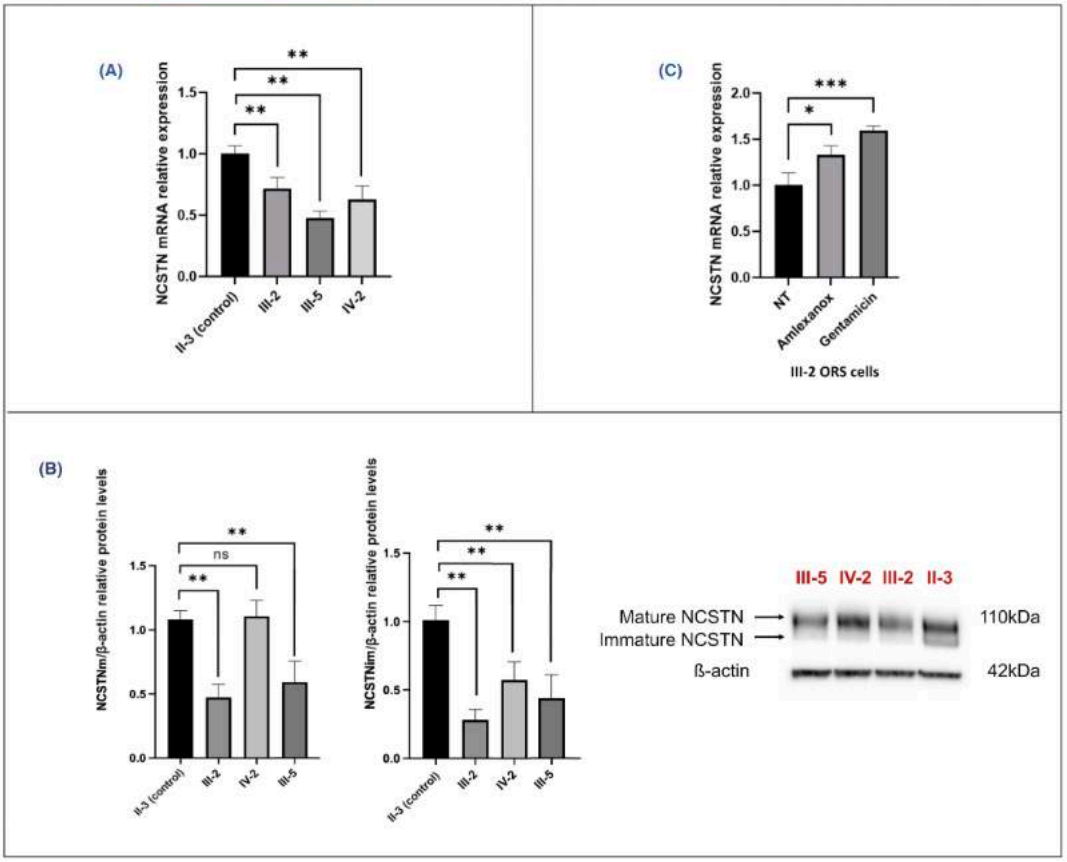

FIGURE 3 Quantification of *NCSTN* mRNA (A) and protein levels (B) present in patients' ORS cells through real-time PCR and western-blot, respectively. Mature (left) and immature (right) NCSTN have been quantified separately. (C) III-2 ORS cells were treated either with Amlexanox (33.4 mM) or Gentamicin A (50 uM). *NCSTN* mRNA levels were then evaluated by qPCR and compared with non-treated (NT) III-2 ORS cells. Expression levels were compared using Student *t*-tests. Bar graphs represent the mean ± standard deviation, $n = 3$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, non-significant.

of the disease.[42,43] Therefore, we can hypothesize that other biological activities of NCSTN may be responsible for DDD in these patients as it is known that NCSTN is localized in melanosome membranes in all their maturation stages[44] and its role in their biogenesis and degradation requires a deeper investigation. We can also conjecture that the deficiency of NCSTN mature form led to the degradation of other gamma-secretase subunits such as PSEN-1, PSEN-2 and PSENEN. Thus, NCSTN deficiency may mimic the effect of *PSENEN* LOF mutations reducing its expression that was recently correlated with increased melanin content in human melanocytes.[45]

Finally, in an attempt to restore NCSTN expression in ORS keratinocytes isolated from the patient with the most severe phenotype, we used two Food and Drug Administration (FDA) approved read-through agents: gentamicin and amlexanox. These 2 compounds have been successfully used to treat severe genodermatoses.[46,47] Gentamicin, in particular, has been shown to reduce infections in HS patients undergoing surgical excision with primary closure without negative side effects.[48]

These findings support the hypothesis that DDD and SCC are complications that may arise in HS patients with mutations in NCSTN and should be paid careful attention by dermatologists during clinical exams. Why some patients with NCSTN mutations do not develop such complications is not yet understood. In our family, the two HS/DDD-affected members analysed by WES harboured a relatively frequent SNV in the KRT5 gene (rs61747180), and we cannot rule out that this polymorphism or other common genetic polymorphisms may account for the co-occurrence of HS and DDD. In this respect, it is intriguing that in the individual IV-2, the NCSTN SNV was associated with a significant decrease of its mRNA but the quantity of the gamma-secretase proteins was comparable to the healthy family member II-3. This indicates that

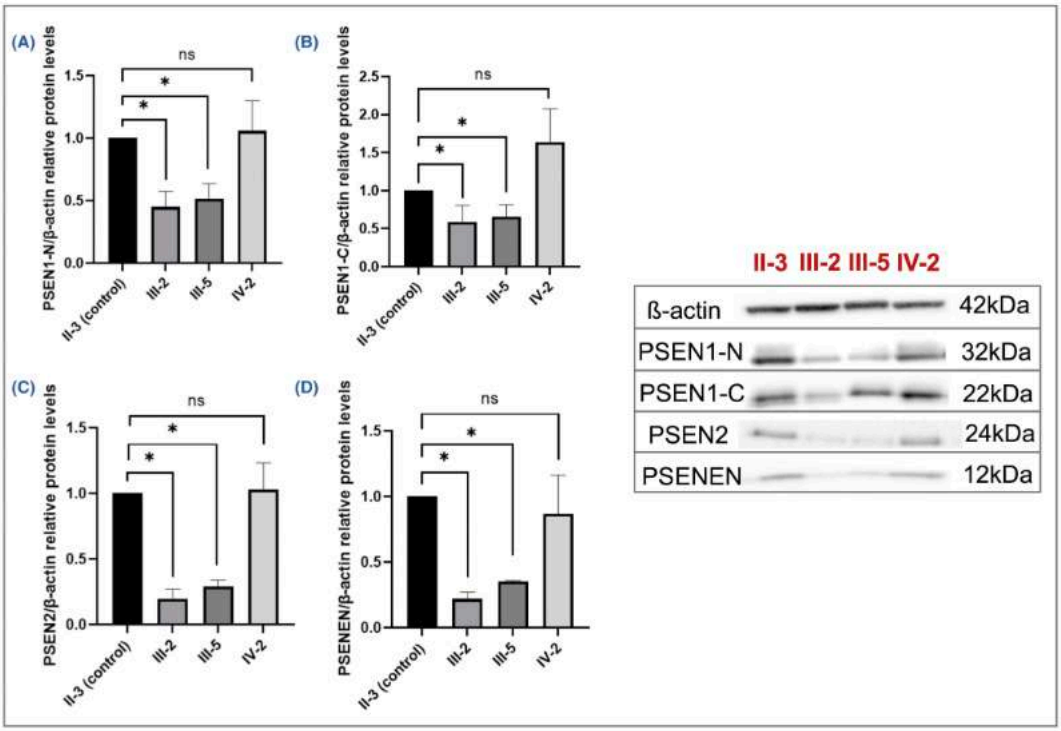

FIGURE 4 (A) Quantification of PSEN1 N-terminal protein; (B) PSEN1 C-terminal protein; (C) PSEN2 protein; (D) presenilin enhancer protein 2 (PEN-2) (encoded by *PSENEN*) levels present in subjects' ORS cells. Expression levels were compared using Student *t*-tests. Bar graphs represent the mean ± standard deviation, $n = 3$. *$p < 0.05$; ns, non-significant.

genetic variants in other biological pathways may affect the quantity of the gamma-secretase subunits, even in the presence of a pathogenic variant.

In conclusion, our study shows the possibilities of genetic and functional analyses for precision medicine in our patients. Herein, *NCSTN* expression in ORS keratinocytes isolated from a severe patient was increased upon treatment with an FDA-approved antibiotic that acts as a readthrough agent. Therefore, to ease the clinical course of the disease, the use of gentamicin should be investigated in individuals bearing this mutation.

AUTHOR CONTRIBUTIONS

Ana Sofia Lima Estevao de Oliveira, Roberta Cardoso de Siqueira, Cécile Nait-Meddour, Michele Boniotto and Lucas André Cavalcanti Brandão designed and performed experiments, analysed the data, and wrote the manuscript. Almerinda Agrelli, Paola Maura Tricarico, Sergio Crovella and Stéphane Jamain reviewed and edited the paper. Stéphane Jamain, Cécile Nait-Meddour and Ronald Moura provided technical support and performed statistical analyses. Paola Maura Tricarico, Maria de Fátima Medeiros Brito, Sergio Crovella and Adamo Pio d'Adamo analysed the data and contributed essential reagents, tools, and funding acquisition. All authors have read and approved the final version of the manuscript. All authors contributed substantially to the conception and the design of the study, data analysis, manuscript writing, and final revision.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are openly available in the Sequence Read Archive (SRA) database, https://www.ncbi.nlm.nih.gov/sra/PRJNA801118. Further enquiries can be directed to the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1 Variants found in genes associated with DDD by WES analysis in the affected patients III-2 and III-4 but absent in the healthy individual II-3.

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APÊNDICE B – Artigo 2: Transcriptome Meta-Analysis Confirms the Hidradenitis Suppurativa Pathogenic Triad: Upregulated Inflammation, Altered Epithelial Organization, and Dysregulated Metabolic Signaling (Fator de impacto 2022 – 4,569)

biomolecules

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Article

Transcriptome Meta-Analysis Confirms the Hidradenitis Suppurativa Pathogenic Triad: Upregulated Inflammation, Altered Epithelial Organization, and Dysregulated Metabolic Signaling

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Abstract: Hidradenitis suppurativa (HS) is an inflammatory skin condition clinically characterized by recurrent painful deep-seated nodules, abscesses, and sinus tracks in areas bearing apocrine glands, such as axillae, breasts, groins, and buttocks. Despite many recent advances, the pathophysiological landscape of HS still demands further clarification. To elucidate HS pathogenesis, we performed a meta-analysis, set analysis, and a variant calling on selected RNA-Sequencing (RNA-Seq) studies on HS skin. Our findings corroborate the HS triad composed of upregulated inflammation, altered epithelial differentiation, and dysregulated metabolism signaling. Upregulation of specific genes, such as *KRT6*, *KRT16*, serpin-family genes, and *SPRR3* confirms the early involvement of hair follicles and the impairment of barrier function in HS lesioned skin. In addition, our results suggest that adipokines could be regarded as biomarkers of HS and metabolic-related disorders. Finally, the RNA-Seq variant calling identified several mutations in HS patients, suggesting potential new HS-related genes associated with the sporadic form of this disease. Overall, this study provides insights into the molecular pathways involved in HS and identifies potential HS-related biomarkers.

Keywords: acne inversa; variant enrichment analysis; OMICs

1. Introduction

Hidradenitis suppurativa (HS), also known as acne inversa, is a chronic inflammatory skin disease involving the pilosebaceous unit, with a worldwide prevalence varying from 0.03% to 4% [1–7]. HS lesioned skin is typically associated with apocrine gland-bearing body areas, such as axillae, breasts, groins, and buttocks [1,2]. HS is clinically characterized by painful and recurrent deep-seated inflammatory nodules and abscesses [3] that, with

disease progression, may evolve into dermal tunnels with malodorous discharge and disfiguring scarring, severely affecting patients' quality of life [4,5].

HS pathogenesis is still poorly understood, although hereditary factors have demonstrated the ability to increase risks associated with the development of the skin condition. [6]. It has been proposed that the primum movens of the disease are represented by keratin follicular plugging progressing to follicle rupture, with subsequent upregulation of the immune system response that leads to an overexpression of pro-inflammatory cytokines/chemokines, as well as several other pro-inflammatory mediators [7–9]. Several genetic changes have been involved in HS pathogenesis, including mutations in the γ-secretase complex genes, both in familial and syndromic forms of HS [10–12]. On the other hand, the γ-secretase gene mutations only occur in roughly 6% of sporadic cases of HS [13]. The γ-secretase complex has the notch signaling pathway as a substrate, which has been shown to alter the keratinocyte differentiation program leading to uncontrolled cell proliferation when downregulated [14]. Moreover, both γ-secretase genes and notch signaling pathway play a crucial role in the development of epidermal cysts and comedones, two phenotypic traits of HS [15]. With several new gene pathogenic variants identified, HS can be considered a multifactorial, polygenic, autoinflammatory condition [12].

In the -omic era, the next-generation sequencing (NGS) approach made major contributions to the study of HS [12,16]. Herein, we performed a meta-analysis of selected RNA sequencing (RNA-Seq) studies, comparing HS lesioned and healthy skin. With this integrated meta-analysis approach, it [17] was possible to combine results from independent studies to increase statistical power and obtain a more precise estimate of differentially expressed genes (DEGs). In addition, we carried out pathway analysis to characterize the DEGs in HS pathogenesis. Lastly, a variant calling was performed allowing the identification of expression quantitative trait loci (eQTL) associated with DEGs. Through these analyses performed we explored HS pathogenesis and created a link between candidate genes and their transcripts, genotypes, and clinical phenotypes.

2. Materials and Methods

2.1. Selection of Publicly Available Studies

We searched Sequence Read Archive (SRA) [18] and Gene Expression Omnibus (GEO) [19] datasets on 11 July 2022, to find studies involving RNA-Seq of HS tissue samples. The search keywords were: ((Hidradenitis Suppurativa) OR (Acne Inversa)) AND ((RNA-Seq) OR (Transcriptome)) [20]. After removing duplicates, we included studies that met the criteria of: experiments carried out in HS patients' skin (*Homo sapiens* organism filter); had matching healthy skin controls; and had raw data (fastq files) available for each sample. Biopsies collected from lesioned skin of HS patients were considered cases, whereas biopsies collected from the skin of patients without HS, or any other skin condition, were accepted as controls.

2.2. RNA-Seq Data Collection, Processing, and Analysis

Figure 1 summarizes the methodology. Data collection, processing, and analysis were conducted as previously described by [21,22]. Briefly, SR Adb package [23] for R software version 4.1.0 [24] was used to download all raw. fastq files. Only the sequencing reads that met the study criteria were downloaded. Then, we reprocessed all reads using the same standard workflow to avoid bias due to heterogeneous pipelines of the original studies. The workflow used Trimmomatic v0.39 [25] to trim Illumina adapters, and to exclude low-quality reads ($Q < 30$) and reads counting shorter than 25 bases (length < 25). Then, the remaining reads were mapped on the National Center for Biotechnology (NCBI) human GRCh38/hg38 reference genome and sorted by coordinates using STAR aligner [26]. Aligned reads (BAM files) were imported into R software and processed with an annotation file from the reference genome with Rsubread package [27]. As a result, a gene counts table was created for each sample. These tables were converted into *DESeq2* package objects,

where we screened for DEGs [28]. Genes with log2(fold change) > 1 and false discovery rate (FDR)-adjusted p-values < 0.05 were acknowledged as statistically significant DEGs.

2.3. Meta-Analysis

We integrated the results produced by the independent groups with a meta-analysis using the RankProd package for R software. The package performs a nonparametric approach based on ranks of fold changes (FC) that detect differentially consistently expressed genes (mDEGs) from independent and replicated experiments. Overall, the meta-analysis approach was based on Lee et al. methodology (2019) [21].

2.4. Pathway Analysis

We performed a pathway enrichment analysis with the mDEGs. We searched the REACTOME database, using ReactomePA package [24] for R software, and PANTHER v17.0 [29,30]. As in the previous analyses, results with FDR-adjusted enrichment test p-value < 0.05 were considered significant.

2.5. RNA-Seq Variant Calling

We performed a variant calling sourcing from the aligned RNA-Seq data. Post-processing was applied using Genome Analysis Toolkit v4.1.2.0 [31] to the aligned reads: duplicates were marked and removed with Mark Duplicates and Split NCigar Reads, and base recalibration was completed with Base Reca librator and Apply BQSR. Variant Calling was performed using Strelka v2.9.10 [32], and single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELs) were separated in two variant calling format (VCF) files using select variants. The resulting variants were removed using Variant Filtration if SNPs had QD < 2.0, FS > 60.0, MQ < 40.0, MQ Rank Sum < −12.5, Read Pos Rank Sum < −8.0 and GQ < 20.0, and if INDELs had QD < 2.0, FS > 200.0, Read Pos Rank Sum < −20.0 and GQ < 20.0. Annotation was performed using Annovar v2019Oct24 [33]. Finally, variants that were present in HS patients were only kept if they had depth (DP > 10) and quality score (QUAL > 30).

To infer the connection between genetic variants and HS pathogenesis, we conducted several approaches. Initially we searched for variants in genes previously associated with HS. Then, we sought variants in new potential HS genes. To unravel the new ones, we selected variants based on some filters detailed as follows. Estimates of HS prevalence vary between 0.03% and 4% among different populations [34,35]. Therefore, it has not been well defined if HS is a rare or a neglected disease [36]. Since rare variants are commonly responsible for disease appearance, herein, for analysis purposes only, we considered HS a rare skin condition (AF $\leq$ 0.01). Thus, potentially deleterious variants found in genes previously associated with HS or with a CADD (phred) score > 15, possessing clinical significance different from benign or likely benign, based on the Clin Var database, and having statistically significant (p-value < 0.05) differences in the genotype were selected as potential HS genetic markers. The median value of the RNA expression level was calculated for each genotype; large differences between the genotypes were considered statistically significant according to the Wilcoxon test.

2.6. Statistics

The Shapiro-Wilk test was used to assess if the counts were normally distributed. To uncover if genotypes were associated with differential RNA expression, we used an appropriate test for normally distributed counts based on the number of observed genotypes (normal and two genotypes: Sztudent's t-test for independent samples; not normal and two genotypes: Wilcoxon-Mann-Whitney test; normal and three genotypes: ANOVA; not normal and three genotypes: Kruskal-Wallis test. In the case of three-genotypes tests, we performed a post-hoc paired Wilcoxon test with FDR correction to identify which genotype was responsible for the overall difference).

