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XXXV CICLO DEL DOTTORATO DI RICERCA IN

Scienze della Riproduzione e dello Sviluppo

Università degli Studi di Trieste

-Omic approaches to unravel Hidradenitis Suppurativa etio-pathogenesis

Settore scientifico-disciplinare: MED/05

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RIASSUNTO

L’idrosadenite suppurativa è una patologia infiammatoria cutanea altamente debilitante caratterizzata dalla presenza di noduli, ascessi e fistole in regioni corporee ricche di ghiandole apocrine. La patogenesi dell’HS è complessa e multifattoriale e in essa è stata identificata una stretta interazione di fattori genetici, immunologici, infettivi e ormonali, sebbene gli esatti meccanismi molecolari alla base di questi fenomeni non siano stati ancora completamente caratterizzati. Un ulteriore livello di complessità è dato dal fatto che comunemente l’HS può manifestarsi anche in combinazione con altri disordini dando luogo a varianti sindromiche in cui l’HS rappresenta una manifestazione cutanea caratteristica. Nonostante siano state di recente identificate delle varianti genetiche potenzialmente associate alla suscettibilità e all'insorgenza di tali condizioni cutanee, la diagnosi di questi disordini è ancora esclusivamente basata sull'obiettività clinica poiché spesso i pazienti non condividono le stesse mutazioni e comunemente le mutazioni identificate non sono informative dei processi biologici che risultano essere alterati nei pazienti stessi. Lo scopo di questo lavoro è stato quello di aumentare le conoscenze relative all’eziopatogenesi dell’HS e delle sue varianti sindromiche (PASH, PAPASH, PASH/SAPHO) tramite approcci di natura -omica. Al fine di scoprire alterazioni genetiche più strettamente correlate all'eziopatogenesi delle sindromi PASH e PAPASH, identificando nuove vie di segnalazione cellulare, abbiamo eseguito un approccio di sequenziamento dell'intero esoma con una nuova pipeline bioinformatica mirata a scoprire geni potenzialmente candidati selezionati dalla densità di mutazioni e coinvolti in pathways rilevanti per le patologie in oggetto. Abbiamo trovato 90 geni portatori di mutazioni, tra cui 14 geni - in comune tra i primi cinque pazienti con HS sindromica esaminati (4 PASH e 1 PAPASH) - che presentavano 237 varianti non sinonime localizzate in regioni codificanti (54 e 183 in omozigosi ed eterozigosi, rispettivamente). Nell'analisi di "pathway enrichment" sono stati inclusi solo dieci geni, che ci hanno portato a scoprire quattro vie di segnalazione cellulare condivise da tutti i pazienti: i) metabolismo della vitamina D, ii) cheratinizzazione, iii) formazione dell'involucro cornificato e iv) metabolismo degli steroidi. L'analisi è stata poi estesa per verificare come queste quattro vie del segnale fossero alterate da tutte le mutazioni funzionali in ciascun paziente; le vie del metabolismo della vitamina D e della cheratinizzazione presentavano un'alta densità di varianti nei nostri cinque pazienti, confermando così che le disfunzioni del metabolismo della vitamina D sembrano essere cruciali nella patogenesi delle sindromi PASH e PAPASH e corroborando la recente opinione secondo cui l'HS e le sue forme

sindromiche possono essere considerate come un potenziale sottotipo di malattia autoinfiammatoria della cheratinizzazione.

Per cercare di stabilire una prima correlazione genotipo-fenotipo in dieci pazienti affetti dalle principali forme sindromiche di HS (PASH, PAPASH e PASH/SAPHO), abbiamo effettuato uno studio dell'intero esoma. Sono stati identificati tre "setting" clinici in base alla presenza/assenza di infiammazione intestinale e articolare. Da un punto di vista genetico abbiamo trovato varianti in geni appartenenti al pathway dell'autoinfiammazione e della cheratinizzazione: quattro pazienti con PASH, che presentavano anche un'infiammazione intestinale, mostravano tre diverse varianti nel gene *NOD2*, due varianti in *OTULIN* e una variante in *GJB2*, mentre tre pazienti con PAPASH e tre con PASH/SAPHO, con concomitante infiammazione articolare, presentavano due diverse varianti nel gene *NCSTN*, una in *WDR1* e *PSTPIP1* e due varianti in *NLRC4*, una delle quali era presente anche in un paziente con fenotipo misto caratterizzato sia da infiammazione intestinale che articolare. Questo studio - anche se le validazioni funzionali di alcuni geni di interesse sono in corso - rappresenta un primo passo preliminare per una correlazione genotipo-fenotipo e conferma la natura autoinfiammatoria poligenica dell'HS sindromica, in questo contesto strettamente correlata all'infiammazione articolare e intestinale. Inoltre, il coinvolgimento di geni legati alla via della cheratinizzazione ci ha permesso di confermare che una via di cheratinizzazione geneticamente disturbata può contribuire alla patogenesi dell'infiammazione cutanea nell'HS sindromica.

Al giorno d'oggi, per comprendere meglio la complessità di alcune patologie, sono necessari strumenti di analisi -omica che si concentrino sui pathways perturbati piuttosto che sulle varianti geniche associate. Abbiamo così sviluppato "Variant Enrichment Analysis" (VEA), uno strumento applicabile ai dati ottenuti dal sequenziamento dell'intero esoma, in grado di rivelare le differenze tra il numero di varianti genetiche presenti in un determinato pathway rispetto a un set di dati di riferimento. Abbiamo quindi utilizzato questo metodo per scoprire nuovi pathways alterati in dodici pazienti con disturbi cutanei autoinfiammatori complessi, quali PASH, PAPASH e PASH/SAPHO, identificando pathways correlati all'omeostasi/attivazione dei neutrofili e delle cellule endoteliali, come perturbati nei nostri pazienti. Questi risultati ci hanno portato a ipotizzare che la migrazione transendoteliale sregolata dei neutrofili possa provocare un aumento dell'infiltrazione neutrofilica e del danno tissutale, riflettendo la classificazione dell'HS e delle sue forme sindromiche nelle dermatosi neutrofiliche, condizioni caratterizzate da un accumulo di neutrofili nella cute e, raramente, negli organi interni, che condividono aspetti clinicopatologici con le malattie autoinfiammatorie.