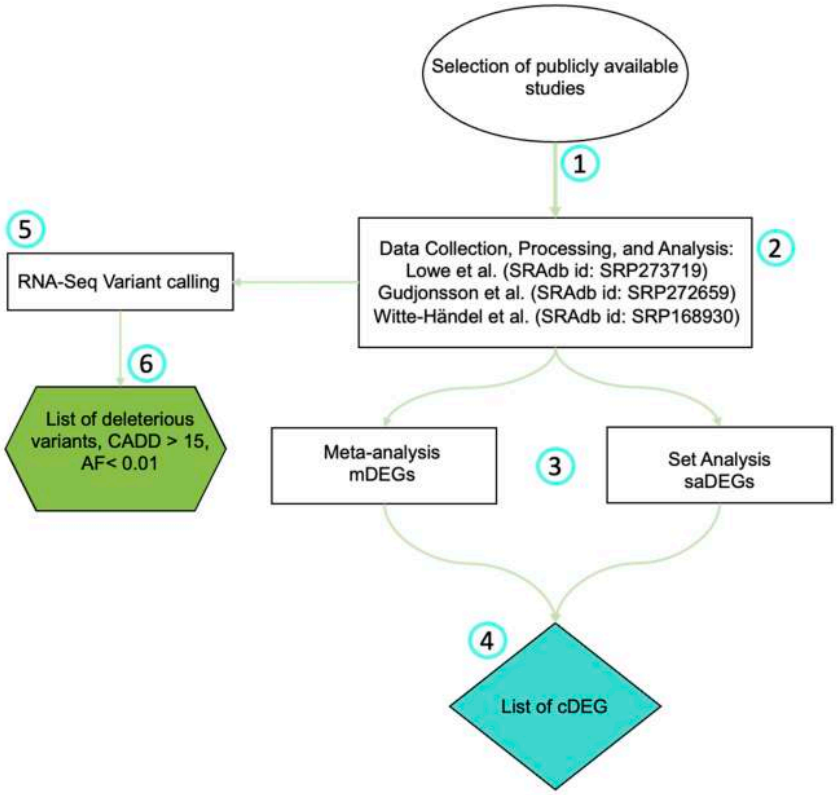

Figure 1. Analysis flowchart: 1: Publicly available studies were selected from the Sequence Read Archive (SRA) and Gene Expression Omnibus (GEO); 2: Three studies passed our selection criteria and were downloaded. SRAdb package for R software version 4.1.0 was used to download all raw. fastq files. Trimmomatic v0.39 was used to trim Illumina adapters and to exclude reads. Resulting gene counts tables were converted into *DESeq2* package objects [37–39]; 3: Lists of DEGs found through the meta-analysis (mDEGs) or the set analysis (saDEGs) (See supplementary data) were created; 4: List of common DEGs (cDEGs) found in both analyses was made (See supplementary data); 5: RNA-Seq variant calling analysis was performed by Strelka v2.9.10; 6: A list of potential HS-related variants originated based on deleterious capacity, allele frequency, and significant genotypic variation based on Wilcoxon Test (p value < 0.5).

3. Results

3.1. Dataset Selection

The search strategy conducted by three authors independently (A.S.L.E.O, G.B., and A.A.) resulted in 25 datasets. Among these, seven studies used microarrays; eight used RNA-Seq; six used quantitative real time polymerase chain reaction (PCR); three used single cell (sc) RNA-Seq; and one used target capture. Since we were searching for RNA-Seq studies only, 17 studies were excluded. Out of the eight RNA-Seq studies, six contained analysis of skin biopsies. Of these six studies, two of them did not have matching healthy skin control, and another analyzed familial HS cases only. Therefore, only three studies were included in our meta-analysis and set analysis. Herein, a total of 51 unique HS lesioned samples, and 30 unique healthy skin samples were considered (Table 1).

Table 1. Detailed information regarding the three selected studies matching our study criteria.

SRA	Title	Samples Included in Our Study	Main Findings
SRP273719	Immunopathogenesis of hidradenitis suppurativa and response to anti–TNF-α therapy	42 samples (HS skin lesion pre-TNF = 19: HS skin lesion mild-moderate HS = 7: healthy skin control = 16)	Highly enriched pathways in HS lesioned skin are immune related. Signatures of complement activation, B cell signaling, and pathways involving phagocytosis were found to be unique to HS. TNF-α–regulated genes, *IFN-γ*, and *IL-1β* were selected as the major drivers of the inflammatory pathways in HS skin lesions. Nonetheless, IL-1 receptor antagonist, *IL-1RN*, and *IL-10RA*, 2 potent immunoregulatory molecules, were relatively reduced in HS skin. Alongside, α-catenin and sirtuin 1, both important for regulation of cell proliferation and survival, were reduced in HS skin.
SRP272659	Contribution of plasma cells and B cells to hidradenitis suppurativa pathogenesis	32 samples (HS skin lesion = 22: healthy skin control = 10)	Several upregulated genes in the skin were associated with B cell responses, including immunoglobulin genes such as *IGLV3-27*, *CD19*, and *CD79a*. Other important genes found were the antimicrobial gene *DEFB4A*; *CXCL13*, a B cell chemoattractant, and the neutrophil chemokine *CXCL1*. In summary, they found B cells, and in particular plasma cells, as a potential therapeutic target in HS.
SRP168930	The IL-1 pathway is hyperactive in hidradenitis suppurativa and contributes to skin infiltration and destruction	7 samples (HS skin lesion = 3: healthy skin control = 4)	*IL-1β* is highly active in HS, contributing to local and systemic inflammation. *IL-1β* induces expression of many molecules involved in extracellular matrix destruction including MMPs, ADAM12, serpinA1, COL3A1, and COL10A1, and immune cell infiltration such as CXCL1, CXCL6, CCL7, CXCL10, CXCL16, CXCL13, CCL24, CCL2, CCL8, and CCL20. *IL-1β* and, therefore, *MMP1*, *MMP3*, *MMP9*, *MMP10*, *CCL2*, *CXCL1*, *IL-6*, *and IL-32* were upregulated when compared with healthy control skin and psoriasis lesions.

In the first study, Lowe et al. (SRAdb id: SRP273719) [37] aimed to uncover genes related to HS immunogenesis and their influence on patients' responsiveness to anti-tumor necrosis factor alpha (TNF-α) therapy. This study was conducted through transcriptome sequencing of lesioned and non-lesioned skin samples before and after anti-TNF-α therapy. Herein, we focused our attention on the RNA-Seq from samples collected before anti-TNF-α therapy. In summary, 26 HS lesioned skin samples and 16 healthy skin controls were included in our analyses.

In the second study, Gudjonsson et al. (SRAdb id: SRP272659) [38] focused on outlining the major dysregulated cell types and inflammatory pathways in HS. In order to evaluate abnormal inflammatory pathways, they performed skin and whole blood RNA-Seq, and scRNA-Seq. In addition, they compared their findings with RNA-Seq data from 28 psoriasis patients and 32 atopic dermatitis patients. In summary, they found 4797 DEGs within the HS skin RNA-Seq compared to healthy controls, and 332 DEGs within HS patients' whole blood. From this study, we incorporated into our analysis 32 skin RNA-Seq samples; 22 from HS lesioned skin and 10 from healthy skin controls.

In the third study, Witte-Händel et al. (SRAdb id: SRP168930) [39] investigated HS skin lesions' cytokine milieu and found *IL-1β* to be highly expressed. First, they quantified the expression levels of approximately 30 mediators in HS lesioned skin and compared the results with the expression patterns from these same mediators in healthy skin control and in psoriasis lesioned skin. After finding *IL-1β* to be upregulated in HS, they compared the results of HS lesioned skin's RNA-Seq with the transcriptome of various cell types isolated from healthy patients' biopsies that were exposed to IL-1β and observed transcriptomic overlapping profiles. RT-qPCR and ELISA analyses were also conducted. Our analysis included samples from three HS patients' lesioned skin biopsy and four healthy individuals skin biopsy not previously exposed with IL-1β.

3.2. Meta-Analisis

We performed a meta-analysis, which resulted in 6445 annotated genes, and 3628 mDEGs with an FDR < 0.05 and | log2(fold change) | > 1. From these mDEGs, 1699 were upregulated and 1929 were downregulated (Table S1). Briefly, upregulated mDEGs that play a role in the maintenance of skin inflammation and structure, namely *DEFB4A/B* [40,41], *TCN1* [9], *MMP1* [42], *S100A7A* [43], and *MMP3* [44] appeared at the top of the overall meta-analysis rank product list. Alongside these mDEGs, other immune-related genes such as *TNF-α*, *IFN-γ*, *IL-1β*, *IL-36*, *IL-17*, *MZB1*, *CD19*, *CD79A*, *CXCL13*, *RETN* [45], and *RARRES2* [46], and skin structure-related genes particularly *PI3* [47], *SERPINB3/4* [48], *SERPINA1* [49], *KRT6/16* [50], and *SPRR1/2/3* [51] were observed. Conversely, the highest positions on the overall rank product list for the down-regulated mDEGs included *THRSP*, *AWAT2*, *DCD*, *UGT3A2*, and *WIF1*, genes associated with energetic metabolism and skin's barrier function [52–56]. Down-regulation of these mDEGs as well as other genes, especially *AQP2/4/5/6/7/8*, *FOXA1*, and *ADIPOQ*, may impair these pathways.

Pathway enrichment analysis of the mDEGs by reactome resulted in 76 statistically significant enriched pathways (Table S2). We then selected the 20 pathways with the lowest FDR values and summarized them in Table 2. Pathways such as keratinization (FDR = 0.0049, gene ratio = 54/1663), extracellular matrix (ECM) organization (FDR = 2.42×10^{-14}, gene ratio 109/1663), neutrophil degranulation (FDR = 5.25×10^{-9}, gene ratio = 132/1663), PD-1 signaling (FDR = 1.5532×10^{-9}, gene ratio = 18/1663), interferon gamma signaling (FDR = 0.00003, gene ratio = 58/1663), signaling by interleukins (FDR = 2.3270×10^{-6}, gene ratio = 118/1663), complement cascade (p value = 0.00003, gene ratio = 25/1663), antimicrobial peptides (p value = 3.476×10^{-6}, gene ratio = 30/1663), cell surface interactions at the vascular wall (p value = 8.1106×10^{-6}, gene ratio = 47/1663), and g-protein coupled receptor (GPCR) ligand binding (p value = 5.2463×10^{-9}, gene ratio = 126/1663) appeared enriched.

In addition, a pathway enrichment analysis by PANTHER database was conducted. Summarily, pathways with the highest percentage of upregulated mDEGs enclosed inflammation mediated by chemokine and cytokine, integrin and interleukin signaling, T and B cell activation, angiogenesis, and apoptosis (Figure S1). Where, pathways containing the highest percentage of down-regulated mDEGs included wnt signaling, angiogenesis, G-protein signaling, gonadotropin-releasing hormone receptor, and cadherin signaling (Figure S2).

We then performed a set analysis to extract DEGs commonly found in all three selected studies (saDEGs), as well as in both analyses (cDEGs). These analyses can be located in the supplementary data.

Table 2. Reactome analysis highlighting the 20 pathways with the lowest FDR, which are associated with upregulated mDEGs.

Pathway Identifier	Pathway Name	Entities Found	FDR
R-HSA-198933	Immunoregulatory interactions between a lymphoid and a non-lymphoid cell	81	7.2839×10^{-31}
R-HSA-1474244	Extracellular matrix organization	109	2.4259×10^{-14}
R-HSA-380108	Chemokine receptors bind chemokines	35	1.4862×10^{-11}
R-HSA-373076	Class A/1 (Rhodopsin-like receptors)	106	2.2223×10^{-11}
R-HSA-202430	Translocation of ZAP-70 to immunological synapse	16	1.2195×10^{-9}
R-HSA-389948	PD-1 signaling	18	1.55×10^{-9}
R-HSA-6798695	Neutrophil degranulation	132	5.2463×10^{-9}
R-HSA-500792	GPCR ligand binding	126	5.25×10^{-9}
R-HSA-909733	Interferon alpha/beta signaling	33	5.2463×10^{-9}
R-HSA-202433	Generation of second messenger molecules	22	5.2463×10^{-9}
R-HSA-202427	Phosphorylation of CD3 and TCR zeta chains	17	5.2463×10^{-9}
R-HSA-1474228	Degradation of the extracellular matrix	55	1.2723×10^{-8}
R-HSA-877300	Interferon gamma signaling	40	3.5328×0^{-8}
R-HSA-1442490	Collagen degradation	32	6.6811×10^{-8}
R-HSA-375276	Peptide ligand-binding receptors	67	7.395×10^{-8}
R-HSA-6809371	Formation of the cornified envelope	50	8.368×10^{-8}
R-HSA-6785807	Interleukin-4 and Interleukin-13 signaling	44	1.1813×10^{-7}
R-HSA-6783783	Interleukin-10 signaling	25	2.22×10^{-7}
R-HSA-1474290	Collagen formation	37	7.0322×10^{-7}
R-HSA-418594	G alpha (i) signaling events	108	1.4245×10^{-6}

3.3. RNA-Seq Variant Calling

We performed the variant calling in the HS lesioned samples and controls. The pipeline identified 323,827 unique variants across all samples. Among them, 190,845 variants were annotated as known SNPs and Indels.

After filtering, a total of 73,134 genetic variants present in 12,665 genes were found only in patients, and thus selected as candidate variants for HS pathogenesis. Up to 39,709 novel variants not present on dbSNP and Gnomad were found and marked by an asterisk at the end of the information regarding amino acids change and reference gene at the column "AAChange.refGene". These results are summarized in Table S3. Briefly, 63,235 single nucleotide variants (SNVs) and 9899 insertion/deletion variants (Indels) were detected across the patients' transcriptomes. They were distributed as follows: 39,577 in 3′-UTR, 3577 in 5′-UTR, 2325 in splicing sites, 25,577 exonic, 22 non-coding RNA splicing, and 2056 non-coding RNA exonic. Among the exonic variants present only in patients, 11,890 were synonymous, 12,266 were nonsynonymous, 35 were start-loss, 140 were stop gain, 203 were non-frameshift deletions, 205 were non-frameshift insertions, 120 were frameshift deletions, and 651 were frameshift insertions.

The variant calling analysis identified 34 variants in genes of the γ-secretase complex. This complex, composed of 4 genes (presenilins (*PSEN*), nicastrin (*NCSTN*), anterior pharynx defective 1 (*APH1*), and presenilin enhancer 2 (*PSENEN*)), modulates NOTCH pathway by cleaving and releasing NOTCH's intracellular domain [14], thus, being important for skin and immune response's homeostasis and HS pathogenesis [57,58]. The 34 mutations were distributed as follows: 12 in the *NCSTN* gene, 3 in the *APH1A*, 6 in the *APH1B*, 8 in

the *PSEN1*, 3 in the *PSEN2*, and 2 in the *PSENEN*. One variant was already reported in HS patients: a stop gain mutation NM_172341.4: c.168T>G (p.Tyr56X) of the *PSENEN* gene previously associated with concomitant HS and Dowling Degos Disease (DDD), a rare genodermatosis, classically characterized by acquired reticular hyperpigmentation in flexural sites [59]. Another non-synonymous variant NM_000243.2:c.2177T>C (p.Val726Ala) in the *MEFV* gene, previously associated with HS, has also been spotted [60]. In Table 3 we summarized these two mutations, along with other variants found in coding regions of genes already linked with HS [60–62].

Apart from variants in genes already considered related to the disease, a second table containing potential HS new genetic markers was created (Table 4). Overall, variants were observed in genes associated with cellular metabolism, immune response, and skin homeostasis. For instance, *ACSF3* plays a crucial role in fatty acid synthesis [63], and its dysregulation may affect the ability of the body to properly process certain proteins and lipids. In conjunction, we also observed mutations in genes, such as *BTN2A1*, *FNIP2*, *AKR1C3*, *ALDH6A1*, *YTHDF1*, and *GALNT7* that are similarly involved in the metabolism of lipids, proteins, fatty-acids, and sterols. Variants associated with immune response were spotted in genes such as *GSDMD*, *SIT1*, *WDR92*, and *GPANK1*. *GSDMD*, a gene that encodes protein Gasdermin D, is a positive regulator of *IL-1β* involved in pyroptosis cell death [64] and was found upregulated by our meta-analysis. SIT1 is involved in the regulation of T cell activation [65], while *WDR92* is involved in apoptosis via activation of caspase-3 [66]. Variants that impair apoptosis of keratinocytes may be associated with the hyperkeratosis responsible for hair follicle occlusion [67]. In the context of skin regulation, variants were detected in genes such as *KLF4*, *FLOT2*, *PHACTR4*, and *CORO1B*. *KLF4* is required for the normal development of the barrier function of skin. Moreover, KLF4 is also part of the NOTCH super pathway. *FLOT2* may act as a scaffolding protein and may be involved in epidermal adhesion, structure, and function, while *PHACTR4* and *CORO1B* behave as actin-binding proteins. Thus, variants in these genes could explain dysfunction of the skin barrier and structure. Finally, *MYOF* plays a role in the cell membrane repair mechanism of endothelial cells that permits rapid resealing of membranes disrupted by mechanical stress [68]. A decreased number of circulating progenitor endothelial cells and endothelial dysfunction in HS has been previously described [69,70].

The variants found by the variant calling corroborated abnormal pathways found by the meta-analysis. Therefore, loss or gain of function of these genes should be closely investigated to better understand their influence in the HS pathogenesis.

Table 3. Distribution of potential pathogenic variants with AF ≤ 0.01 among genes that have been already associated with HS.

Gene	DEGs	SNP ID	Ref	Alt	Distribution of Genotypes among HS Patients	Wilcoxon Test	Exonic Function	HGVS	AF
NCSTN	-	rs35603924	G	C	GG (50)/GC (1)	0.1087	nonsynonymous SNV	c.G231C:p.E77D	0.00432
APH1A	-	rs996158631	A	T	AA (50)/AT (1)	0.5630	nonsynonymous SNV	c.T123A:p.D41E	0.00010
APH1B	-	rs142676640	C	T	CC (50)/CT (1)	0.2229	nonsynonymous SNV	c.C640T:p.R214	0.000699
PSEN2	-	rs143912759	C	A	CC (50)/CA (1)	0.4288	nonsynonymous SNV	c.C1139A:p.T380K	0.00026
PSEN1	-	rs1174374799	-	T	−(50)/−T (1)	0.4542	frameshift insertion	c.526dupT:p.S178Ffs * 10	0.000006573
PSENEN	-	rs751542345	T	G	TT (50)/TG (1)	0.1519	stopgain	c.T168G:p.Y56X	0.000008
FGFR2	-	rs56226109	G	A	GG (49)/GA (2)	0.2413	nonsynonymous SNV	c.C170T:p.S57L	0.003722
MEFV	up	rs28940579	A	G	AA (50)/AG (1)	0.0215	nonsynonymous SNV	c.T2177C:p.V726A	0.001440
MEFV	up	rs104895094	T	C	TT (50)/TC (1)	0.0996	nonsynonymous SNV	c.A2084G:p.K695R	0.005245
NOD2	-	rs104895452	C	A	CC (50)/CA (1)	0.9148	nonsynonymous SNV	c.C2672A:p.A891D	0.000707
NOD2	-	rs5743279	G	A	GG (50)/GA (1)	0.1775	nonsynonymous SNV	c.G2288A:p.R763Q	0.001217
NOD2	-	rs5743272	A	G	AA (49)/AG (2)	0.0699	nonsynonymous SNV	c.A974G:p.H325R	0.000392
NOD2	-	rs35285618	G	A	GG (50)/GA (1)	0.2845	nonsynonymous SNV	c.G2042A:p.R681H	0.00198
NOD2	-	rs2066847	-	C	−(50)/−C (1)	0.6377	frameshift insertion	c.2936dupC:p.L980Pfs * 2	0.015002
NOD2	-	rs34684955	G	A	GG (50)/GA (1)	0.2845	nonsynonymous SNV	c.G337A:p.A113T	0.00251
NOD2	-	rs5743278	C	G	CC (50)/CG (1)	0.1038	nonsynonymous SNV	c.C2093G:p.A698G	0.00371
NOD2	-	rs576658764	C	T	CC (50)/CT (1)	0.2845	nonsynonymous SNV	c.C1540T:p.R514W	0.00007
PSTPIP1	up	rs34240327	G	C	GG (49)/GC (2)	0.4935	nonsynonymous SNV	c.G773C:p.G258A	0.00461

Ref = reference; Alt = altered; HGVS = Human Genome Variation Society; AF = allelic frequency.

Table 4. Potential Clin Var pathogenic non-synonymous variants found by the RNA-Seq variant calling analysis in HS patients with an allele frequency ≤ 0.01, CADD score > 15, and genotypic differences that were statistically significant (p value < 0.05).

Genes	DEGs	SNP ID	Ref	Alt	Distribution of Genotypes among HS Patients	Wilcoxon Test	HGVS	AF	CADD Score
ACSF3	-	rs144681140	G	A	GG (49)/GA (2)	0.0295	c.G1406A:p.R469Q	0.0032	22.9
KLF4	-	rs139237114	G	A	GG (49)/GA (2)	0.0385	c.C859T:p.H287Y	0.0016	23.8
DUSP23	-	rs11544443	A	T	AA (49)/AT (2)	0.0475	c.A371T:p.E124V	0.0022	25.1
BTN2A1	-	rs143104579	G	A	GG (48)/GA (3)	0.045	c.G188A:p.R63H	0.0096	18.01
FLOT2	-	rs3736238	C	T	CC (49)/CT (2)	0.0284	c.G982A:p.A328T	0.0119	18.44
GPANK1	-	rs35265780	G	A	GG (49)/GA (2)	0.04	c.C335T:p.A112V	0.0096	32
FNIP2	-	rs62001914	C	A	CC (49)/CA (2)	0.0476	c.C1653A:p.S551R	0.0092	24.8
CORO1B	-	rs145707942	C	G	CC (49)/CG (2)	0.0462	c.G367C:p.E123Q	0.0002	24.2
ADCY4	-	rs61745073	T	A	TT (49)/TA (2)	0.0357	c.A1358T:p.E453V	0.0022	22.7
AKR1C3	down	rs34186955	C	T	CC (49)/CT (2)	0.0295	c.C538T:p.P180S	0.0086	23.3
ALDH6A1	down	rs139579994	G	A	GG (49)/GA (2)	0.0395	c.C716T:p.P239L	0.0018	27.9
GSDMD	up	rs62000416	C	A	CC (49)/CA (2)	0.0315	c.C556A:p.L186M	0.0056	23.4
YTHDF1	-	rs141487890	G	A	GG (49)/GA (2)	0.0344	c.C437T:p.A146V	0.0008	24
MYOF	-	rs61861290	G	A	GG (48)/GA (3)	0.0207	c.C4576T:p.P1526S	0.0062	26.2
SIT1	up	rs138786883	C	A	CC (49)/CA (2)	0.0496	c.G520T:p.A174S	0.0032	17.54
RBMXL1	-	rs139713926	T	C	TT (49)/TC (2)	0.0242	c.A701G:p.Y234C	0.0022	25.5
GALNT7	-	rs144873913	C	A	CC (48)/CA (2)	0.0496	c.C1585A:p.P529T	0.0014	30
PHACTR4	-	rs72661785	G	C	GG (49)/GC (2)	0.043	c.G1609C:p.A537P	0.0036	26.3
WDR92	-	rs138784630	C	T	CC (49)CT (2)	0.04	c.G841A:p.A281T	0.0072	23.4

Ref = reference; Alt = altered; HGVS = Human Genome Variation Society sequence variant nomenclature; AF = allelic frequency; CADD score = mutation impact prediction.

4. Discussion

Here, we operated an RNA-Seq meta-analysis to explore HS lesioned skin transcription profiles among three independent studies. These studies exploited HS immunopathogenesis, yet the re-evaluation of these transcriptomes resulted in more than immunological regulation, they highlighted other important DEGs in the context of skin homeostasis and energetic metabolism. The main findings are summarized in Figure 2, which briefly represents the three main outcomes of the meta-analysis at the molecular level: immune dysregulation, skin homeostasis perturbation, and abnormal metabolic signaling. In addition, we performed a variant calling from RNA-Seq data that may potentially contribute to novel insights for HS pathogenesis.

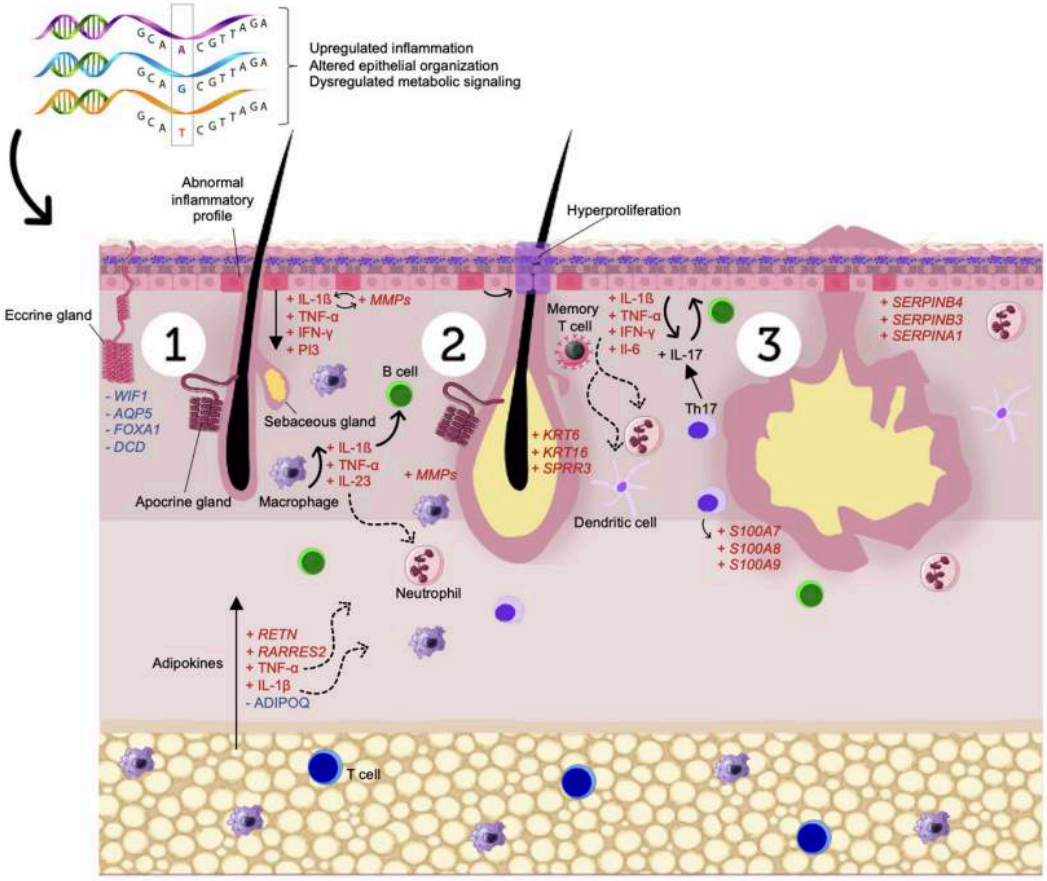

Figure 2. Representation of the HS pathogenesis based on the three main outcomes found by the meta-analysis: upregulated inflammation, altered epithelial organization, and dysregulated metabolic signaling; 1: It is believed that keratinocytes may have an abnormal inflammatory profile in HS lesions. These keratinocytes expressing cytokines such as TNF-α, IL-1β, IFN-γ, alongside pro-inflammatory adipokines, may induce infundibular hyperkeratosis and subsequent perifollicular immune cell infiltration; 2: Hyperkeratosis occludes the hair follicle, resulting in follicular hyperplasia and nodule formation demonstrated by upregulation of *KRT6-KRT16* and *SPRR3*; 3: Non-stop follicular dilatation leads to follicle rupture and impairment of the skin barrier function hinted at due to the upregulation of serpins-family genes, exacerbating inflammation. Recurrent injured nodules may evolve into dermal tunnels with impaired wound healing capacity, a consequence of abnormal eccrine gland function suggested by downregulation of *WIF1*, *AQP5*, *FOXA1*, and *DCD*.

4.1. Immune Dysregulation

The importance of immunological pathways in HS is well accepted and their dysregulation may predispose the development of a strong initial inflammatory reaction commonly seen in HS patients [9]. Here, we provide evidence that many immune-associated DEGs are present in HS lesioned skin and absent in healthy control skin. These DEGs are crucial for commonly HS-associated enriched pathways, namely neutrophil degranulation, signaling by interleukins, complement cascade, immunoregulatory interactions between a lymphoid and a non-lymphoid cell, antimicrobial peptides, and interferon signaling.

Overall, our results were in agreement with the three studies used in our analyses. Lowe et al. [37], when comparing lesioned skin to healthy control, identified *IFN-γ* and *IL-1β* as highly upregulated DEGs. These genes were found to be more than 4 times overexpressed in the meta-analysis. Our results suggested that upregulation of both proinflammatory molecules was essential to local inflammation in the skin condition. In HS lesions, IFN-γ may function as the primary activator of macrophages, which were the most numerous immune cells in the inflammatory infiltrates [71]. In normal conditions, the recruitment of macrophages was crucial not only for the immune response but also for the removal of cellular debris, the promotion of healing, and reorganization of areas within inflammation [72]. However, dysregulated activation and proliferation of macrophages may contribute to an elevated secretion of pro-inflammatory cytokines, such as IL-1β [71]. Upregulation of *IL-1β* exacerbates the inflammation contributing to the recruitment of other immune cells to the lesion, and to the pus formation seen in HS abscess [73]. Indeed, interferon alpha/beta/gamma signaling, as well as neutrophil degranulation pathway appeared enriched by the reactome. Witte-Händel et al. [39], who also found *IL-1β* highly expressed in HS lesions, postulates that its pathways-related are of paramount importance for HS phenotype. Corroborating this theory, a potential gain-of-function mutation in a positive regulator of *IL-1β* (*GSDMD*), was found by the variant calling, and upregulated expression of important molecules for the IL-1 signaling, as well as genes that stimulate or are stimulated by the expression of this cytokine were found in the mDEGs.

Gudjonsson et al. [38] obtained similar results for *IFN-γ*, but not for *IL-1β*. Instead, they found upregulation of *IL36A* and *IL36G* genes. Still, these genes have been recently reported as agonistic cytokines in the interleukin IL-1 superfamily through the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) [74]. *IL-36A* and *IL-36G* were also found upregulated in the meta-analysis. Hessam S. et al. [74] provide evidence for a distinctive IL-36 pro-inflammatory role in the development of an inflammatory loop commonly seen in HS phenotypes. Besides, IL-36 was also found to induce HS like acanthosis and hyperkeratosis in transgenic mice [75].

In our analyses, we found the upregulation of *IL-17A* and *IL-17F*. In HS, the release of follicular debris in the dermis results in the activation of an immune response mediated by Th17 [76]. IL-17 induces proinflammatory cytokine expression by keratinocytes, contributing to immune cell infiltration by neutrophils, dendritic cells, and memory T cells to the lesioned area [77]. However, Kelly et al. proposed that Th17 cells were present in HS skin prior to the formation of an active lesion, indicating that they could participate in lesion development. Indeed, genes that are influenced by *IL-17*, such as *DEFB4A/B*, *MMP1*, and *MMP9* were also found upregulated, and had the highest-ranking product positions in the meta-analysis. Other skin genes, namely psoriasin (*S100A7*) and calprotectin (*S100A8/9*), whose expression augmented in the presence of IL-17, were also found upregulated in the meta-analysis. Since keratinocytes are believed to have an inflammatory profile in HS skin [78], these findings suggested that vicious interactions between keratinocytes and TH17/IL-17 contributed more to chronic inflammation than to enhancing a tissue protective response.

Enhanced B cell signatures were found by Lowe et al. [37] and Gudjonsson et al. [38] when they compared HS lesioned skin with the healthy control and other skin conditions, such as psoriasis. Although Witte-Händel et al. [39] did not explore B cell signaling, they mentioned finding upregulation of *CXCL13*, a B cell chemoattractant [79]. In our analyses,

several genes associated with B cells, such as *MZB1*, *CD19*, CD79A, and CXCL13 were found to be expressed 10 times more in HS lesions when compared to controls. This may suggest that B cell alteration was a common event for HS development. B cells are important for HS pathogenesis since they contribute to fibrosis, stromal remodeling, and therapeutic responses [80]. The latter still remaining controversial; reports using rituximab, a monoclonal antibody that depletes B cells from the circulation targeting CD20 molecules, has shown to ameliorate HS phenotype [81] in some HS patients, but to induce HS-like lesions appearance in others [82]. When we analyzed the upregulated mDEGs in the PANTHER gene analysis tool, the B cell activation (P00010) pathway was enriched with 18 genes, suggesting an important role of B cells for HS lesions maintenance.

4.2. Skin Homeostasis

The main enriched pathways associated with skin regulation were ECM organization, keratinization, formation of the cornified envelope, epidermal cell differentiation, and collagen formation and degradation. Moreover, several variants associated with disruption of these pathways were identified, suggesting potential HS-related genes. Zouboulis C.C. et al. [9] demonstrated that the inflammatory process in HS was linked with abnormalities in these signaling routes. Our results confirmed these connections. For instance, elafin (*PI3*), an elastase-specific inhibitor that acted as an antimicrobial peptide and was expressed by epithelial and certain immune cells, appeared eight times more expressed. Besides acting against gram-positive and gram-negative bacteria, fungal pathogens, and being involved in NF-κB pathway modulation, cytokine secretion and cell recruitment [83], the gene encoding elafin has also been correlated to abnormal epithelial differentiation in a context of hyperproliferation [47,84]. Additionally, we found dysregulation in some genes of the serine protease inhibitor superfamily, serpin. Among them, *SERPINB4*, *SERPINB3*, and *SERPINA1* stood out in our ranking product, corroborating the involvement of the hair follicle unit [85]. Serpins overexpression has been previously spotted at the internal and epithelial root sheath of hair follicles in HS patients [9]. In general, serpins are associated with epidermal barrier homeostasis, and chronic skin inflammation [48]. These roles have been corroborated in atopic dermatitis and psoriasis [49,86], two inflammatory skin diseases that overlap molecular pathways with HS [87,88]. Thus, upregulation of *PI3* and *SERPINs* may be associated with keratinocyte hyperproliferation responsible for hair follicle plugin, a hallmark of HS.

Additionally, several cytokeratin appeared dysregulated, which may affect keratinization and formation of the cornified envelope. *KRT6* and *KRT16* are particularly interesting since they are expressed together in the outer root sheath of the hair follicles under stressful conditions [50]. In contrast, under homeostatic conditions, *KRT6-KRT16* are co-expressed to respond to barrier breach, stimulating hyperproliferation of interfollicular keratinocytes and modulating the inflammatory response to wounds [89]. Dysregulation of these KRTs have been previously assigned as psoriasis biomarkers [90,91]. In parallel, dysregulation of other mDEGs, such as *SPRR3*, and several subtypes of *SPRR2* and *SPRR1* also provided evidence of impairment of barrier functions in HS. SPRRs proteins play a structural role in the cornified envelope, and SPRR3 is generally not detected in normal skin [92]. It had been suggested that an altered expression of *SPRR3* could impact barrier function through altered production of cornified envelope scaffold that impaired the supramolecular organization of lamellar body-derived lipids into normal bilayer structures [93,94]. This type of dysregulation may be associated with thinning of the cornified envelope [95]. Thus, upregulation of these genes may be linked to the impaired structure and fragility of the cornified envelope and follicular infundibulum that easily break and start an immunological reaction.

Equally important to hair follicle fragility is the family of matrix metalloproteinases (MMPs), a zinc-dependent extracellular protease that break down and remodel the ECM [96]. MMPs play an important role in HS pathogenesis because it is involved in inflammation, sinus formation, and in the thinning of the basement membrane surrounding the hair follicle unit [97]. Interestingly, MMPs are also known to play a critical role in the conver-

sion of IL-1β into its active form [98], which respond by enhancing MMP expression [99], potentially creating a long-lasting inflammatory response in HS lesioned skin. Here, we found up to 12 types of MMPs upregulated, of which *MMP1* and *MMP3* composed some of the highest positions in the meta-analysis overall ranking, suggesting that skin intrinsic components may represent a critical step in disease progression.