In conclusione, nel contesto di queste condizioni che presentano un'eziologia multifattoriale con una forte eterogeneità e complessità genetica, il nostro studio evidenzia l'importanza cruciale di eseguire nuovi approcci volti a identificare potenziali nuovi geni e vie di segnalazione cellulare coinvolti nella patogenesi dell'HS e delle sue forme sindromiche. Abbiamo confermato la natura autoinfiammatoria poligenica dell'HS, la sua possibilità di essere definita come una malattia autoinfiammatoria della cheratinizzazione e il ruolo dei neutrofili in queste malattie complesse.

ABSTRACT

Hidradenitis suppurativa (HS) is a chronic-relapsing, debilitating autoinflammatory skin disease of the pilosebaceous unit, whose pathogenesis is very complex and, to date, has not yet been fully elucidated. A close interaction between genetic, epigenetic factors, host-specific aspects, and environmental influences contribute to the susceptibility, onset, severity, and clinical progression of this disease. Furthermore, an innate and adaptive immunity dysfunction leading to autoinflammation has been reported to play a pivotal role with upregulation of proinflammatory cytokines such as IL-1β and IL-17, chemokines, and tumour necrosis factor (TNF)-α both in lesional skin and in serum of HS patients.

Based on genetic heterogeneity and complexity, three different forms can be recognized and considered separately as sporadic, familial, and syndromic. The clinical triad of pyoderma gangrenosum (PG), acne, and suppurative hidradenitis (PASH) represents the prototype of syndromic HS, and it is now considered an autoinflammatory syndrome within the spectrum of neutrophilic dermatoses, conditions hallmarked by an accumulation of neutrophils in the skin, and, rarely, internal organs. PASH with the presence of pyogenic sterile arthritis represents a distinct entity, namely PAPASH - pyogenic arthritis, PG, acne, HS -, while synovitis, acne, pustulosis, hyperostosis, and osteitis (SAPHO) is another autoinflammatory disease in which HS lesions could be a common dermatologic manifestation. Syndromic HS patients are generally young people with poor life quality due to skin involvement, frequent association with gastrointestinal and rheumatological diseases and in most cases, refractoriness to standard treatments.

A range of genetic changes have been involved in syndromic HS pathogenesis, including mutations in Nicastrin (*NCSTN*) and in other genes encoding for the γ-secretase complex subunits, as well as genetic variations in autoinflammatory genes including, among others, *PSTPIP1* or *MEFV*.

Genetic diagnosis of syndromic forms of HS, despite novel variations recently identified, is just descriptive, since most cases are not sharing the same mutations, and the mutations themselves are not informative of the biological signaling pathways commonly disrupted in these patients. As an example, some recent works failed to reveal mutations in genes already described in the literature, leading to assume new pathomechanisms underlying these complex conditions. To date, a main impaired pathway associated to HS is the Notch pathway; a deficiency in Notch signaling has been observed mainly in familial cases of HS, associated with loss of function

pathogenic variants in γ -secretase genes. Nevertheless, functional studies demonstrated that several *NCSTN* missense variants causing HS are functional and promote Notch signalling, counteracting the view that pathogenic variants in genes encoding γ-secretase components cause disease simply as a result of haploinsufficiency that leads to impaired Notch signalling pathway.
In this critical context, - omic approaches can be regarded as fundamental diagnostic and research tool for the detection of rare variants, enriched in syndromic HS patients, providing the identification of several genes as well as signaling pathways potentially associated to the susceptibility, onset, and progression of these diseases, additionally prompting the development of pathogenesis-targeted treatments.

To reveal genetic changes more closely related to PASH and PAPASH etiopathogenesis, identifying novel common biological signaling pathways involved in these skin diseases, we performed a whole exome sequencing (WES) approach with a novel bioinformatic pipeline aimed at discovering potentially candidate genes selected from density mutations and involved in pathways relevant to the diseases. We found 90 genes carrying damaging mutations, among which, 14 genes were in common among the 5 patients (4 PASH and 1 PAPASH) and bared 237 non-synonymous (ns) variants located in the coding regions (ExonVar) (54 and 183 in homozygosis and heterozygosis, respectively). In the pathway enrichment analysis, only 10 genes were included, leading us to discover four pathways shared by all patients: i) vitamin D metabolism, ii) keratinization, iii) formation of the cornified envelope and iv) steroid metabolism. Then, the analysis has been extended to check how these four pathways were altered by all functional mutations in each patient; vitamin D metabolism and keratinization pathways presented high variant density in our five patients, thus confirming that vitamin D metabolism dysfunctions appear to be crucial in PASH and PAPASH pathogenesis and corroborating the recent view that HS and its syndromic forms can be regarded as a potential subtype of autoinflammatory keratinization disease (AIKD).

To describe clinical features and genetic signature of ten patients with the main syndromic HS forms (PASH, PAPASH, and PASH/SAPHO overlapping syndrome), we conducted a WES approach. Three clinical settings have been identified by the presence/absence of gut and joint inflammation. From a genetic point-of-view we found variants in both autoinflammatory and keratinization genes: four PASH patients, also presented with gut inflammation, showed three different variants in *NOD2* gene, two variants in *OTULIN*, and a variant in *GJB2*, respectively,

whereas three PAPASH and three PASH/SAPHO overlapping patients, with concomitant joint inflammation, exhibited two different variants in *NCSTN* gene, one in *WDR1* and *PSTPIP1*, and two variants in *NLRC4*, one of whom was present in a patient with a mixed phenotype characterized by gut and joint inflammation. This study – although functional validations of some genes of interest are in progress - represents a first preliminary step for a genotype-phenotype correlation and confirms the polygenic autoinflammatory nature of syndromic HS, in this context, closely related to joint and gut inflammation. Additionally, the involvement of genes related to keratinization pathway allowed us to confirm that a genetically disrupted keratinization pathway may contribute to the pathogenesis of cutaneous inflammation in syndromic HS.