Finally, sweat glands are believed to regulate epidermal homeostasis and wound repair [100,101]. Genes that are relevant for a proper sweat-gland function namely *WIF1*, *AQP5*, *FOXA1*, and *DCD* were found highly decreased in HS skin by our meta-analysis. Respectively, the genes appeared approximately 10, 2, 6, and 24 times less expressed when compared to healthy skin. In normal conditions, sweat glands' multipotent progenitor cells contribute to repair and skin re-epithelialization [100,101]. Thus, down-regulation of these genes may affect the capacity of skin repair, contributing to the clinical HS phenotype of non-healing wound-like environment. Similar results were found by Coates et al. (2019) [102], corroborating the possibility of impaired sweat gland function contributing to HS pathogenesis. Moreover, alongside *AQP5*, several other aquaporin-related genes such as *AQP7*, *AQP6*, *AQP8*, *AQP2*, *AQP4*, *AQP7P3*, *AQP7P1*, and *AQP4-AS1* were also found down-regulated in the mDEGs. Aquaporins are membrane channel proteins that through the bidirectional transport of water, glycerol, and small solutes across the membrane, serve as critical players of the skin barrier [103]. Besides their essential role for correct skin barrier activity, AQPs, mainly AQP7, also operated in the modulation of skin's inflammatory responses [104]. In this context, the downregulation of several aquaporins may be associated with the impaired skin barrier function evidenced by our analyses and commonly seen in inflammatory skin diseases, as well as atypical skin immune surveillance.

4.3. Energy Metabolism

Metabolic dysregulation in HS was detected through several downregulated DEGs and pathways including the metabolism of lipids, triglyceride and fatty acid, and glucocorticoid biosynthesis. Additionally, variants found by the RNA variant calling may be associated with several metabolic-related disorders, including metabolic syndrome (MetS). MetS concomitant with HS had already been described [105–107]. Nevertheless, it is not clear if inflammation induced by MetS leads to the initiation of HS or if the systemic inflammation in HS leads to manifestations of MetS [106]. MetS is characterized by a combination of clinical conditions including central obesity, hyperglycemia, dyslipidemia, and/or hypertension [108,109]. Although not necessarily present, central obesity plays a critical role in MetS' development [109], and phenotype severity in HS [110,111]. In fact, at the molecular level, metabolic disorders and obesity may be considered a primary risk factor in HS [112].

Body mass index (BMI) data was available for two studies. The data confirmed that most of the patients had a BMI > 25. Obesity is supposed to favor HS skin alteration in a few manners [113]. From the molecular point of view, obesity induces low levels of systemic inflammation and metabolic changes [97]. Inflammatory immune cells including M1-type macrophages and T cells infiltrate the hypertrophic and damaged adipose tissue, producing inflammatory cytokines and inducing a dysregulated pattern of soluble mediators called adipokines [114]. Adipokines seem to drive metabolic alterations at the same time it represents a mechanistic link in the interaction between skin and metabolic comorbidities [115]. They are regularly expressed by several skin cells, such as keratinocytes, melanocytes, sebocytes, and fibroblasts, and their abnormal expression has been associated with various inflammatory skin conditions [116]. A few types of adipokines have been described and can have good and bad properties regarding the skin and body's homeostasis [116]. The "bad" adipokines include resistin (RETN), chemerin (RARRES2), and classical pro-inflammatory cytokines, such as TNF-α, and IL-1β [115]. These metabolic villains were found upregulated in our meta-analysis. Besides their role in skin inflammation with immune cell tissue infiltration and cell dysfunction, they also drive insulin resistance, disturbance of glucose and lipid metabolism, and vascular dysfunction [117–120]. The dysregulation of these

pathways was corroborated by our findings, suggesting concomitant HS and metabolic-related disorders in the patients. On the other hand, "good" adipokines such as adiponectin (ADIPOQ) that have properties, such as control of fat metabolism and insulin sensitivity, with direct anti-diabetic, anti-atherogenic, and anti-inflammatory activities [117,121–123], were found ten times less expressed in HS lesions. This may represent an association between the downregulation of *ADIPOQ* and susceptibility of HS and metabolic comorbidities. The exploration of adipokines' role in skin and cellular metabolism is still in an early phase.

5. Limitations

Not all the studies provided clinical information of the patients, such as weight, smoking history, and race, nor general information about lifestyle, comorbidities, and different HS phenotypes. Therefore, clinical information was not taken into consideration in our analysis. Still, independent studies provided similar results, confirming the importance of the DEGs found here for HS pathogenesis. Moreover, whole exome or genome sequencing data from the same samples were not available. Thus, we could not confirm the results found by our variant calling, nor could we confirm them by PCR or sanger sequencing. However, known HS-related variants were identified substantiating our findings. Ultimately, our study design involves re-analyzing previously published transcriptomic data and aggregating evidence of transcriptional changes, thus we were not able to confirm our results on the translational level. Notwithstanding, we hope our results and conclusions stimulate new functional studies that focus on confirming our findings at the protein/tissue level. In general, our results are in compliance with what is reported in the literature.

6. Conclusions

Herein, we confirm the HS pathogenic triad composed by upregulated inflammation, altered epithelial differentiation, and dysregulated metabolism signaling. The variant calling and meta-analysis verify this highly interconnected network. The upregulation of *PI3*, *TNF-α*, *IL-1β*, and *IFN-γ* suggest that the inflammatory process in HS may be linked with keratinocyte hyperproliferation. Moreover, the upregulation of *KRT6*, *KRT16*, serpin-family genes, and *SPRR3* alongside downregulation of aquaporin-family genes confirm the involvement of hair follicles and the impairment of barrier function in early phases of HS pathogenesis. Finally, our results demonstrate that HS and metabolic-related syndromes such as MetS and obesity may share transcription profiles and suggest that adipokines may be potential biomarkers for this interaction; down-regulation of *ADIPOQ* may be associated with diseases' co-occurrence susceptibility. Several mutations associated with the HS pathogenic triad were found, highlighting potentially new HS-related genes associated with the sporadic form of this disease.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biom12101371/s1, Figure S1: Percent of upregulated gene hits found in the mDEGs against total pathway hits, p-value < 0.05; Figure S2: Percent of down-regulated gene hits found in the mDEGs against total pathway hits, p-value < 0.05; Figure S3: Venn Diagram analysis representing saDEGs found in the individual studies; (a) saDEGs of the included studies; (b) upregulated saDEGs; (c) downregulated saDEGs. Figure S4: Percent of upregulated gene hits against total pathway hits; Table S1: Total mDEGs; Table S2: Reactome enriched pathways analysis associated with mDEGs (FDR < 0.05); Table S3: Variants found in patients only by the RNA-Seq variant calling; Table S4: Enriched pathways associated with saDEGs, and therefore, cDEGs, filtered by FDR < 0.05 found in the set analysis. Among the upregulated cDEGs, 91 genes had major roles in immunological pathways. For instance, 34 genes, such as *CYBB* (p-value = 1.758×10^{-20}, logfc = 3.1303), *CD3E* (p value = 2.421×10^{-15}, logfc = 2.6583), *CD8A* (p value = 3.734, logfc = 2.2508), and *CD19* (p value = 2.485×10^{-30}, logfc = 5.5543) were associated with adaptive immune response and their cellular signaling networks, while 44 genes, including Matrix Metallopeptidases (MMPs) family genes, *DEFB4* (p value = 2.278×10^{-58}, logfc = 7.6490), *TLR8* (p value = 3.003×10^{-19}, logfc = 2.8510), and *BIN2* (p value = 2.563×10^{-13}, logfc = 2.4414), were linked with innate immune system. In other important pathways, such as extracellular matrix organiza-

tion, 23 genes such as *ADAM12* (p value = 1.492×10^{-22}, logfc = 4.1200), *ADAMTS2* (p value = 2.6091, logfc = 1.111×10^{-14}), and *COL3A1* (p value = 1.433×10^{-17}, logfc = 2.7178), were found. On the other hand, down-regulated cDEGs, such as *FA2H* (p value = 5.394×10^{-20}, logfc = −3.7949), *FADS2* (p value =1.073×10^{-24}, logfc = −4.61), *GUCY2EP* (p value = 5.673, logfc = −1.9362), *HSD3B1* (p value = 4.861×10^{-30}, logfc = −5.2634), *IYD* (p value = 1.337×10^{-20}, logfc = −2.4972), *MOGAT2* (p value = 1.59×10^{-23}, logfc = −3.7538), *MPPED1* (p value = 1.519×10^{-25}, logfc = −3.1882), *PNPLA5* (p value = 3.314×10^{-29}, logfc = −4.2126), *SOX9-AS1* (p value = 8.702×10^{-17}, logfc = −2.3383), and *THRSP* (p value = 4.101×10^{-48}, logfc = −5.4498) play a crucial role in metabolic pathways. In particular, *FA2H*, *FADS2*, *MOGAT2*, *HSD3B1*, *THRSP*, and *PNPLA5* are important for the metabolism of lipids while *IYD* participates in thyroxine biosynthesis and metabolism of amine-derived hormones.

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APÊNDICE C – Artigo 3: Pleiotropic Role of Notch Signaling in Human Skin Diseases (Fator de impacto 2020 – 5,523)

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Review

Pleiotropic Role of Notch Signaling in Human Skin Diseases

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Abstract: Notch signaling orchestrates the regulation of cell proliferation, differentiation, migration and apoptosis of epidermal cells by strictly interacting with other cellular pathways. Any disruption of Notch signaling, either due to direct mutations or to an aberrant regulation of genes involved in the signaling route, might lead to both hyper- or hypo-activation of Notch signaling molecules and of target genes, ultimately inducing the onset of skin diseases. The mechanisms through which Notch contributes to the pathogenesis of skin diseases are multiple and still not fully understood. So far, Notch signaling alterations have been reported for five human skin diseases, suggesting the involvement of Notch in their pathogenesis: Hidradenitis Suppurativa, Dowling Degos Disease, Adams–Oliver Syndrome, Psoriasis and Atopic Dermatitis. In this review, we aim at describing the role of Notch signaling in the skin, particularly focusing on the principal consequences associated with its alterations in these five human skin diseases, in order to reorganize the current knowledge and to identify potential cellular mechanisms in common between these pathologies.

Keywords: Notch pathway; skin disorder; proliferation; differentiation

1. Introduction

Notch signaling is a ubiquitous and evolutionarily conserved intracellular pathway involved in the regulation of many diverse cellular functions and is implied in mediating responses that might be significantly variable and strictly linked to the activation context and the cellular type [1]. Despite the fact that the Notch pathway operates primarily in the regulation of crucial cellular processes, including proliferation, differentiation, migration, cellular fate and death, both during normal development and pathological conditions, it possesses a relatively simple signal transduction route [2].

Canonical Notch signaling occurs through a cell-to-cell communication in which transmembrane receptors are activated by transmembrane ligands located on the adjacent cell; subsequently, a few molecular components are involved in the transmission of signals from the cellular surface to the transcriptional machinery [3].

Nevertheless, other mechanisms of Notch signaling have been disclosed and are known to occur in a ligand- or transcription-independent manner, generally referred to as non-canonical Notch signaling [4].

To date, a deep characterization of Notch transduction is quite appealing since a progressively expanding spectrum of human diseases has been found to be associated with alterations in Notch signaling [3]. These variations are either due to aberrant regulation or direct mutations leading to both hyper- or hypo-activation of the transcription of target genes or of the core components of the Notch signaling route [3].

In humans, up to 40 different proteins (45 genes) are reported to be involved in Notch signaling [5]. In this review, we will describe the core components, key steps and principal modulators of Notch transduction to then disclose the impact and consequences of their alterations in human skin diseases. In this context, we intend to reorganize the current knowledge on the principle exerted functions of Notch pathway and to identify potential mechanisms in common between these diseases.

1.1. Core Components of Notch Signaling

Mammalian cells are known to express four different Notch receptors—Notch1–4, each encoded by a different gene—that together constitute the core of Notch signaling. The four Notch paralogs exhibit both unique and redundant functions and show well-conserved structural homology regions [6]. In general, Notch1–4 receptors are known to exert fundamental roles during cell fate determination by affecting differentiation, proliferation and apoptotic programs. Nevertheless, each receptor is also involved in responses that go beyond cell fate determination and that might depend on their tissue distribution.

All Notch receptors are single-pass type I integral membrane proteins composed by a series of complex domains and organization motifs [7]. Notch extracellular domain (NECD) comprises the N-terminal extracellular region of the receptors acting as the site of interaction with the ligand located on the signal-sending adjacent cell. NECD is formed by 29 up to 36 epidermal growth factor-like (EGF-like) tandem repeats, sites directly involved in associations with ligands [8] and carries a subset of calcium ions binding sites known to modulate the affinity and conformation of the receptor during ligand binding [9]. Always within the extracellular region, EGF-like tandem repeats are directly followed by the negative regulatory region (NRR), composed by three cysteine-rich Lin12-Notch repeats (LNR-A, -B, -C) and a heterodimerization domain (HD) [6,9]. The NRR is defined as an activation switch for the receptor since it allows a context-specific activation and prevents the induction of a ligand-independent response [10,11]. Receptors possess a single transmembrane domain (TMD) and an intracellular domain (NICD) consisting of a Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (RBP-Jκ) association module (RAM), seven ankyrin repeats (ANK), two nuclear localization signals (NLS) and a transactivation domain (TAD) that contains conserved proline/glutamic acid/serine/threonine-rich motifs (PEST) [9,12].

Following the induction of Notch signaling, the RAM module, together with the ANK motifs and the TAD, is indispensable for the modulation (induction or inhibition) of the appropriate expression pattern of target genes by interacting with transcriptional activators in the nucleus [13]. The NLS motif is essential for the nuclear targeting of the NICD [14], while the PEST domain modulates the proteolytic degradation of this active fragment [13].

Differences between the four Notch receptors reside primarily in structural dissimilarities in the NICD. This region is known to affect both the binding affinity between the NECD with its ligand and the interactions of receptors with the transcriptional factors in the nucleus, therefore influencing the cellular and temporal expression of the receptors and of downstream target genes. Structural variations in the NICD imply that the γ-secretase cleaves the intracellular partition of receptors in correspondence of diverse amino acid sequences, therefore generating and releasing from the cellular membrane peptides with distinct amino-termini [15]. The formation of structurally diverse active NICD moieties allows combinatorial interactions with transcription complexes and local/tissue-specific activator proteins bound to regulatory elements of target genes, thus permitting the induction of transcription in a selective manner [15,16]. These specific interactions are known to be essential for the activation of the expression of distinct subsets of target genes; indeed, upon Notch pathway induction,

in absence of local/tissue-activators, an insufficient initiation of gene transcription patterns has been reported (Figure 1A) [15,17].

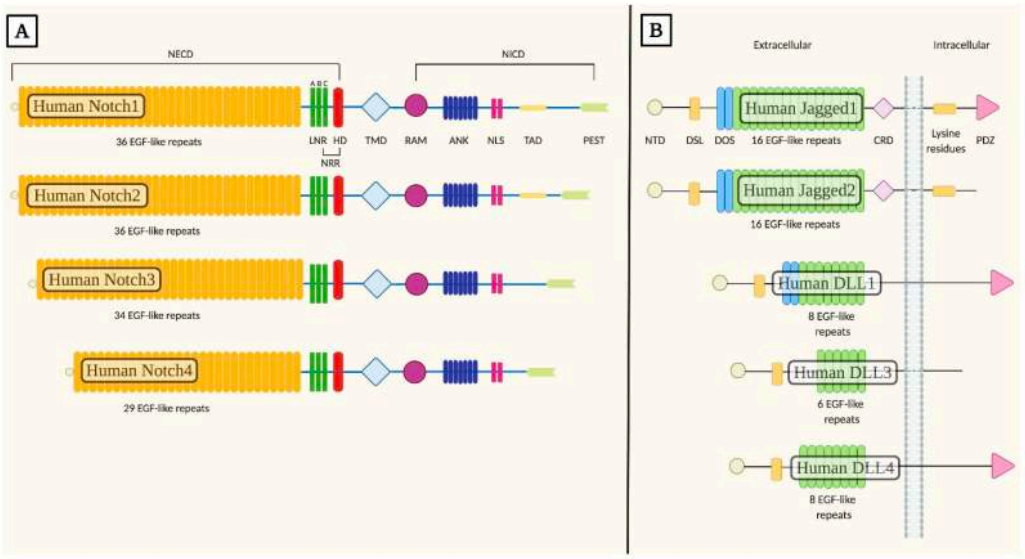

Figure 1. Structural organization of human Notch receptors and human canonical Notch ligands. (**A**) Mammalian cells express four different Notch receptors, Notch1–4. All receptors are single-pass type I transmembrane proteins. The notch extracellular domain (NECD) comprises 29 up to 36 epidermal growth-like factor tandem repeats (EGF-like repeats), followed by the negative regulatory region (NRR), composed by three cysteine-rich Lin12-Notch repeats (LNR-A, -B, -C) and a heterodimerization domain (HD). The single transmembrane domain (TMD) is directly followed by the Notch intracellular domain (NICD) consisting of a Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (RBPjκ) association module (RAM), seven ankyrin repeats (ANK), two nuclear localization signals (NLS) and a transactivation domain (TAD) that retains conserved proline/glutamic acid/serine/threonine-rich motifs (PEST). (**B**) Five canonical Notch ligands have been described in mammals and they are generally referred to as canonical Delta/Serrate/Lag-2 (DSL) ligands. These proteins belong to the Serrate family of ligands (Jagged1 and Jagged2) and to the Delta-like family of ligands (DLL1, DLL3 and DLL4). The extracellular region possesses a conserved structural organization with an N-terminal domain (NTD), followed by a Delta/Serrate/Lag-2 (DSL) domain flanked by the Delta and OSM-11-like region (DOS) and multiple epidermal-growth-factor-like tandem repeats (EGF-like repeats). The NTD, together with the DSL, DOS and EGF-like motifs, is required for ligand binding to Notch receptors. Jagged1 and Jagged2 possess a juxtamembrane cysteine-rich domain (CRD) not present in Delta-like ligands. In the intracellular region, most Serrate ligands present numerous lysine residues involved in ligand signaling. Always within the intracellular C-terminal region, most DSL ligands express a PSD-95/Dlg/ZO-1 (PDZ) motif that is required for interactions with the cytoskeleton.