Nowadays, genomic analysis tools focusing on disrupted pathways rather than associated gene variants are required to better understand the complexity of certain diseases. We developed the Variant Enrichment Analysis (VEA) workflow, a tool applicable for WES data, capable of revealing differences between the numbers of genetic variants present in a given pathway in comparison with a reference dataset. We thus applied VEA to discover novel pathways impaired in twelve patients with complex autoinflammatory skin disorders, namely PASH, PAPASH and PASH/SAPHO overlapping, identifying pathways related to neutrophils and endothelial cells homeostasis/activation, as disrupted in our patients. These findings lead us to assumed that unregulated neutrophil transendothelial migration could elicit increased neutrophil infiltration and tissue damage, reflecting the classification of HS and its syndromic forms in neutrophilic dermatoses, conditions hallmarked by an accumulation of neutrophils in the skin and, rarely, internal organs, sharing clinicopathological aspects with the autoinflammatory diseases.
Our VEA approach is applicable in situations of individual reports or in cases with low numbers of subjects; moreover, although skin transcriptomics and proteomics of our patients is not, to date, available, this novel VEA approach - based only on WES data - contributes to changing the vision of genetic analysis and its objectives (i.e., genetic variants associated with certain diseases), thus representing a modern tool for translating exome variants in a broader pathological context.

In conclusion, in the context of these disorders presenting a multifactorial aetiology with a strong genetic heterogeneity and complexity, our study highlights the crucial importance of performing novel -omic approaches, aimed at identifying potential novel genes and biological

signaling pathways involved in the pathogenesis of HS and its syndromic forms. We confirmed the polygenic autoinflammatory nature of HS, its possibility to be defined as an autoinflammatory keratinization disease and the role of neutrophils in these complex diseases.

Further studies in this direction are necessary to shed more light on the pathophysiology of these diseases to bring us closer to pathogenesis-targeted treatments.

This PhD dissertation has been conducted at Dermatology Unit of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milan with the close collaboration of the immunology genetics laboratories of the IRCCS Burlo Garofolo of Trieste and the Department of Pathology of the Federal University of Pernambuco (Recife, Brazil)

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1.INTRODUCTION

1.1 Hidradenitis suppurativa

Hidradenitis suppurativa (HS) is a chronic-relapsing, debilitating autoinflammatory skin disease of the terminal follicular epithelium, clinically characterized by deep-seated nodules, usually ending in abscesses and draining fistulous tracts, with disfiguring hypertrophic scarring of apocrine gland-bearing skin, such as axillary, inguinal, and mammary folds as well as the anogenital areas [Johnston et al., 2022].

Neutrophilic dermatoses (NDs) encompass a wide spectrum of conditions that are hallmarked by an accumulation of neutrophils in the skin and, rarely, internal organs; they share clinicopathological aspects with the autoinflammatory diseases, which present with recurrent episodes of sterile inflammation in the affected organs, in the absence of high title of circulating autoantibodies and autoreactive T cells [Marzano et al., 2019]. Neutrophilic diseases may be categorized into three main groups: (i) hypodermal forms whose paradigms are pyoderma gangrenosum and hidradenitis suppurativa, (ii) dermal forms whose prototype is Sweet's syndrome and (3) epidermal forms among which amicrobial pustulosis of the folds may be regarded as the model. A fourth subset of epidermal/dermal/hypodermal forms has been recently added to the classification of neutrophilic diseases due to the emerging role of the syndromic hidradenitis suppurativa variants, whose pathogenesis has revealed a relevant autoinflammatory component [Marzano et al., 2019; Tricarico et al., 2019].

The prevalence of HS is estimated to range between 0.05-4% in Europe, showing a female predominance with a female-to-male ratio of 3:1 [Deckers et al., 2014] and a positive family history in one-third of patients [Elkin et al. 2020]. HS generally occurs after puberty, during the second or third decade of life, although a prepubertal and post-menopausal onset has been also reported in the literature [Seyed Jafari et al., 2020; Seyed Jafari et al., 2018]. The diagnosis of HS is still problematic mainly due to the absence of specific pathognomonic tests and the numerous possible differential diagnoses with other skin diseases with overlapping clinical manifestations, thus leading to a diagnostic delay [Lindhardt Saunte and Jemec, 2017; Harvey and Fortson, 2021].

This improper management of the disease can have a negative impact on the patient's quality of life [Elkin et al., 2020; Lindhardt Saunte and Jemec, 2017]; indeed, although the skin is the

primary affected site, HS is known to lead to the appearance of severe psychological and physiological sequelae. Psychological comorbidities, including depression and anxiety, have been seen to be common amongst HS cases and generally stem from chronic pain, movement limitations and malodour due to purulent secretions [Elkin et al., 2020; Sabat et al., 2020; Vossen et al., 2018]. Furthermore, HS patients are often affected by other cutaneous and systemic conditions such as metabolic syndrome, androgen dysfunction and autoinflammatory disorders [Frew et al., 2019], suggesting the coexistence of a pathogenetic link between HS, metabolic and hormonal dysfunction, and impairment of the inflammatory network.

To date, HS patients are not subjected to a standard therapy and therapeutic strategies are strongly influenced by the severity of the disease. Future treatments should be guided by personalized therapies, biomarkers, and pharmacogenomics to maximise clinical outcomes and limit adverse events according to specific patient characteristics at both genetic and phenotypic levels [Scala et al., 2021].

1.2 Clinical presentation

The diagnosis of HS is based on the clinical aspects and history of disease. Based on the modified Dessau definition, three diagnostic criteria should be fulfilled: presence of characteristic lesions, typical location, and chronicity [Zouboulis et al., 2015].

These three criteria alone generally give high diagnostic sensitivity and specificity.

Typical lesions include deep-seated painful nodules, commonly ending into abscesses and sinus tracts – these latter composed by invasive proliferative gelatinous material - multiple comedones and disfiguring hypertrophic scars (**Figure 1**) [Revuz J., 2009].

Nodules and subcutaneous abscesses can rupture, bleed, and produce purulent secretions, resulting in a chronic process that causes scarring adhesions and fibrosis of the injured skin. Nodules are typically inflammatory, warm, painful and deep; they may be solitary or multiple and persist for several days or months [Jemec G., 2012]. They are often the first manifestation observed in a patient with HS and their regression often occurs following the discharge of purulent secretion. Abscesses, in most cases, represent the evolution of inflammatory nodules; they may open towards the skin surface releasing a purulent or serous secretion, resulting in subjectively improved pain and skin tension.