Notch signaling in mammals is primarily induced by five functional canonical Notch ligands, all of which are single-pass integral membrane proteins belonging to the Delta-like family of ligands (Delta-like 1 ligand (DLL1), Delta-like 3 ligand (DLL3) and Delta-like 4 ligand (DLL4)) and to the Serrate family of ligands (Jagged1 and Jagged2). These ligands are members of the Delta/Serrate/Lag-2 (DSL) family and are therefore generally referred to as DSL ligands [18].

DSL ligands present a distinctive distribution and expression patterns in the various organs of the adult human body. Different DSL ligands are able to induce differential responses by regulating the levels of the active NICD fragment; this aspect is strictly driven by the ligand identity and the cellular context [19]. Indeed, structural differences between the extracellular domains of DSL ligands define dissimilarities in their signaling strengths, resulting in distinct NICD levels [19].

Human DSL ligands have a common structural organization in their extracellular domain comprising an N-terminal domain (NTD), followed by a DSL motif and a variable number of EGF-like repeats ranging from 16 in the Serrate family to 5–9 in the Delta-like family of ligands [20,21]. The various structural motifs found in the extracellular domain are known to be strictly required to guarantee ligand binding to Notch receptors [22].

Specifically, the DSL region is defined as a degenerate EGF-like repeat which is known to be essential but not sufficient to mediate ligand binding to Notch receptors, but it is of crucial importance since mutations in the conserved residues of this domain have been associated with a loss of function in Notch signaling both in invertebrates and vertebrates [23].

Another required structural and functional component found in the extracellular region is given by the Delta and OSM-11-like region (DOS) flanking the DSL motif. DOS is composed of the first two EGF-like tandem repeats that are thought to be implied in the activation of the ligand by interacting with the adjacent DSL region [21,22] region.

Furthermore, a relevant structural difference in the extracellular region resides in the presence of a juxtamembrane cysteine-rich domain (CRD) in Serrate ligands which is absent in the Delta-like family members [21].

The C-terminal intracellular region of DSL ligands is poorly conserved and lacks in highly homologous motifs except for numerous lysine residues required primarily for ligand signaling activity, which are found in most but not all DSLs [24]. Regions rich in lysine residues are crucial sites involved in the addition of ubiquitin by the E3 ligase [22]. In addition, Jagged1, DLL1 and DLL4 posses a PSD-95/Dlg/ZO-1 (PDZ) motif that is crucial for interactions with the cytoskeleton (Figure 1B) [24].

Amongst the members of the Delta-like family of ligands, DLL1 and DLL4 present almost the same structural organization, but only about 60% of their protein sequence is the same [25]. The impact of these differences influences primarily their affinity for Notch receptors, which differs more than 10-fold and therefore affects their mutual signaling strengths [26]. Moreover, in the case of Jagged1 and Jagged2 ligands, their constitutive domains and organization motifs are almost the same, though they possess 53% of protein sequence identity. If compared to the other DSL ligands, DLL3 is the most structurally divergent since it possesses a degenerate DSL domain. In addition, DLL3 is predominantly localized in the Golgi apparatus, and it seems to be exposed on the cell surface only when overexpressed primarily under pathologic conditions and acts by inactivating Notch signaling [22,27]. DLL3 inhibitory activity is exerted by maintaining or redirecting Notch receptors and DLL1 in the Golgi apparatus or in the lysosomal and/or late endosomal compartments, thus impeding their insertion in the cell membrane [28].

Non-canonical ligands of Notch receptors have also been identified lately, and they comprise proteins lacking DSL and DOS domains and include secreted proteins as well as integral and glycosylphosphatidylinositol (GPI)-linked membrane proteins. Nevertheless, the physiological functions and molecular mechanisms underlying their activity in the context of non-canonical Notch signaling are not yet fully understood [9].

1.2. Notch Signaling

As Notch signaling exerts a fundamental role in many cellular processes and in a vast variety of tissues, it is not surprising that a loss or gain of Notch pathway has been directly linked to many human disorders including developmental syndromes and adult-onset diseases.

To date, the main steps of a canonical Notch signaling are well established and include a ligand induction of Notch receptor, proteolysis of the NICD, migration of NICD to the nucleus and subsequent activation of downstream target genes. Nevertheless, Notch receptors are able to function non-canonically, in a ligand- or transcription-independent manner, through cellular and molecular mechanisms that are still under investigation and collectively known as non-canonical Notch signaling [29].

The progression of both canonical and non-canonical Notch pathways requires a series of events ranging from the maturation of receptors to their activation. The maturation of Notch receptors occurs

through a series of proteolytic cleavages that arise in proximity of the TMD during trafficking to the cell surface. Specifically, by advancing through the secretory route, Notch receptors are cleaved by furin-convertases within the trans-Golgi network at site 1 (S1), generating heterodimers composed by the NECD and a Notch transmembrane and intracellular domain (NTMIC) joined by a non-covalent linkage [30]. Before the final exportation of Notch receptors to the cellular surface, the extracellular domain of the cleaved polypeptide undergoes O-linked and N-linked glycosylations, post-translational modifications known to promote proper protein folding and subsequent interactions with extracellular ligands [31,32].

The activation of Notch signaling following ligand binding causes an irreversible dissociation between the ligand-binding partitions of the receptor from the NICD. As a consequence, each receptor molecule can only signal once; therefore, the advancement of Notch pathway is guided by a strict regulation of Notch proteins in order to obtain a balance between their production and degradation [33].

1.2.1. Canonical Notch Signaling

Canonical Notch signaling is triggered by the binding of Notch receptors to a DSL ligand exposed on a neighboring cell, which leads to a proteolytic processing of the membrane-bound receptor at cleavage sites located in the HD domain, referred to as site 2-4 (S2-4), to ultimately allow the release of an intracellular active fragment, the NICD.

Following ligand binding, signaling is initiated when the trans-endocytosis of ligand-receptor complexes in the neighboring cell induce a conformational change in the juxtamembrane NRR, ultimately leading to the exposition of S2 and therefore rendering this region susceptible to the proteolytic cleavage catalyzed by a disintegrin and metalloprotease, namely ADAM protease, which removes the extracellular domain bound to the DSL ligand. As a consequence, Notch cleavage at S2 on the receptor-expressing cell generates a membrane-tethered partition of Notch comprising a transmembrane and an intracellular region, namely Notch extracellular truncation (NEXT). The cleavage ultimately leads to the exposure of S3 and S4 sites, which are recognized and cleaved by the γ-secretase intramembrane complex, therefore releasing the NICD [9,34].

Once freed from the membrane, NICD migrates to the nucleus and binds to a conserved DNA-binding protein, CBF1/RBP-Jκ/Su(H)/Lag-1 (CSL or RBP-Jκ in vertebrates), that under basal conditions is known to function as a transcriptional repressor by associating with ubiquitous co-repressor (Co-R) proteins [3,16]. Interactions between NICD and RBP-Jκ are thought to promote conformational changes in RBP-Jκ that facilitate its dissociation from transcriptional repressors and furthermore allow the recognition of transcriptional activators of the Mastermind-like (MAML) family, generating the ternary Notch transcription complex (NTC). The NTC is further involved in the recruitment of general transcription factors to induce the transcription of downstream primary target genes, including the hairy/enhancer of split (*HES*) genes and the hairy/enhancer of split with YRPW motif (*HEY*) genes [9,12,35,36]. HES and HEY proteins are basic helix–loop–helix transcription repressors that act as Notch effectors by negatively regulating the expression of downstream target genes such as tissue-specific transcription factors that affect critical cellular and developmental decisions regarding neurogenesis, blood vessel formation, heart development and somitogenesis [37].

A peculiar feature of Notch signaling resides in the fact that upon ligand binding, the proteolytic cleavage of the NICD induces an irreversible dissociation of the intracellular active signaling unit from the ligand-interacting unit [33]. As a consequence, each receptor can only signal one time, and once released, the NICD fragment can no longer be subjected to regulatory processes mediated by ligand binding or other cell-surface interactions; thus, the turnover of NICD is strictly regulated in order to avoid the presence of a sustained and long-lasting or excessively high signaling [33]. Specifically, the MAML component of the NTC regulates the duration of the transcriptional event by interacting with the cyclin-dependent kinase 8 (CDK-8). The phosphorylation of the PEST domain of NICD fragment by CDK-8 causes the disassembly of the NTC and results in an attenuation of the response. The final step comprises the ubiquitination of NICD by the E3 ubiquitin ligase Fbw7, which leads to its

proteasome-dependent degradation [38]. Under physiological conditions, unbound Notch receptors are recycled or degraded through the lysosomal machinery (Figure 2) [38].

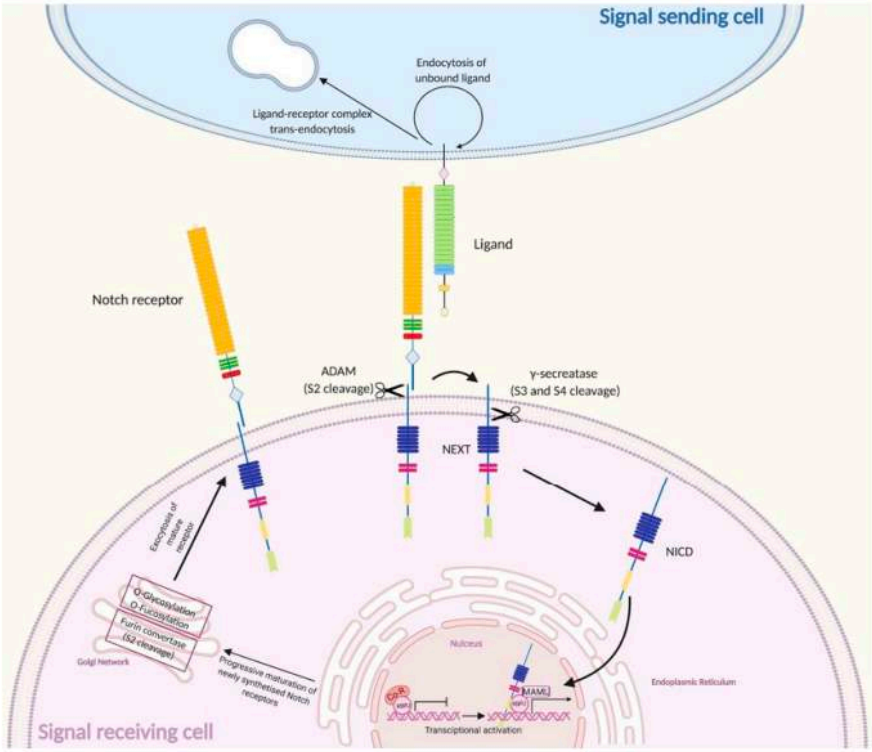

Figure 2. Overview of canonical Notch signaling. Notch receptors are newly translated within the endoplasmic reticulum, processed by a furin convertase (S1 cleavage) and subjected to O-linked and N-linked glycosylations in the Golgi compartment. Once on the cell surface, the receptor is activated by binding to a ligand on a juxtaposed cell. Following ligand binding, Notch signaling is initiated when the trans-endocytosis of ligand-receptor complexes in the neighboring cell induces a conformational change in the receptors, leading to the exposure of S2, site cleaved by ADAM metalloproteases (S2 cleavage) to generate a membrane-tethered partition of Notch, namely Notch extracellular truncation (NEXT). The cleavage in S2 exposes the S3 and S4, allowing the subsequent proteolytic cleavage in these sites by the γ-secretase complex (S3/S4 cleavage), ultimately leading to the release of an intracellular active fragment, the NICD. The NICD migrates in the nucleus and binds to CBF1/RBP-Jκ/Su(H)/Lag-1 (CSL), known as RBP-Jκ in vertebrates, that under basal conditions is known to function as a transcriptional repressor by associating with ubiquitous co-repressor (Co-R) proteins, and to transcriptional activators of the Mastermind-like (MAML) family, generating the ternary Notch transcription complex that initiates the transcription of downstream target genes. Membrane trafficking and endocytosis is crucial to regulate the availability of receptors and ligands on the cell surface. In the signal receiving cell, pointed arrows (→) define the orderly succession of events ranging from the synthesis and processing of Notch receptor, to its activation on the plasma membrane and subsequent induction of the transcriptional machinery. In the signal sending cell, the → the trans endocytosis of the ligand-receptor complex, while the rounded arrow (↺) describes the endocytosis of the unbound ligand.

1.2.2. Non-Canonical Notch Signaling

Canonical and non-canonical Notch signaling need to be considered as two strictly integrated pathways triggered by a single receptor [39]. Indeed, the precise interplay between the principle activities exerted by canonical Notch signaling during cell fate determination and non-canonical Notch

signaling, which primarily include cell adhesion, cytoskeletal remodeling and cell motility, needs to be taken into account to unravel the complex functions of Notch signaling cascade [39,40].

Nevertheless, while the molecular mechanisms and cellular functions of a canonical Notch pathway have been well established, the signaling events underlying a non-canonical Notch cascade have yet to be fully elucidated.

Recent studies highlighted the necessity to undertake a thorough characterization of the non-canonical mechanisms of the Notch pathway since its alterations have been seen to be potentially associated with different pathological conditions including cancer and immune deregulations [4,41]. Indeed, it might be possible that a deep understanding of the non-canonical Notch signaling could be useful to assess specific strategies able to block pathological Notch signaling while simultaneously maintaining intact many other physiological processes mediated by the canonical Notch route [4].

The better-characterized modes of non-canonical Notch signaling include regulated activation of Notch route by the γ-secretase occurring independently from ligand interaction, RBP-Jκ-independent activity of NICD, induction of Notch signaling induced by a membrane-bound form of Notch receptor in absence of a proteolytic cleavage mediated by the γ-secretase protease or in some cases of ligand interaction [4].

The ligand-independent activation of Notch receptors is known to occur through endosomal trafficking. The levels of Notch proteins are strictly regulated in order to obtain a balance between production and degradation. In the absence of an interaction with a ligand, the NICD is marked for internalization and degradation by ubiquitination mediated by the E3 ubiquitin ligase Deltex [42]. Indeed, the turnover of Notch receptors under physiological conditions requires that the ubiquitinated Notch proteins are sorted from the endosomal vesicles into the intraluminal vesicles of the multivesicular bodies (MVBs). Once in the lumen of the MVBs, Notch receptors are transferred into the lumen of lysosomes for subsequent degradation. Nevertheless, it might occur that while being directed towards the lysosomal compartments, a disturbance of the endosome-mediated sorting of Notch receptors might result in the triggering of a ligand-independent activation of the receptor [43]. In this context, Notch receptors are retained in the endosomal membrane site, in which they may be subjected to an accidental intracellular activation following the removal of the NICD, which mimics the modes of a ligand-dependent Notch induction [42,43].

Notch signaling might also occur through an RBP-Jκ-independent mechanism that seems to be crucial in cellular processes including primarily immune dysfunctions and oncogenesis [4]. In this context, the released NICD active fragment might regulate transcription principally through two alternative mechanisms: the first contemplates the interaction of the active NICD with transcription factors not belonging to the CSL/RBP-Jκ family, including hypoxia-inducible factor (HIF) and monocyte enhancer factor-2 (Mef2) [44]; the second involves the delivery into the cytoplasm of a slightly different fragment from NICD resulting from the cleavage catalyzed by a distinct protease from presenilin of the γ-secretase complex [41,44].

The protease-independent activation of Notch signaling occurs when Notch receptors are not processed by the γ-secretase complex [41]. It is widely accepted that the cleavage of Notch receptor by the catalytic moiety of the γ-secretase constitutes a hallmark of canonical Notch activation. Nevertheless, different studies, showing limited evidence so far, suggest the presence of a non-canonical Notch signaling that is activated in absence of the cleavage catalyzed by the γ-secretase complex, revealing that, in limited specific occasions, Notch can be non-canonically processed [41].

2. Skin

The skin is the most extended human organ, and it is partitioned into two layers: the epidermis, the uppermost layer constituted by a stratified squamous epithelium primarily composed of keratinocytes and dendritic cells; and the derma, the bottom layer composed of connective tissue bearing vascular and nervous networks, resident fibroblasts, mast cells and macrophages, amorphic collagen and epidermal appendages [45].

Specifically, the epidermis is defined as a multi-layered epithelium, comprising the interfollicular epidermis (IFE) and associated epidermal appendages (hair follicles, sebaceous and sweat glands). The IFE is primarily composed of progressively differentiated keratinocytes organized in specific layers that, from the deepest to the most superficial one, are given by the basal layer, spinous layer, granular layer and the stratum corneum (Figure 3) [46].

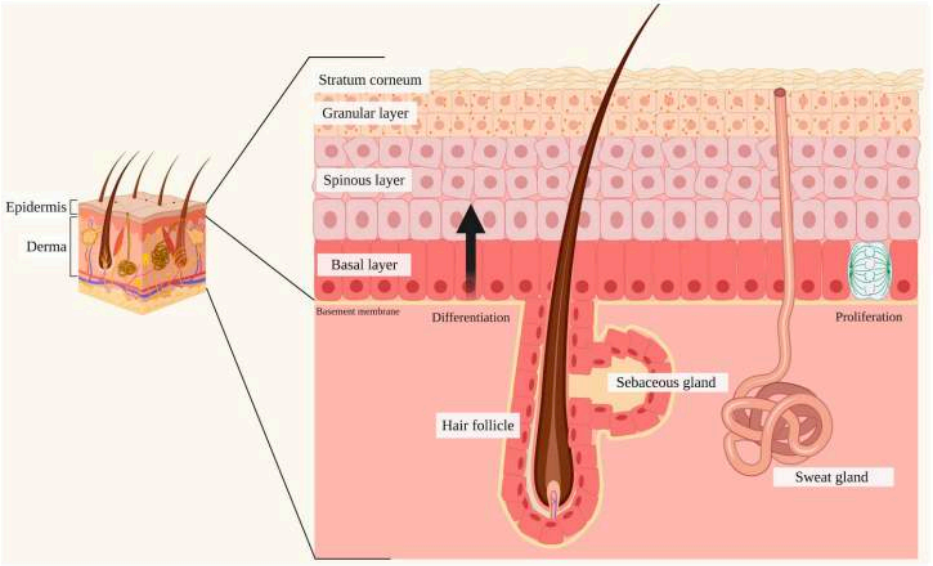

Figure 3. Schematic representation of the skin. The skin is composed of two layers: the epidermis and the dermis. The epidermis, the uppermost partition, is defined as a stratified squamous epithelium comprising the interfollicular epidermis (IFE) and various skin appendages (hair follicles, sebaceous glands and sweat glands). The IFE is primarily composed of progressively differentiated keratinocytes organized in specific layers, which include the basal layer (the deepest portion of the epidermis), stratum spinosum, stratum granulosum and stratum corneum (the most superficial portion of the epidermis). The basal layer comprises mitotically active cells that generate during every cell division process, either stem cells that self-renew or transient amplifying cells that gradually undergo terminal differentiation by migrating upwards towards the stratum corneum. This process requires specific signals released by the various skin appendages and contemplates the acquisition of layer-specific characteristics by keratinocytes, including the expression of epidermal keratins and transcriptional activators. Once in the stratum corneum, keratinocytes are completely keratinized, metabolically inactive and are released through desquamation. The epidermis is physically and functionally separated from the surrounding dermis by the basement membrane. The dermis is a connective tissue layer interposed between the epidermis and the subcutaneous tissue, involved in the protection and support of the skin and the deeper layers, in aiding sensation and in assisting thermoregulation.

The epidermal stem cells (ESC) reside in the basal layer and are represented by mitotically active cells that yield at every division cycle either more stem cells that self-renew or transient amplifying cells, defined as a cellular progeny that undergo terminal differentiation strictly driven and regulated by local microenvironmental signals, to ultimately generate IFE, hair follicles and apocrine and sebaceous glands [46]. During differentiation, the transient amplifying cells stop proliferation and progressively migrate upwards to the stratum corneum. During this migration, the keratinocytes acquire layer-specific characteristics, including the expression of peculiar proteins such as specialized epidermal keratins like keratin 1 (K1), keratin 5 (K5) and keratin 10 (K10); transcriptional activators including NF-κB and peroxisome-proliferator activated receptor (PPARγ); involucrin (IVL); transglutaminase (TGM) 1; periplakin (PPL), and loricrin [47–50]. Subsequently, once in the stratum corneum, cells cease their

metabolic activity, and as stratification occurs, epidermal appendages such as hair follicles and associated sebaceous or sweat glands are formed [51,52].

2.1. Notch Signaling and Skin

Notch activation regulates skin homeostasis by balancing primarily growth arrest and progressive differentiation processes of keratinocytes; indeed, Notch signaling is known to act as a molecular switch that strictly modulates the advancement of cells through the various skin layers during epidermal differentiation [53]. Recent studies highlighted the presence of a specific crosstalk activity between the different Notch receptors, ligands and downstream signaling molecules, including p21, involved in inducing growth arrest of keratinocytes, in the initiation of terminal differentiation and requiring Notch for the induction of its expression [54]; and p63, which acts by promoting keratinocyte differentiation and whose activity is counteracted by Notch to maintain immature cell characteristics [55].

2.2. Notch and Skin

Throughout time, the Notch pathway in the skin has been proven to be essential for skin homeostasis [56]. Indeed, Notch receptors and ligands could influence the maintenance of epidermal homeostasis, by providing the correct control of proliferation and differentiation programs within the epidermal cells, during adult and embryonic development [48,52,57].

In detail, Blanpain et al. presented molecular and functional in vivo evidence of overall skin homeostasis mechanisms [58]. Their work highlighted the pleiotropic role of Notch signaling in the proliferation and differentiation of the epidermal cells by observing that Notch operates to govern the balance between proliferative basal progenitors and their terminally differentiating progeny, culminating in epidermal barrier formation [58].

When ligands bind to Notch receptors, NICD is released and translocates to the nucleus, where it induces the activation of downstream target genes including HES and HEY family of genes, p21 gene inducing keratinocytes' growth arrest and p63 expression that acts by promoting keratinocyte differentiation [9,12,35,36,59]. Once activated, target genes mediate the regulation of signaling pathways aimed at committing the ESC of the basal layer towards two distinct fates: proliferation with self-renew purposes or differentiation [60–62].

Notch1–4 receptors and their ligands play an important role in regulating epidermal proliferation and differentiation, and their distinctive expression in the epidermal layers seems to correlate with the activation of layer-specific target genes in a well-determined and distinct manner, therefore strongly impacting the subsequent IFE development [50,57].

Notch1, Notch2, Notch3 and Notch4 receptors have been well documented to be expressed in the IFE, predominantly in the suprabasal cells, and the expression of receptors has been seen to occur in proliferating cells or cells that are initiating or undergoing terminal differentiation [59]. Notch ligands detected in the epidermis comprise Jagged1, Jagged2 and DLL1 [59]. Jagged1 expression has been predominantly identified in the suprabasal layer, while Jagged2 and DLL1 ligands have been principally identified in the basal layer (Figure 4) [50,57].

Jagged1 and Jagged2 ligands bind to Notch family members with similar specificities and act by promoting cellular terminal differentiation [50].

A differential outcome is given by Notch interactions with DLL1 ligand. DLL1 have been reported to be exclusively expressed in the basal layer, where interactions with Notch receptors results in the maintenance of ESCs in an undifferentiated state [58]. Nevertheless, it is known that upon receptor-ligand binding, DLL1 functions also by blocking Notch signaling [57,58,62]. Notably, the role of DLL1 in the modulation of Notch activity is extremely intriguing. DLL1 counteracts the activity of basal-layer-localized Notch1 receptors by upregulating the cell cycle regulator p21, an inhibitor of cell cycle progression, therefore leading to the downregulation of ESCs proliferation [56,63]. Therefore, keratinocytes are committed to exit from the stem cell compartment, to their subsequent detachment from the basal layer and to initial differentiation [58].

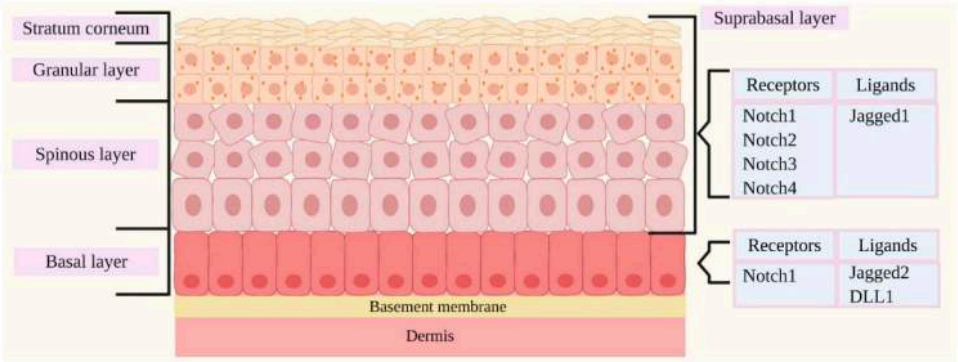

Figure 4. Distribution of Notch ligands and receptors in human skin. Notch1–4 receptors and their ligands are involved in the maintenance of epidermal homeostasis principally by regulating proliferation and differentiation programs within the epidermal cells, mediating the balance between proliferating basal progenitors and the terminally derived differentiated progeny, ultimately leading to the formation of the epidermal barrier. The distinctive expression patterns of Notch receptors and their ligands within the different epidermal layers seem to be associated with the activation of layer-specific target genes. Notch1–4 receptors have been reported to be expressed in the suprabasal layers, while Notch1 receptor seems to be widely expressed in the basal layer. Jagged1, Jagged2 and Delta-like 1 (DLL1) are the Notch ligands detected in the epidermis. The expression of Jagged1 has been reported to be predominant in the suprabasal layer, while Jagged2 and DLL1 have been primarily identified in the basal layer.

Further agreeing with this idea, Negri et al. noted an incremented proliferation of the ESCs in knockdowns for Notch1 [57]. Rangarajan et al. also detected epidermal hyperproliferation and reduced differentiation in a mouse model with conditional ablation of Notch1 in skin epithelium [56].

Notch1 is known to activate caspase 3, to minimize stem cell proliferation and to increase cellular differentiation [64,65]. In addition, the receptor negatively regulates p63, a p53 family member, in order to prevent an inhibition of differentiation [66]. It is important to note that, also in this case, p63 is expressed in cells of the basal layer where proliferation occurs, while it is strongly down-modulated in the upper layers where differentiation happens [66]. Remarkably, proliferation or differentiation of keratinocytes may be connected to a delicate balance between Notch1 and p63 levels [67].

In contrast to Notch1's well-reported activity, in several studies using mice carrying conditional mutations in Notch2, -3 and -4, it was observed that the ablation of these 3 molecules does not cause any overt phenotype in the skin [68–70].

It is interesting to note that mice deficient in Notch1 or Notch2 gene are embryonic lethal [71,72], while mice deficient in Notch3 or Notch4 are born without any apparent altered phenotype in the skin [68,69].

Mazur et al. detected a reduction of p21 expression in a mouse model with ablation of Notch1 that is not found in a model with ablation of Notch2; in fact, they speculated that Notch2 signaling might not be required for skin differentiation since it is expressed exclusively in suprabasal keratinocytes, mainly represented by already differentiated cells [65].

Except for the cellular localization of Notch3 and Notch4, no information is available regarding their involvement in skin physiology.

Furthermore, excluding differentiation and proliferation, Notch signaling also regulates other important outcomes in the epidermis such as wound healing through the regulation of vascular endothelial cell proliferation, tube formation and migration of keratinocytes and fibroblasts [73,74].

2.3. The Main Proteins Related to Notch and Skin

Other molecules play a key role in the correct assembly and functioning of Notch signaling. The activity of these molecules is essential for the regulation of many diverse cellular functions. Indeed, their alterations can induce signal failure and, as a consequence, health disorders.

The principal molecules related to Notch signaling in the skin are the following:

γ-secretase: The maturation and activation of Notch receptors is strictly regulated by a series of proteolytic processings. The γ-secretase complex is a transmembrane protease that exerts a fundamental role during Notch activation by catalyzing the ultimate cleavage of the receptor, causing the release of the intracellular and active fragment NICD, that once migrated in the nucleus, induces the expression of genes involved in epidermal and follicular differentiation and proliferation [75]. The γ-secretase consists of four protein subunits, comprising: presenilins (PSEN), the catalytic subunit; and three cofactor subunits, represented by presenilin enhancer-2 (PSENEN), nicastrin (NCSTN) and anterior pharynx defective-1 (APH1) [76]. These proteins are encoded by six genes: *PSEN1*/*PSEN2*, encoding for PSEN1 and PSEN2; *NCSTN*, encoding for NCSTN; *APH1A*/*APH1B*, encoding for APH1; and *PSENEN*, encoding PSENEN [77]. Mutations in the genes encoding for the different subunits of the γ-secretase complex have been linked to various skin disorders and primarily act by affecting Notch signaling [77].

GDP-fucose protein O-fucosyltransferase 1 and Protein O-glucosyltransferase 1: Glycosylation of Notch ECD, by the addition of O-glycans, has progressively emerged as a fundamental post-transcriptional modification able to strictly regulate the receptors' activity [78]. In the context of canonical Notch signaling, a pivotal role in ECD glycosylation is given by two enzymes: the first is the GDP-fucose protein O-fucosyltransferase 1 (POFUT1), encoded by *POFUT1* gene, a protein involved in catalyzing the addition of an O-linked fucose moiety [79] to the EGF-like repeats of the ECD domain; the second is the protein O-glucosyltransferase 1 (POGLUT1), encoded by *POGLUT1* gene, that functions by adding an O-linked glucose [80] to the EGF-like repeats of the ECD domain. These post-transcriptional modifications allow the binding between Notch ligands and receptors, which ultimately leads to the proteolytic release of NICD. Though the localization of POFUT1 is not uniform in the epidermis, recent evidence strongly suggests that this enzyme may influence melanin synthesis and transport in melanocytes [81]. A high expression of POGLUT1 has been registered in the matured layers of the epidermis, and this aspect may indicate the involvement of POGLUT1 in the differentiation and the development of the epidermis [82].

EGF domain-specific O-linked N-acetylglucosamine transferase: The epidermal growth factor domain-specific O-linked N-acetylglucosamine transferase (EOGT), encoded by *EOGT* gene, is an O-linked N-acetylglucosamine (O-GlcNAc) transferase known to transfer O-GlcNAc moieties to consensus sequences in EGF-like repeats of few membrane-bound and secreted proteins, including Notch receptors [83,84]. EGF-specific O-GlcNAc glycosylation is a rare form of functional post-transcriptional modification occurring exclusively in the lumen of the endoplasmic reticulum, and it seems to be essential for Notch regulation and ligand-induced Notch signaling [83]. Pathogenic variants on *EOGT* impair the glycosyltransferase activity of the enzyme, resulting in a defective post-translational modification of Notch receptors [83]. Nevertheless, the impact of the activity of EOGT on Notch receptor and signaling has still to be fully understood, and currently, no specific data are available regarding its involvement and activity in skin physiology.

Filaggrin: Filaggrin (FILA), encoded by the *FLG* gene, is a late epidermal differentiation protein that plays an important role in the skin's barrier function. FILA interacts exclusively with intermediate filaments and is specifically known to possess a strong keratin-binding activity; indeed, once FILA binds to intermediate keratin filaments, it causes their dense aggregation into microfibrils, ultimately rendering intermediate filaments tightly packed in parallel arrays. The resulting crosslink between keratin intermediate filaments leads to the formation of highly insoluble keratin, which acts as a protein scaffold for the subsequent attachment of lipids and proteins, necessary to guarantee the progressive differentiation of keratinocytes [85]. In the IFE, terminally differentiated keratinocytes express keratin-bundling protein FILA, in the upper granular layer and in the cornified envelope [85,86].

Notch signaling is required for the late-stage granular layer differentiation and correct filaggrin processing in the epidermis [48].

3. Notch Signaling and Skin Diseases

Any disruption of canonical and non-canonical Notch cascade, resulting in a gain or loss of function, can induce a health disorder as a consequence of signal failure, as Notch signaling orchestrates the regulation of cell proliferation, differentiation, migration and apoptosis.

The molecular mechanisms through which Notch contributes to the pathogenesis of skin diseases are multiple and are still far from being fully understood. Despite the initial efforts focused on skin cancer, attention has recently turned also on the correlation between Notch signaling and skin diseases other than malignancies.

To date, the skin diseases correlated to alterations in Notch signaling are: Hidradenitis Suppurativa, Dowling Degos Disease, Adams–Oliver Syndrome, Psoriasis and Atopic Dermatitis.

3.1. Hidradenitis Suppurativa (HS)

Hidradenitis Suppurativa (HS) is a chronic inflammatory skin disease affecting the hair follicle.

It has been estimated that the pathology presents a prevalence of 0.05–4% in Europe with a female predominance [87] and with an onset after puberty. Interestingly, in familial cases of HS, the first manifestations can occur in younger children, who often tend to develop a more severe form of the disease [88].

The events underlining the early onset of HS phenotype are given by infundibular hyperkeratosis of the terminal hair follicles, perifolliculitis and hyperplasia of follicular epithelium, events that collectively anticipate the follicular occlusion and disruption. The rupture of hair follicles releases cellular debris and keratins into the surrounding dermis, therefore activating an inflammatory immune response [89,90].

The primary visible lesions of HS are recurrent, painful, subcutaneous and inflamed nodules that can rupture, leading to deep and purulent dermal abscess [91]. With the progression of the disease, the connection of the lesions can result in dilated sinus tracts (i.e., skin tunnels) [92], fibrosis and scarring [91]. HS clinical manifestations occur primarily in apocrine-gland bearing regions at inverse body sites including the axilla, genito-femoral area, perineum, perianal and gluteal region and are characterized by recurrence and chronicity that significantly impact the patients' quality of life [91].

The exact etiology of HS is yet not completely unraveled; however, both genetic and environmental factors are known to trigger the development of the disease. A recent article by Tricarico et al. reported an exhaustive overview of the genes involved in HS susceptibility [93], highlighting that about 35% of HS patients present a family history of HS. Mutations in *NCSTN*, *PSENEN* and *PSEN1* genes, respectively encoding for NCSTN, PSENEN and PSEN1, have been identified as the most common genetic variants involved in HS familial cases. These three proteins are essential components of the γ-secretase multiprotein complex [94], and their haploinsufficiency is probably linked to nonsense-mediated decay of their mRNA, resulting in a dysfunction of γ-secretase activity and ultimately in alterations of Notch signaling (Figure 5) [94].

In an in vitro model of HS, developed through *NCSTN* silencing in HaCaT cells, Xiao et al. observed that *NCSTN* inhibition induced cell proliferation and cell cycle progression, probably through a modulation of phosphoinositide 3-kinase (PI3K)/AKT pathway. The transcriptome profile of these *NCSTN*-silencing HaCaT cells underlined the expression of different genes related to biological processes such as epidermis development, epidermal cell differentiation and keratinocyte differentiation and keratinization. Moreover, a downregulation of genes involved in the Notch signaling pathway was also detected. Similarly, in epidermidal biopsies derived from an HS patient with *NCSTN* mutations, characterized by psoriasiform hyperplasia of the interfollicular epidermis, Notch pathway molecules, such as Notch1-3 and HES-1, were found to be decreased in lesional regions with respect to normal areas [95].

Studies on animal models gave some interesting results regarding the role of Notch pathway in the epidermal environment. For instance, sebaceous gland development was blocked in mice with γ-secretase deficiency [70], whereas hair follicles were replaced by epidermal cysts in mice with Notch deficiency [96].