The formation of multiple nodules in the same skin area can lead to the formation of intercommunicating sinus tracts and fistulae. Hypertrophic scars, not infrequently, represent the evolution of nodules, abscesses, and fistulae; sometimes the scarring evolution may reduce, due to tissue retraction, the mobility of certain body districts, or cause lymphatic obstruction, with possible evolution into lymphoedema, especially in the vulvar or scrotal area.

Anatomical areas commonly affected include the axillary, infra- and intermammary, inguinal, perineal, perianal, and gluteal regions [Revuz and Jemec, 2016]. Localisations to the lower abdomen, suprapubic, retro auricular, neck, eyelids and scalp are reported less frequently [Agut-Busquet et al., 2019].

Patients may experience pain, itching, burning sensation and local heat.

Chronicity, defined as the presence of at least two recurrences within six months, is a distinctive feature of HS.

Other factors supporting the diagnosis are the following: i) a family history of HS, ii) history of other follicular occlusion diseases (e.g., pilonidal cysts), iii) presence of furuncle-like lesions in the rubbing sites (e.g., belt or inner thigh) and iv) absence of pathogenic microbes to explain a primary infection.

However, the phenotypic heterogeneity of HS is high, probably due to the multifactorial aetiologies underlying this disease, which to date are still not fully understood.

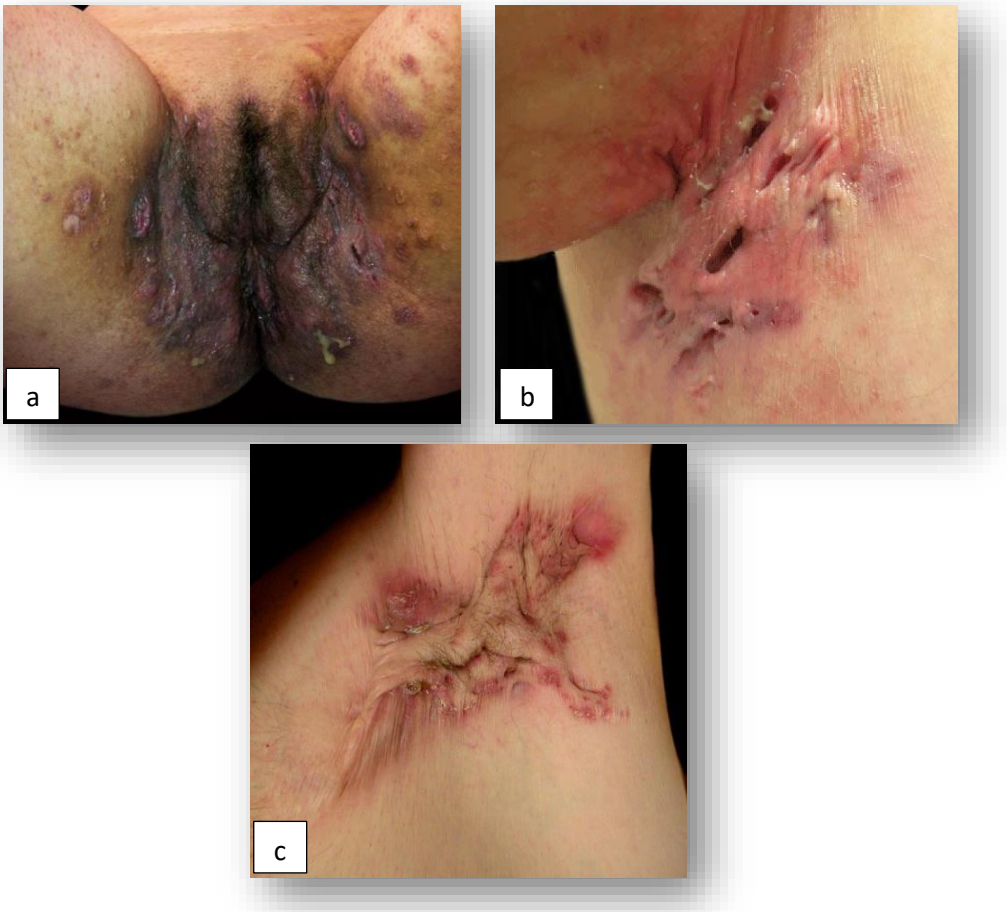

Figure 1: Clinical features of HS. a) inflammatory nodules, sinus tracts with purulent exudate and scarring; b) fistulae; c) retracting scars involving the axillary region

1.3 Disease scoring systems

The severity of the disease can be evaluated using various scoring systems.

Hurley staging [Hurley, 1989] is a 3-stage classification of disease severity:

- Stage I: single or multiple, isolated lesions without fistulae and hypertrophic scars; it is the most frequent stage affecting 68% of patients;
- Stage II: recurrent, unconnected abscesses with formation of fistulae and hypertrophic scars; it occurs in 28% of cases;
- Stage III: disseminated/widespread disease or confluent/interconnected abscesses/fistulae; it is present in the remaining 4% of cases.

Subsequently, a revised version of Hurley's staging system has been proposed; this latter divides stages into mild (A), moderate (B) and severe (C), thus reflecting more accurately the severity of the disease [Horvath et al., 2017].

In 2003, the staging of Sartorius et al. [Sartorius et al., 2003] has been introduced, later updated in 2007 in Modified Sartorius score, a more dynamic and detailed severity classification tool, in which i) the anatomical region involved – by awarding 3 points for each area -, ii) the number of lesions, iii) the distance between lesions, and iv) the presence or absence of healthy skin between lesions have been considered [Sartorius et al., 2009].

The Hidradenitis Suppurativa Physician's Global Assessment scale (HS-PGA) [Pascoe et al., 2015] categorizes HS into six degrees of progressive severity (clear, minimal, mild, moderate, severe or very severe) based on number of nodules, abscesses and fistulae:

1. Clear: absence of nodular lesions;
2. Minimal: only non-inflammatory nodules;
3. Mild: <5 inflammatory nodules or 1 abscess/draining fistula without inflammatory nodules;
4. Moderate: <5 inflammatory nodules or 1 abscess/draining fistula with at least 1 inflammatory nodule or 2-5 abscesses/draining fistulae and <10 inflammatory nodules;
5. Severe: 2-5 abscesses/draining fistulae and ≥ 10 inflammatory nodules;
6. Very severe: >5 abscesses or draining fistulae.

Since it has been observed that these clinical measures may not be optimal in assessing treatment effectiveness, the Hidradenitis Suppurativa Clinical Response (HiSCR) has been developed to evaluate clinical response in HS patients. This instrument is exclusively designed for assessing treatment response, based on reduction of inflammatory nodules and abscesses, but not to evaluate disease severity cross-sectionally [Kimball et al., 2015].
The attempt to introduce a novel tool that could be easily used in daily clinical practice led to the creation of the International HS Severity Score System (IHS4), in which the final IHS4 score (points) corresponds to number of nodules multiplied by 1 + number of abscesses multiplied by 2 + number of draining tunnels (fistulae/sinuses) multiplied by 4; a score of 3 or less corresponds to mild HS, a score of 4–10 signifies moderate HS, and a score of 11 or higher implies severe HS. The IHS4 score system represents a quick and useful tool for assessing

disease severity both in clinical trials and in real-life, and to monitor clinical and treatment outcomes [Zouboulis et al., 2017] (**Figure 2**).

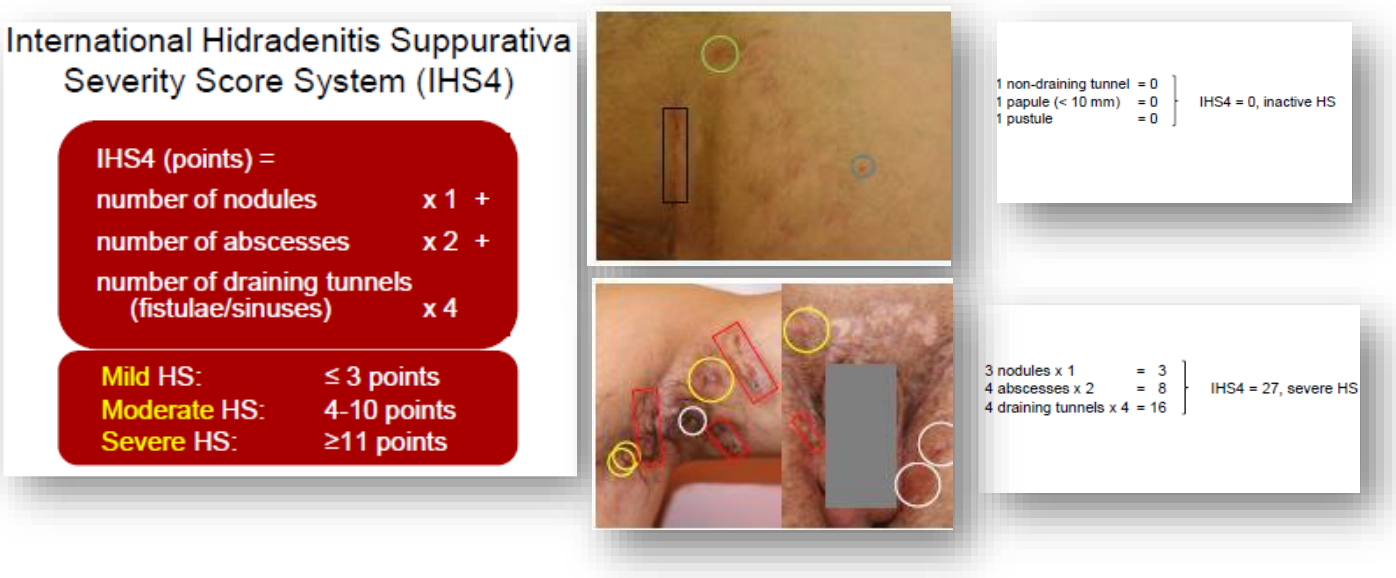

Figure 2: International Hidradenitis Suppurativa Severity Score System (IHS4) [Zouboulis et al., 2017]

IHS4-55, a novel dichotomous IHS4 version, based on a 55% reduction of the total score, was developed for both clinical trials and daily clinical practice [Tzellos et al., 2022].

All these tools are based only on physician-assessed clinical parameters and lack a patient-centred measure able to evaluate the overall burden of HS on patient quality of life.
A recently developed HS severity score, the Severity Assessment of Hidradenitis Suppurativa (SAHS) score, includes two patient-reported outcomes: the number of new boils or number of existing boils that flared up during the past 4 weeks and the assessment of current severity of pain of the most symptomatic lesion during the patient's daily activities, that are ranked on a numerical rating scale [Hessam et al., 2018].

In line with this unmet need, HIDRAscore, a novel and integrated tool has been assessed. Along with the clinical parameters considered critical to assess disease severity according to experts in HS research, this tool includes a specific quality-of-life instrument, the HIDRAdisk, which

covers different aspects of the effect of HS on patients in a comprehensive clinical severity and quality-of-life measure, thus adding more complete information on the effect of HS on patient's quality of life and enabling a more in-depth assessment of the psychosocial condition of the patient with HS [Marzano et al., 2020].

1.4 Phenotypic classification

The above-mentioned scoring systems could not reflect the wider clinical spectrum of HS; for these reasons, considering the clinical variability of the disease and the lack of a homogeneous classification, it has been proposed a phenotypic classification of patients affected by HS.
HS patients can be stratified into different groups in relation to the presence of comorbidities and to the type and localisation of lesions.
Van der Zee et al. [Van der Zee et al., 2015] proposed to classify HS in six clinical phenotypes:

- Regular type, comprising most cases of HS which satisfy all the fixed diagnostic criteria;
- Frictional furuncle type, characterized by deep-seated nodules and abscesses in body areas subjected to enhanced mechanical friction (buttocks, legs, and abdomen), which rarely evolve into fistulae;
- Scarring folliculitis type, in which HS patients, who are commonly smokers and overweight, present classical superficial lesions (Hurley stage I) frequently followed by cribriform scarring and double-ended comedones, mainly localized in the pubic region, buttocks and the groin area;
- Conglobata type, that includes moderate and severe HS cases bearing a positive family history of the disease that are characterised by cysts and acne conglobata lesions primarily localised on the face and upper back;
- Ectopic type, in which the main area involved is the face
- Syndromic type: in patients with concomitant diseases such as inflammatory bowel disease (IBD), arthritis, and autoinflammatory syndromes. In this context, it is possible to identify a spectrum of syndromes associated with HS including: PASH syndrome, caused by the clinical triad of pyoderma gangrenosum (PG), acne, and suppurative hidradenitis (PASH); PASH with pyogenic arthritis (PA), namely PAPASH; psoriatic arthritis, PG, acne and HS (PsAPASH); pustular psoriasis, arthritis, PG, synovitis, acne and HS (PsAPSASH), and PG, acne, HS, and ankylosing spondylitis (PASS) and

finally, the syndrome of synovitis, acne, pustulosis, hyperostosis, and osteitis (SAPHO), which incorporates a combination of cutaneous and articular symptoms including HS as a common cutaneous manifestation.

Subsequently, Frew et al., [Frew et al., 2019] assessed the inter-rater reliability of HS phenotypes described in the literature and based on genotype-phenotype correlation, proposing a revision of the classification limiting it into a: (i) typical HS, corresponding to the regular type, (ii) atypical HS, including scarring folliculitis and conglobata types, and (iii) syndromic HS.

Finally, Dudnik et al., [Dudnik et al., 2021] based on real-life observation on a Dutch cohort of patients, suggested that the ectopic and syndromic phenotypes are not specific, lacking distinctive clinical features and could be categorized as one of the other phenotypes; interestingly, a positive family history did not differ between the phenotypes.

1.5 PATHOGENESIS

1.5.1 Genetics

The genetic scenario of HS in its different forms is still to be unravelled, as is considering recent growing evidence provided by the multiplicity of Next Generation Sequencing (NGS) tools and functional cell and organoids models for the validation of genetic variants.
Despite the high heritability estimates of HS (77–80%), only a minority of HS patients demonstrate a strong monogenic aetiology in the context of familial or syndromic HS (5%) [Kjaersgaard Andersen et al., 2021]. Nevertheless, common forms of the disease demonstrate familial segregation but to date, the exact nature of the genetic variants driving these common forms of HS remains unelucidated.

Familial Cases of HS

Familial forms of HS have been reported in one-third of cases (OMIM: # 142690 ACNE INVERSA, FAMILIAL, 1; ACNINV1 and # 613736 ACNE INVERSA, FAMILIAL, 2, ACNINV2) and an autosomal dominant inheritance pattern with an incomplete penetrance has been described, although the described causative monogenic mutations are just explained by/associated with approximately 5% of familial HS cases [Jemec GB, 2012; Kjaersgaard Andersen et al., 2021].
The first genetic study on HS has been performed by Fitzsimmons et al., in 1984 on a total of 21 members deriving from 3 UK families showing a transmission of the disease through three generations [Fitzsimmons et al., 1984]. The next year, the study has been extended to other 23 families that allowed to conclude, based on the familial segregation and the number of affected individuals, that the disease's transmission was possibly indicative of a single gene (mendelian) disorder inherited as an autosomal dominant trait [Fitzsimmons et al., 1984]. Their observations were not strictly consistent with the designed inheritance pattern since the frequency of affected first-degree relatives has been found to be of 34% against the expected 50% for an autosomal dominant disease. In addition, some of the analysed families revealed that more women were affected than man, showing the 3:1 female-to-male ratio that is currently confirmed by several epidemiological studies [Garg et al., 2017], while in other families a male-to-male transmission

resulted to be favoured. Taken together, these findings seemed to strongly suggest the impossibility of defining HS as a 'single gene, single trait' disorder.

In 2000, J M Von Der Werth et al., [Von Der Werth et al., 2000] performed a re-examination of the familial cases identified 15 years earlier by Fitzsimmons et al., with a more accurate clinical characterization, highlighting the concept that HS is a mendelian disorder with an autosomal dominant inheritance pattern.

In 2006, M Gao et al., [Gao et al., 2006], on a HS Chinese four-generation family, composed by nine HS patients (four females and five males), described a locus for HS located on chromosome 1p21.1–1q25.3, but no specific gene has been identified due to the considerable size of the genomic region.

Only in 2010, B. Wang et al. [Wang et al., 2010] recognized Nicastrin (*NCSTN*) as a specific gene located at 1q23.2 and subsequently, in all available HS familial patients, independent heterozygous loss-of-function pathogenic variants in three different genes *NCSTN*, *PSENEN* (Presenilin Enhancer, 19q13.12) and *PSEN1* (Presenilin 1, 14q24.2) have been found, thus discovering the first typical pool of mutations occurring in familial forms of HS (**Figure 3**).