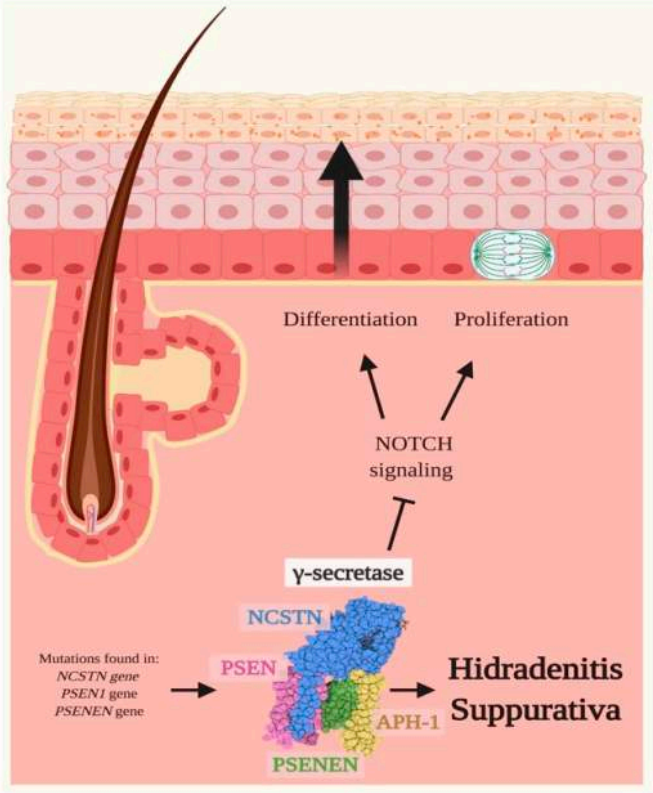

Figure 5. Hidradenitis Suppurativa and Notch signaling. Hidradenitis Suppurativa (HS) is a chronic inflammatory skin disease affecting the pilosebaceous unit, in which up to 35% of cases present a positive family history. These patients frequently carry mutations in *NCSTN*, *PSEN1* and *PSENEN* genes, respectively encoding for nicastrin (NCSTN), presenilin 1 (PSEN) and presenilin enhancer 2 (PSENEN) (encoding for protein). These genes encode for three of the four subunits of the γ-secretase multiprotein complex, a transmembrane protease involved in the cleavage of Notch receptors, and their haploinsufficiency results in the dysfunction of the complex. As a consequence, the γ-secretase is not able to cleave and activate Notch receptors and therefore induces an impairment of Notch signaling, ultimately resulting in augmented levels of epidermal cell proliferation and differentiation. Therefore, alterations in Notch signaling might underlie, at least partially, the initial steps of HS onset, which includes infundibular hyperkeratosis and hyperplasia of follicular epithelium that anticipate the follicular occlusion and disruption. Pointed arrows (→) define the induction of the indicated process or associated function, while the truncated arrow (T) designates the inhibition of the described process.

3.2. *Dowling Degos Disease (DDD)*

Dowling Degos Disease (DDD) is a rare autosomal dominant skin genodermatosis [97].

DDD is very rare, since to date few cases have been reported in the literature, and it possesses a post-pubertal age of onset from the third to fourth decade of life [98].

DDD is characterized by reticulate hyperpigmentation, by small hyperkeratotic dark-brown papules and lentigo-like brown macules [99]. The hyperpigmentation tends to increase progressively over time from flexural sites to intergluteal and inframammary areas, neck, trunk, arms and thighs [99]. The hyperpigmentation occurs in the epidermal basal layer with the thinning of the suprapapillary epithelium. Moreover, melanophages and infiltrates of lymphocytes and histiocytes are present [100], together with melanosomes and keratinocytes with irregular appearance [82].

Classical DDD is caused by mutations in the *KRT5* gene, encoding for keratin 5 (K5) that is normally paired with keratin 14 (K14) to assemble intermediate filaments; in DDD, K5 is truncated, resulting in abnormal intracellular perinuclear architecture of intermediate filaments [99]. Actually, in a breast cancer cell line (MCF7) transfected with plasmid carrying DDD mutation in *KRT5*, K5 was not embodied in cytoskeletal intermediate filament network persisting in its soluble form [99]. Normally, this fraction is small, but it is essential during differentiation for dynamic cytoskeleton rearranging [101]. Therefore *KRT5* haploinsufficiency could negatively impact cell adhesion, organelle movement, nuclear anchoring and melanosome transport into keratinocytes, triggering epithelial remodeling [99]. K5 is linked to Notch pathway; indeed, a loss of K5 expression during epidermal differentiation is concurrent with an increased activation of Notch1 [102].

Other genes involved in DDD are *POFUT1*, *POGLUT1* and *PSENEN* [82,103]. Both POFUT1 and POGLUT1 are components of the canonical Notch signaling pathway.

POFUT1 performs O-fucosylation of Notch receptors driving ligand-receptor binding and the proteolytic release of NICD; in turn, NICD, complexing with RBP-Jκ, impacts the expression levels of NOTCH1, NOTCH2 and HES1 [81,104]. Indeed, in shRNA *POFUT1* HaCaT cells, the gene expression of these three genes was downregulated [81]. *POFUT1* mutations in DDD lead to nonsense-mediated decay of the transcript and impaired Notch signaling with production of abnormal skin pigments [81].

POGLUT1 catalyzes the O-glycosylation of Notch receptors, therefore impacting the receptors' conformation and allowing the activation of intracellular pathways [80]. DDD patients carrying heterozygous nonsense or splice site *POGLUT1* mutations probably present a nonsense-mediated mRNA decay resulting in haploinsufficiency. The immunohistochemistry showed a weak expression of POGLUT1 in the epidermal layers in comparison to healthy donors, where the staining is more prominent, especially in the stratum spinosum and granulosum, indicating the role of this protein for the correct differentiation of the epidermidis [82]. Interestingly, in DDD biopsies, an increment of K5 was also observed if compared to healthy controls, possibly suggesting an interplay between Notch signaling and K5 during epidermal maturation [82].

Individuals carrying *PSENEN* mutations present a different phenotype if compared to classical DDD patients. Indeed, genetic variations in *PSENEN* are pathogenetic for the development of HS. Therefore, co-manifestations of DDD and HS were reported for these patients that develop reticulate pigmentation, comedones, follicular hyperkeratosis, nodules and scars [105]. In our recent study, we also described a patient with familial HS and concomitant DDD harboring a novel nonsense mutation in *NCSTN* gene associated with a reduced quantity of subunits of γ-secretase [106].

PSENEN is a cofactor of gamma-secretase that is involved in the canonical Notch pathway, by cleaving Notch intracellular domain and activating it [107]. In keratinocytes derived from patients carrying *PSENEN* mutations, the expression of *PSENEN*, *POGLUT1* and other Notch related genes were decreased and associated with an abnormal Notch signaling (Figure 6) [107].

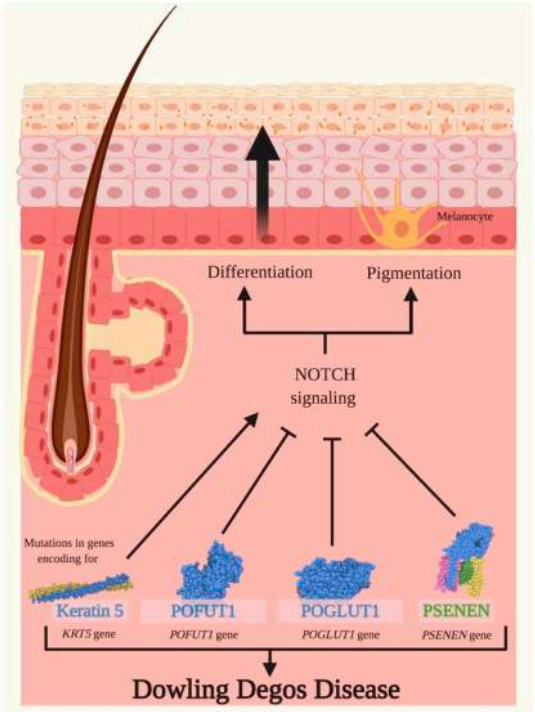

Figure 6. Dowling Degos Diseases and Notch signaling. Dowling Degos Disease (DDD) is a rare skin genodermatosis characterized by hyperkeratotic papules and reticulate hyperpigmentation. Classical DDD is induced by mutations in *KRT5* gene encoding for keratin 5, and the latter is a fundamental protein required for the synthesis of intermediate filaments, principally in the epidermal cells of the stratum basale. The loss of expression of *KRT5* during epidermal differentiation seems to be linked to an activation of Notch signaling, therefore negatively impacting epithelial remodeling. Other genes involved in DDD are *POFUT1*, *POGLUT1* and *PSENEN*, encoding for GDP-fucose protein O-fucosyltransferase 1 (POFUT1), Protein O-glucosyltransferase 1 (POGLUT1) and presenilin enhancer protein 2 (PSENEN), respectively. *POFUT1* mutations lead to nonsense-mediated decay of the transcript, to impaired Notch signaling and to an abnormal pigmentation of the skin. Mutations in *POGLUT1* gene presumably cause a nonsense-mediated decay of mRNA resulting in haploinsufficiency, in a blockade of the Notch pathway and in promoting cell differentiation. Pathogenic *PSENEN* variants impair Notch signaling and cause an aberrant differentiation and pigmentation of the epidermis, leading to reticulate pigmentation, comedones, follicular hyperkeratosis, nodules and scars. Pointed arrows (→) define the induction of the indicated process or associated function, while the truncated arrow (T) designates the inhibition of the described process.

3.3. *Adams–Oliver Syndrome (AOS)*

Adams–Oliver Syndrome (AOS) is a rare inherited disorder, with an estimated incidence of 1 in 225,000 live births, characterized by the combination of aplasia cutis congenita (ACC) of the scalp vertex and terminal transverse limb defects (TTLD) including hypoplastic nails, brachy/oligodactyly and amputation defects [108].

Additional major features of AOS comprehend vascular anomalies such as cutis marmorata telangiectatica congenita (CTMC), pulmonary and portal hypertension and retinal hypervascularization.

Autosomal dominant or sporadic forms of AOS are linked to mutations in *ARHGAP31* (Rho GTPase Activating Protein 31), *DLL4*, *Notch1* or *RBP-Jκ* genes, while mutations found in *DOCK6* (Dedicator of Cytokinesis Protein 6) or *EOGT* genes characterize an autosomal recessive inheritance of the syndrome [109,110].

Each of these genes is involved in tightly regulated processes occurring during embryonic development that specifically involve Notch signaling pathway and the organization of actin cytoskeleton. Mutations and/or alterations in any one of them can impair these mechanisms, therefore leading to the onset of the AOS phenotype [110].

Notch pathway seems to play a determinant role in the pathogenesis of AOS; indeed, 71% of identified mutations found in cases affect genes directly correlated to Notch signaling, including *Notch1*, *DLL4*, *RBP-Jκ* and *EOGT*, primarily through haploinsufficiency or loss of function mechanisms, by impacting the maturation of receptors, receptor-ligand binding and the transcription of target genes [110].

The activation of Notch receptors ultimately leads to the migration of the NICD active fragment in the nucleus and immediately interacts and activates the transcriptional activator RBP-Jκ. Once activated, RBP-Jκ functions by recruiting chromatin remodeling complexes containing histone deacetylase/acetylase proteins to promote the expression of downstream target genes [111].

EOGT is an enzyme that catalyzes the addition of O-GlcNAc moieties to consensus sequences in the EGF-like repeats of the ECD domain of Notch receptors during their maturation occurring through the secretory pathway. EOGT has been seen to be particularly relevant for the glycosylation of Notch1 receptors in mammalian cells [112]. EOGT pathogenetic variants cause impaired glycosyltransferase activity, resulting in a defective post-translational modification of targeted Notch proteins directed towards the plasma membrane [113]. However, the impact of these variants on Notch signaling remains still poorly understood.

Notch1 mutations are frequent and likely constitute the primary single genetic origin of the disease [114]. *Notch1* can present deleterious genetic variants, including deletions, frameshifts, missense, nonsense and splice site mutations. Truncating mutations, which are generally spread throughout the entire length of the gene, result in the degradation of the mutant transcript through the nonsense-mediated decay mechanism. Clusters of missense mutations occurring in the regions encoding for EGF-like tandem repeats 11–13 have been identified, and they have been seen to impair the binding of Notch1 to DSL ligands [115]. Furthermore, missense variants often cause the addition or removal of cysteine residues, and the presence of an abnormal amount of this amino acid causes an alteration in the formation of disulfide bonds, therefore disrupting the tertiary structure of the receptor [115].

DLL4 is a critical Notch ligand, encoded by the *DLL4* gene, that is capable of binding and promoting the activation of Notch1 and Notch4 receptors. Recent studies identified heterozygous pathogenic variants and nonsense and missense mutations in *DLL4*. Specifically, most of these missense variants have been characterized and seen to cause a replacement or creation of cysteine residues, and as a consequence negatively impact the structural integrity of the ligand [108]. These findings strongly suggest that sequence changes in this gene are responsible for the onset of AOS [108]. Another relevant function of this gene comprises its involvement in the negative regulation of endothelial cell proliferation, angiogenic sprouting and retinal progenitor proliferation [116].

Mutations in *RBP-Jκ* are commonly given by missense variants that affect the DNA-binding region of the transcriptional activator, which is no longer able to bind to the promoters of target genes, specifically of *HES1*. The resulting impaired binding ability of RBP-Jκ leads to a disruption of the transcriptional regulation of Notch signaling on downstream target genes [117].

On the contrary, the remaining 29% of patients carry causative variants in *ARHGAP31*, encoding for the Rho GTPase-activating protein 31 (ARHGAP31), and *DOCK6*, encoding for guanine nucleotide exchange factor (GEF), which are not directly associated to Notch signaling. These genes encode for regulatory proteins that are actively involved in actin cytoskeleton formation and therefore exert a primary role in cell morphology, cellular migration, cell division and survival [110,118]. Despite six genes underlying AOS having been well-established and characterised to date, recently it has been observed that some probands do not possess mutations in any of these genes (Figure 7) [110].

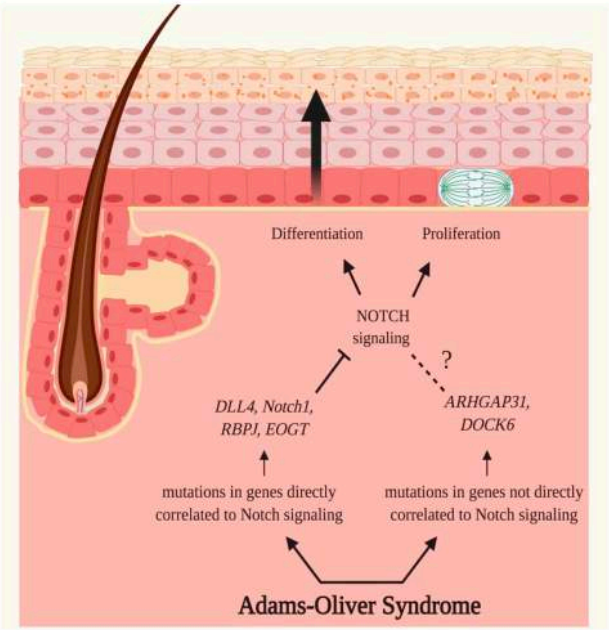

Figure 7. Adams–Oliver Syndrome and Notch signaling. Adams–Oliver Syndrome (AOS) is a rare inherited disorder characterized by aplasia cutis congenita of the scalp, terminal transverse limb defects, vascular anomalies, pulmonary and portal hypertension, and retinal hypervascularization. 71% of identified mutations in AOS cases have been identified in genes directly correlated to Notch signaling, therefore suggesting the crucial role of this pathway in the onset of AOS. Mutations have been registered in *Notch1*, *DLL4*, *RBP-Jκ* and *EOGT* genes, encoding, respectively, for Notch1, Delta-like 4 ligand (DLL4), Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (RBP-Jκ) and EGF domain-specific O-linked N-acetylglucosamine transferase (EOGT). The identified genetic variants impact the maturation of receptors, receptor-ligand binding and interaction, and ultimately the transcription of target genes. As a consequence, these mutations cause a disruption of Notch signaling, therefore negatively impacting proliferation and differentiation of epidermal cells. The remaining 29% of AOS cases carry causative variants in *ARHGAP31*, encoding for the Rho GTPase-activating protein 31 (ARHGAP31), and *DOCK6*, encoding for guanine nucleotide exchange factor (GEF), which are not directly associated with Notch signaling. The products of these genes are regulatory proteins implied in the formation of actin cytoskeleton and are consequently involved in cell morphology, cell migration, survival and division; therefore, variants in these genes are thought to negatively regulate these fundamental processes. Pointed arrows (→) define the induction of the indicated process or associated function, while the truncated arrow (T) designates the inhibition of the described process. The dotted line (—) indicates a proposed association that still needs to be clarified.

3.4. Psoriasis

Psoriasis is a chronic immune-mediated, proliferative and inflammatory disorder with primary cutaneous manifestations and a strong genetic predisposition. This skin disorder is characterized by papules and plaques in demarcated areas of affected skin with variable morphology, distribution and severity. The scalp, elbows, knees, lower back, hands, feet and body folds are commonly affected sites, and the lesions have typically a symmetrical distribution [119].

The pathogenesis of Psoriasis is complex and multifactorial and is the result of an interplay between disturbances in the innate and adaptive cutaneous immune responses, complex genetic and epigenetic backgrounds and alterations in the skin microbiome [120].

The major events underlying Psoriasis' etiology comprise an abnormal differentiation and proliferation of keratinocytes causing epidermal hyperplasia, dermal infiltration by various immune

cells, and increased dermal capillary density leading to an augmented permeability in wide-caliber vessels [121–123].

To date, the major accredited immunopathogenic mechanism of Psoriasis asserts that the crosstalk between keratinocytes and autoreactive T cells leads to the development of inflammatory and immune-driven responses that are necessary for the onset, progression and persistence of the disease [124]. The ongoing infiltration in the epidermis and dermis of different leukocyte populations causes hyperproliferation of the epidermis and an altered keratinization, which results in a thickened epidermis, altered constitution of the cornified envelope of the skin and elongated protrusions in the dermis [124,125]. Nevertheless, the sole T cell-induced immune response targeting keratinocytes cannot justify the phenotype of Psoriasis. Indeed, growing evidence suggests that intrinsic alterations in epidermal keratinocytes also have a relevant impact in the development of the disease [126]. The intrinsic alterations found in keratinocytes of Psoriasis cases have been seen to affect primarily the expression of cytokines and growth factors that directly convert T cell-derived signals in a dysregulated hyperproliferation and differentiation; induce the activation, recruitment and retention of T cells in the epidermal compartment; and promote angiogenesis [124,126,127].

Bearing in mind that the proliferation of ESCs and the progressive differentiation of keratinocytes are strictly regulated by the Notch signaling pathway, it is not surprising that alterations in this intracellular route have been found to be crucial in the onset of Psoriasis [128].

Further sustaining the potential crucial role of Notch cascade in the pathogenesis of this disorder is a study conducted by Thélu et al. [128], in which authors registered a decrement in Delta-like/Notch signaling in conditions of altered cell proliferation and differentiation in Psoriasis skin lesions and in in vivo experiments that involved the grafting of normal human skin on a nude mouse [128].