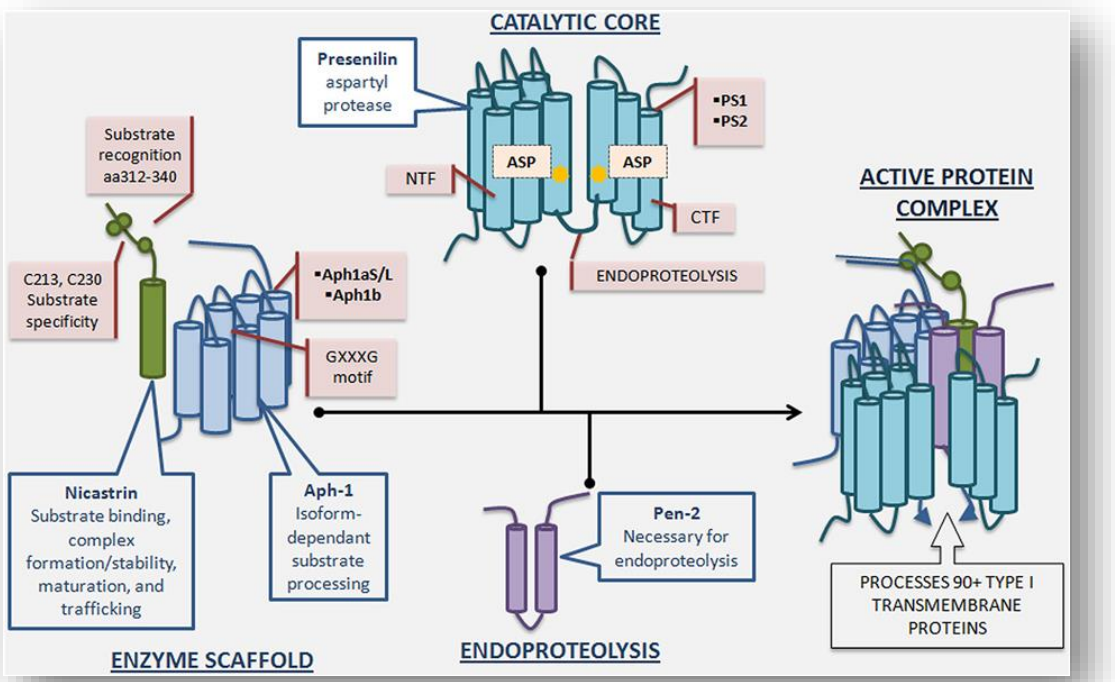

Figure 3: Secretase complex formation and regulatory roles of individual essential subunits. The γ-Secretase complex is formed by the sequential assembly of Aph1, nicastrin, presenilin, and Pen-2. First, Aph-1 and nicastrin come together to form the scaffold. Next, full length presenilin is incorporated. Last, Pen-2 is recruited and full length presenilin is endoproteolysed into presenilin-NTF/CTF, activating the enzyme (Gertsik et al., 2015).

Interestingly, these genes encode for three of the four subunits of the γ-secretase complex (GSC), even though some studies failed to reveal the causative role of γ -secretase gene mutations in HS familial cases. Pink et al. in 2011 [Pink et al., 2011], conducted a UK study focusing on 53 individuals from seven multigenerational pedigrees; the screening of *NCSTN*, *PSEN1*, and *PSENEN* genes did not reveal pathogenic variants in five out of the seven pedigrees examined. Furthermore, in a French study [Miskinyte et al., 2012], only three out of fourteen pedigrees revealed the causative involvement of GSC genes, and similarly, mutational analysis of *NCSTN*, *PSEN1*, and *PSENEN* in twenty familial HS patients referred to a tertiary U.K. clinic, revealed only two pathogenic variants in the coding region and splice site of *NCSTN* gene [Pink et al., 2012]. In a South Wales cohort of twelve familial HS subjects, sequencing analysis of all GSC genes, including the seven-transmembrane-domain protein, anterior pharynx defective 1 homolog A (APH1A) and the paralogous anterior pharynx defective 1 homolog B (APH1B), failed to reveal HS-associated mutations; most of the variants identified

were intronic or synonymous substitutions without a functional impact on protein and correlation with the clinical phenotype. Taking together all these findings, it could hypothesize that in several HS familial individuals, the contribution of GCS genes is not pivotal or at least should be more deeply elucidated.

As evidence of this Jiffri et al. [Jfri et al., 2020] reported two novel causal pathogenic variants in *MEFV* (MEditerraneanFeVer) and *NOD2* (Nucleotide Binding Oligomerization Domain Containing 2) genes in a two-generation HS family, contributing to the evidence for a polygenic presentation mode for this disease, although further studies on familial HS are needed to highlight the pathophysiological mechanisms underlying this condition.

γ-secretase complex and Notch signaling in the skin

Notch signaling has been shown to exert crucial functions in the skin, mainly by mediating the proper maintenance of epidermal homeostasis, strictly regulating the balance between cell differentiation and proliferation programmes in epidermal cells. [Rangarajan et al., 2001; Gratton et al., 2020]. Notch 1-4 receptors and functional ligands (Jagged 1, Jagged 2 and Delta Like Canonical Notch Ligand 1 - DDL1) have a differential distribution throughout the layers of the skin, thus strongly indicating that their distinctive localisation pattern is specifically linked to the expression of layer-specific genes [Watt et al., 2008]. The binding of Notch receptors to Jagged 1 and Jagged 2 promotes keratinocyte terminal differentiation [Blanpain et al., 2006], while interactions between Notch and DLL1 occur in the basal layer and are involved in the maintenance of epidermal stem cells (ESCs) in an undifferentiated state [Blanpain et al., 2006]. Notch signalling has also been reported to play a pivotal role in maintaining the homeostasis of skin appendages. Specifically, in hair follicles, high expression levels of Notch receptors (Notch1-3) and ligands (Jagged 1 and Jagged 2) are found mainly at the base of the follicle in clusters of cells undergoing active proliferation and more distally in cells engaged in terminal differentiation [Watt et al., 2008] (**Figure 4**).

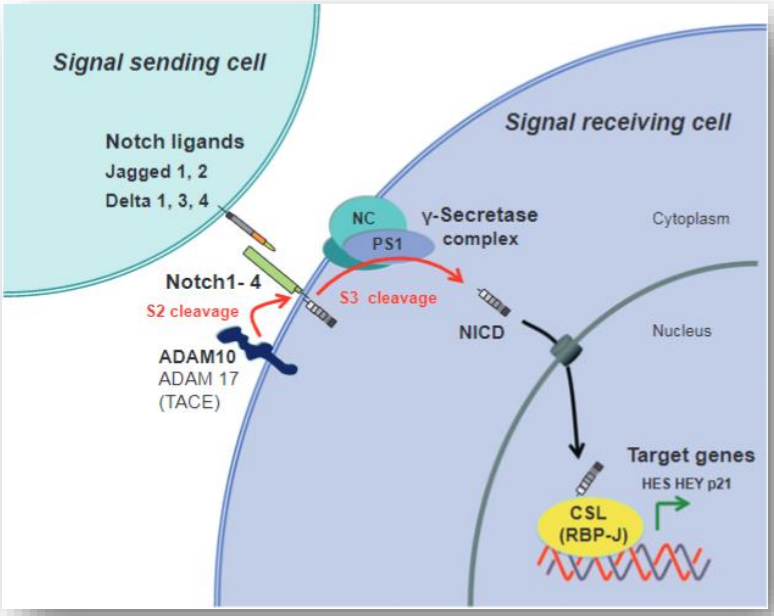

Figure 4: Notch signaling. After proteolytic cleavage (S2) of the extracellular domain of the Notch receptor by ADAM metalloproteinase, the following intramembrane proteolytic cleavage (S3) of Notch by the γ-secretase complex releases Notch intracellular domain (NICD), which enters the nucleus and assembles the CSL family transcription factor complex (also designated as RBP-J). This complex activates the HES (Hairy and enhancer of split) and HEY (Hairy and enhancer of split related) target genes involved in epidermal differentiation and immune regulation. NC, nicastrin; PS1, presenilin1; ADAM17=TACE=TNFα-converting enzyme (Melnik et al., 2013).