Ota et al. [129] observed a decrement in Notch molecules in psoriatic skin that might cause an aberrant expression and localization of K10 and K14, leading to abnormal differentiation of the epidermis. Moreover, they observed a decrease in Notch1 and Notch2 expression that caused hyperproliferation of keratinocytes through the induction of *p21* gene expression. Psoriatic skin is characterized by hyperproliferation and aberrant differentiation; in fact, these events comprise the typical histopathological basis for the formation of epidermal hypertrophy and parakeratosis [130].

Instead, Skarmoutsou et al. [131] have registered a hyperactivation of the Notch pathway in skin samples derived from psoriatic patients. In particular, Skarmoutsou et al. showed a significantly higher protein expression level of Notch1, Notch2, Jagged1 and hairy/enhancer of split 1 (Hes1) in psoriatic skin lesions if compared to normal controls. Abdou et al. [132] also recently observed an upregulation and not a downregulation of Notch1 in 35 lesional biopsies of psoriatic patients in comparison with normal skin biopsies. In addition, in a study conducted by Jiao et al. [133], authors found a nuclear form of Notch1 that guarantees the activation of the cascade of Notch signaling and whose levels are significantly associated with the severity of Psoriasis diseases. Furthermore, in another recent work conducted by Rooney et al. [134], the role of Notch1 signaling was investigated in the context of Psoriasis by studying the tissue expression of several effectors of this route such as Notch1, DLL4, Jagged1, vascular endothelial growth factor (VEGF) and other proteins. The results of this study highlighted an increased expression of Notch1, DLL4, Hrt-1 (Ring-box protein HRT1) and A-SAA (Acute-phase Serum Amyloid A) in Psoriasis lesional skin if compared to unaffected skin. Moreover, the authors observed that A-SAA plays a role in the transcriptional regulation of *Notch1*, and it induces angiogenesis and vascular invasion, a process negatively regulated by Notch1 siRNA. The authors concluded that vascular dysfunction in Psoriasis is mediated by Notch1 signaling route and that this pathway could represent a novel therapeutic target (Figure 8).

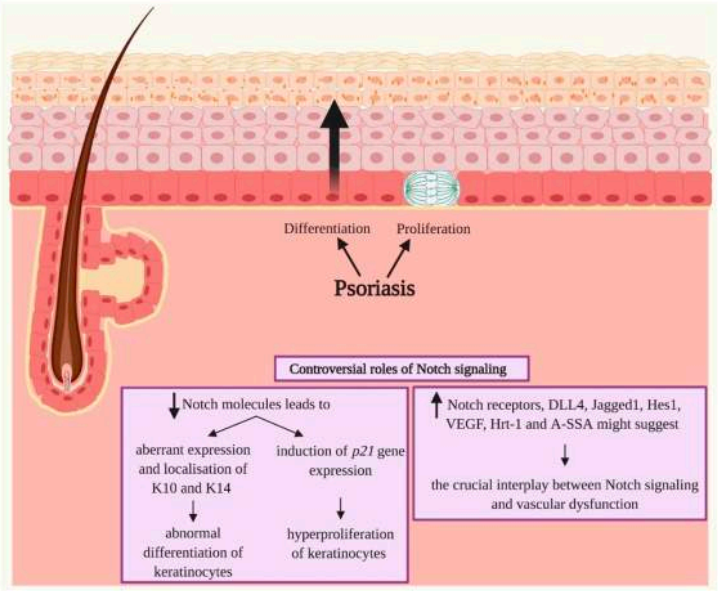

Figure 8. Psoriasis and Notch signaling. Psoriasis is defined as a chronic immune-mediated and inflammatory skin disease characterized by hyperproliferation and aberrant differentiation of epidermal cells, primary cutaneous manifestation and a strong genetic predisposition. The role of Notch signaling in Psoriasis seems to be controversial since both downregulations and upregulations of Notch molecules might be responsible for the onset of the skin disease. A decrement in Notch molecules results primarily in an abnormal differentiation of keratinocytes due to an aberrant expression and localization of keratin 10 (K10) and keratin 14 (K14) in the epidermis, and in the induction of *p21* gene expression leading to hyperproliferation of keratinocytes. A hyperactivation of Notch signaling has also been registered in the skin of psoriatic patients. High expression of Notch receptors, Jagged1, hairy/enhancer of split 1 (Hes1), vascular endothelial growth factor (VEGF), Ring-box protein HRT1 (Hrt-1) and Acute-phase Serum Amyloid A (A-SSA) have been registered, and they suggest the tight link between Notch signaling and vascular dysfunction in Psoriasis. Pointed arrows (→) define the induction of the indicated process or associated function. The arrows facing upwards (↑) indicate an upregulation, while the arrows facing downwards (↓) designate a downregulation of Notch molecules.

3.5. *Atopic Dermatitis (AD)*

Atopic Dermatitis (AD), also called Atopic Eczema, is one of the most common chronic pruritic inflammatory diseases and affects up to 20% of children and adolescents worldwide and oscillates between 2% and 17% in the adult population [135].

The clinical presentation of AD varies widely; indeed, AD patients may exhibit various symptoms including pruritus, xerosis, pain and sleep disturbance, which lead to a severe impairment in quality of life. Furthermore, the progression of the disease is chronic but intermittent [136].

AD is associated with comorbidities such as asthma, allergic rhinitis, food allergies and an increased risk of other inflammatory diseases, such as arthritis and inflammatory bowel disease, though to date the link between these diseases is not yet known [137].

The pathogenesis of AD is multifactorial, and it is thought to occur via a combination of skin barrier abnormalities; immune dysregulation, including excessive T helper-2 cell activity; and genetic and environmental factors [138,139].

A family history of AD is the strongest known risk factor for the development of the pathology [140]. Mutations in the *FLG* gene have been identified as the most common genetic variations involved in AD familial cases (20–30% of AD patients compared with 8–10% of the general population without AD) [86]. *FLG* encodes for a large protein called profilaggrin that is cleaved to produce multiple

copies of the functional FILA protein, which plays an important role in the skin's barrier function [86]. Loss-of-function mutations in the *FLG* gene lead to the truncation of profilaggrin and to a loss of FILA expression.

Major insights into AD reveal an important role for disturbed epidermal differentiation with impaired skin barrier function in the pathophysiology of the disease [141]. Interesting evidence shows that Notch signaling is required for late-stage granular layer differentiation and correct FILA processing in the epidermis [48]. Confirming the close correlation between FILA and Notch signaling, Dumortier et al. observed a downregulation of all Notch receptors expression in the epidermis of lesional skin of AD, whereas healthy control patients exhibited significant Notch expression confined to suprabasal epidermal layers [142]. The same authors confirmed the main role of Notch signaling in AD with skin-specific simultaneous inactivation of Notch1 and Notch2 that induced the development of an AD-like disease in a mouse model. In fact, these adult mice were characterized by acanthosis, dry skin, spongiosis, hyperkeratosis and massive dermal infiltration of eosinophils and mast cells (Figure 9) [142].

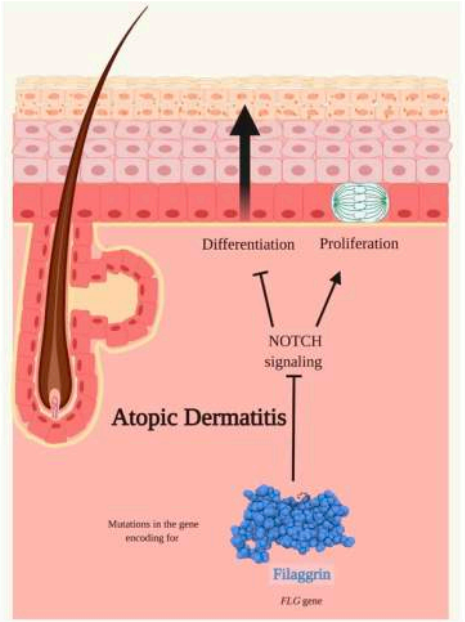

Figure 9. Atopic Dermatitis and Notch signaling. Atopic Dermatitis (AD) is a common, chronic and pruritic inflammatory disease. The pathogenesis of AD is multifactorial and occurs through a combination of disturbed epidermal differentiation with abnormalities in the skin barrier functions, immune dysregulation, and genetic and environmental factors. Mutations in the *FLG* gene encoding for filaggrin have been registered to be the most common variants in AD familial forms. Filaggrin is a late epidermal differentiation protein that possesses a high keratin-binding affinity, interacts exclusively with intermediate filaments to form highly insoluble keratin scaffolds necessary to guarantee the progressive differentiation of keratinocytes and ultimately form the skin barrier. Loss-of-function mutations in the *FLG* gene are common in AD and cause a loss of filaggrin expression and therefore to a disturbed epidermal differentiation. Notch signaling is required for the late-stage granular layer differentiation and correct filaggrin processing in the epidermis. A downregulation of Notch receptors expression has been identified in lesional skin of AD. The inactivation of Notch receptors seems to promote the proliferation of epidermal cells leading, amongst other manifestations, to hyperkeratosis and to impair the correct differentiation program in keratinocytes. Pointed arrows (→) define the induction of the indicated process or associated function, while the truncated arrow (T) designates the inhibition of the described process.

4. Conclusions

With the collaboration of other cellular pathways, Notch signaling orchestrates the regulation of cell proliferation, differentiation, migration and apoptosis of the epidermal cells. Any disruption of canonical and non-canonical Notch signaling, resulting in an up- or downregulation of Notch, either due to aberrant regulation or direct mutations that might lead to both hyper- or hypo-activation, can induce skin diseases. Indeed, the role of Notch signaling can be considered as pleiotropic in light of its involvement in proliferation as well as in differentiation of epidermal cells. In the previously described skin diseases, the relationship between Notch activity, keratinocyte proliferation and differentiation has been thoroughly described, and they gave rise to contradictions: Notch has been reported to be downregulated in all diseases discussed herein, with the exception of Psoriasis and DDD, in which either an upregulation or a downregulation of Notch signaling has been reported. The identified down- or upregulation induced hyperproliferation of keratinocytes in HS, Psoriasis, AOS and AD and abnormal differentiation of keratinocytes in HS, DDD, Psoriasis and AOS; instead, in AD there is a disturbed differentiation in association with decreased epidermal barrier function (Figure 10).

	Notch signaling	Keratinocytes proliferation	Keratinocytes differentiation	References
Hidradenitis Suppurativa	↓	↑	↑	[94,95]
Dowling Degos Disease	↑ ↓		↑	[82,99,101,102]
Adams-Oliver Syndrome	↓	↑	↑	[110,116,117]
Psoriasis	↑ ↓	↑	↑	[124,125,128,130, 131,132]
Atopic Dermatitis	↓	↑	↓	[48,142]

Figure 10. Schematic representation of the interactions between Notch signaling, proliferation and differentiation of keratinocytes in skin diseases. The role of Notch signaling, proliferation and differentiation of keratinocytes in Hidradenitis Suppurativa, Dowling Degos Disease, Adams–Oliver Syndrome, Psoriasis and Atopic Dermatitis. The arrows facing upwards (↑) indicate an upregulation, while the arrows facing downwards (↓) designate a downregulation, of Notch signaling, keratinocyte proliferation and differentiation in the various skin diseases.

Accumulating evidence suggests that alterations of Notch signaling play a crucial role in the pathological features of these skin disorders. The understanding of the exact roles exerted by Notch signaling in all the single skin diseases is important to characterize potential common mechanisms between them, in order to better correlate cell signaling dysfunctions with clinical features and to contribute to the identification of targeted pharmacological intervention.

Unfortunately, apart from skin malignancies, to date, the clinical and preclinical studies regarding skin diseases are still very few and surely need to be implemented in order to apply the knowledge and clarify the various critical and contradictory findings that characterize many skin disorders. For instance, Ma et al. suggest that Notch1 inhibition obtained by γ-secretase blockade induced by *N*-*S*-phenylglycine t-butylester (DAPT) can effectively alleviate the severity of mouse Psoriasis-like skin inflammation [143]. Nevertheless, in Psoriasis, as widely explained in the previous paragraphs, both upregulation or downregulation of Notch signaling have been reported. Therefore, we are convinced that this contribution will be significant for the integration of pathogenetic aspects and its functional consequences on the clinical phenotypes. The progressive definition of this integration is increasingly essential to create a tailored therapeutic approach.

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Abbreviations

NECD	Notch extracellular domain
EGF-like	Epidermal growth factor-like
NRR	Negative regulatory region
LNR	Cysteine-rich Lin12-Notch repeats
HD	Heterodimerization domain
TMD	Transmembrane domain
NICD	Intracellular domain
RBP-Jκ	Recombination Signal Binding Protein for Immunoglobulin Kappa J Region
RAM	Recombination Signal Binding Protein for Immunoglobulin Kappa J Region association module
ANK	Seven ankyrin repeats
NLS	Nuclear localization signals
TAD	Transactivation domain
PEST	Proline/glutamic acid/serine/threonine-rich motifs
DSL	Delta/Serrate/Lag-2
DLL1	Delta-like 1 ligand
DLL3	Delta-like 3 ligand
DLL4	Delta-like 4 ligand
NTD	N-terminal domain
DOS	Delta and OSM-11-like region
CRD	Cysteine-rich domain
PDZ	PSD-95/Dlg/ZO-1
GPI	Glycosylphosphatidylinositol
NTMIC	Notch transmembrane and intracellular domain
NEXT	Notch extracellular truncation
Co-R	Co-repressor
MAML	Mastermind-like
NTC	Notch transcription complex
HES	Hairy/enhancer of split
HEY	Hairy/enhancer of split with YRPW motif
CDK-8	Cyclin-dependent kinase 8
MVBs	Multivesicular bodies
HIF	Hypoxia-inducible factor
Mef2	Monocyte enhancer factor-2
IFE	Interfollicular epidermis
ESC	Epidermal stem cells
K1	Keratin 1
K5	Keratin 5
K10	Keratin 10
PPARγ	Peroxisome-proliferator activated receptor
IVL	Involucrin
TGM	Transglutaminase

PPL	Periplakin
PSEN	Presenilin
PSENEN	Presenilin enhancer-2
NCSTN	Nicastrin
APH1	Anterior pharynx defective-1
POFUT1	GDP-fucose protein O-fucosyltransferase 1
EOGT	Epidermal growth factor domain-specific O-linked N-acetylglucosamine transferase
POGLUT1	Protein O-glucosyltransferase 1
O-GlcNAc	O-linked N-acetylglucosamine
FILA	Filaggrin
FLG	Filaggrin gene
HS	Hidradenitis Suppurativa
PI3K	Phosphoinositide 3-kinase
AKT	Protein Kinase B
DDD	Dowling Degos Disease
KRT5	Keratin 5 gene
K14	Ketatin 14
AOS	Adams–Oliver Syndrome
ACC	Aplasia cutis congenital
TTLD	Terminal transverse limb defects
CTMC	Cutis marmorata telangiectatica congenita
ARHGAP31	Rho GTPase Activating Protein 31
DOCK6	Dedicator of Cytokinesis Protein 6
GEF	Guanine nucleotide exchange factor
Hrt-1	Ring-box protein HRT1
A-SAA	Acute-phase Serum Amyloid A
AD	Atopic Dermatitis
DAPT	*N-S*-phenylglycine t-butylester

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ANEXO A – Resumo publicado "A novel NCSTN mutation is associated with Dowling-Degos Disease and hidradenitis suppurativa in a 4-generation Brazilian family"

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ABSTRACTS

Experimental Dermatology WILEY

11th EHSF Conference 2022 Abstracts

Posters

3. Education, Basic and Translational Research

P-118 | A novel NCSTN mutation is associated with Dowling-Degos disease and hidradenitis suppurativa in a 4-generation Brazilian family

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Background: Dowling-Degos disease (DDD) is a rare autosomal dominant genodermatosis of unknown prevalence characterized by acquired slowly progressive reticulated pigmented lesions primarily involving flexural skin areas, such as axilla, groin, nape, and genital skin. Mutations in the gene encoding for the presenilin enhancer (PSENEN) have been associated with co-occurrence of DDD and hidradenitis suppurativa (HS) in several patients whilst nicastrin (NCSTN) role in DDD is still undervalued [1–3]. We have clinically identified a 4-generation family with six members affected by HS and DDD. DDD was later confirmed by biopsy. The HS+DDD patients have a severe HS phenotype characterized by inflammatory nodules, pyogenic granuloma, fistulas, and single and double comedones in different body areas, and reticulated pigmentations in the armpits and crateriform / cribriform scars on the back, nasal dorsum and lip philtrum commonly seeing in DDD. Whole exome sequencing (WES) in two affected patients and in one unaffected family member identified a novel nonsense mutation of the NCSTN gene. Sanger sequencing confirmed the segregation of this mutation in all affected family members.

Objectives/Methods: To study the activity of this nonsense mutation, we isolated outer root sheath cells (ORS) from hair follicles of the patients and performed real-time PCR and Western Blot analyses.

Results: We showed that mutated patients have a diminished NCSTN mRNA and protein levels thus confirming that the premature stop codon leads to NCSTN nonsense mediated mRNA decay.

Conclusion: Our findings suggest that NCSTN haploinsufficiency could be associated with DDD and HS in some patients and the co-occurrence should be carefully investigated in NCSTN mutated HS patients.

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ANEXO B – Resumo publicado "PRS analysis in patients with hidradenitis suppurativa suggests a shared genetic vulnerability with psoriasis and Crohn's disease"

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PRS analysis in patients with hidradenitis suppurativa suggests a shared genetic vulnerability with psoriasis and Crohn's disease

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Hidradenitis Suppurativa (HS) is a chronic inflammatory skin disease clinically characterized by recurrent painful deep-seated nodules and abscesses commonly found in the apocrine-bearing localization of the body. This cumbersome disorder has a heritability estimated at 80%. While more than one-third of patients has a family history of HS, less than 7% of them showed mutations in one of the three genes encoding the gamma-secretase subunits that have been associated with autosomal dominant forms of the disease. This suggests that many other genes largely contribute to the disease vulnerability. Here, we used an ongoing Genome-Wide Association study (GWAS) in an Italian population of 152 HS patients and 189 unaffected controls to calculate Polygenic Risk Scores (PRS) for HS-associated comorbidities, including psoriasis, atopic dermatitis, Crohn's disease, and type 2 diabetes. We observed that individuals with HS had a higher PRS for psoriasis than controls (p-value = 0.014). Although not statistically significant, PRS for Crohn's disease was also more elevated in HS patients than in healthy individuals (p-value = 0.08). These results suggest that a genetic predisposition to HS may be mediated by common variants associated with psoriasis and Crohn's disease.