It was seen that functional alterations of the γ-secretase complex in the skin did not impair Notch signaling during early embryonic morphogenesis, whereas in postnatal skin, loss of function of this complex had a direct impact on cell proliferation and growth, eventually leading to hyperproliferation of epidermal cells, absence of mature sebocytes and conversion of hair follicles into cysts [Watt et al., 2008; Pan et al., 2004]. Overall, these characteristics strongly resemble the clinical features occurring in HS patients, thus strongly corroborating the hypothesis that interactions between the γ-secretase and Notch transduction might shed light on one of the crucial pathogenic mechanisms involved in HS (**Figure 5**).

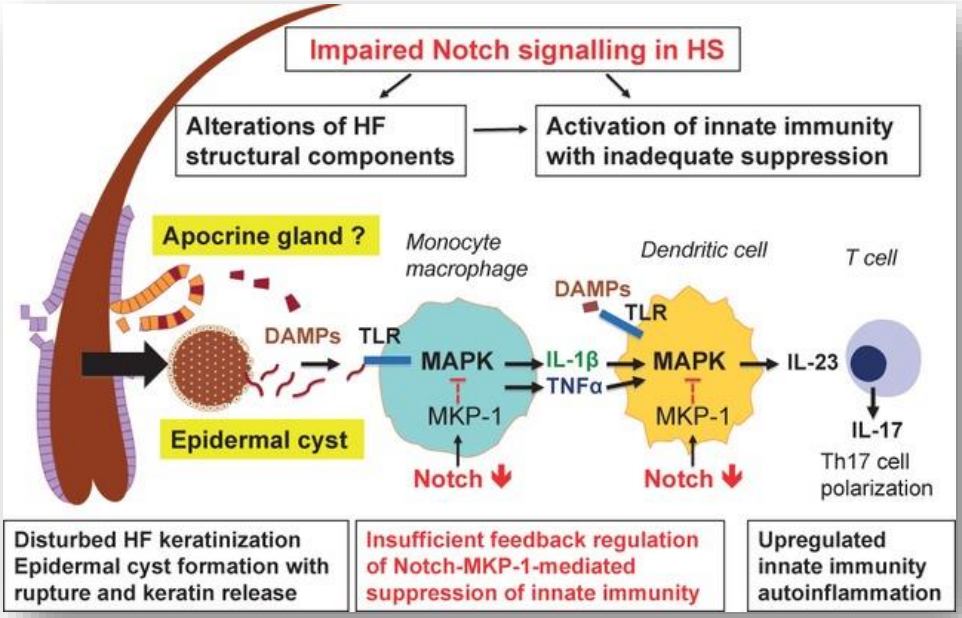

Figure 5: Alteration of Notch signalling in HS. This impairment disturbs terminal HF homoeostasis and results in instability of outer root sheath cells and formation of keratin-rich epidermal cysts. Notch deficiency may also affect apocrine gland integrity. Released DAMPs activate TLRs on macrophages and dentritic cells (DCs). Activated macrophages secrete increased amounts of proinflammatory cytokines (TNF-α, IL-1b) and activate DCs, which secrete IL-23 and thus promote Th17 cell polarization. Due to deficient Notch signalling, impaired Notch-mediated feedback inhibition of innate immunity by reduced activation of MAPK phosphatase-1 (MKP-1) results in chronic inflammation and Th17-driven autoinflammation leading to progressive tissue destruction and IL-17-mediated neutrophil attraction (Melnik et al., 2013).

Nevertheless, as mentioned above, conflicting findings concerning Notch signaling pathway involvement in HS pathogenesis have also been reported; for example, Phosphoinositide-3-Kinase Regulatory Subunit 3 (PIK3R3) and AKT Serine/Threonine Kinase 3 (AKT3), two downstream signaling pathway components of NCSTN and Notch, have been found markedly overexpressed both in lesional and perilesional skin of HS patients when compared to control [Hessam et al., 2021]; the levels of these proteins, in addition to NCSTN and Notch, were significantly higher in patients with mild disease when compared to those with moderate and severe HS. Additionally, functional studies demonstrated that several *NCSTN* missense variants associated with HS are functional and promote Notch signaling, counteracting the view that pathogenic variants in genes encoding gamma-secretase components cause disease simply as a result of haploinsufficiency that leads to impaired Notch signaling [Xiao et al., 2016].

Sporadic Cases of HS

Despite the absence of fully penetrant variants, van Straalen et al. [van Straalen et al., 2020] suggested that sporadic forms of HS possess a strong genetic background that contributes to their causality; furthermore, environmental/epigenetic factors have been shown to contribute to HS susceptibility, supporting a multifactorial aetiology. To date, the nature of the genetic variants driving these forms of HS remains to be clarified since gamma-secretase gene mutations occur only in around 6% of sporadic HS [Zouboulis et al., 2020].

Based on the growing evidence of a central role of a deregulated immune system in the HS pathogenesis, various studies have been conducted with the aim of unravelling the potential causative role of variants in genes encoding for proteins involved in immune responses (**Figure 6**).

Notably, Savva et al. conducted the first study aimed at evaluating the presence of single nucleotide polymorphisms (SNPs) in genes related to immune responses in 190 sporadic HS patients compared with 84 healthy subjects. Their results revealed the presence of only one relevant SNP occurring in the promoter region of *TNF* (Tumour Necrosis Factor) gene (-238G/A, rs361525) associated with susceptibility to HS as well as with response to TNF antagonists [Savva et al., 2013].

Dysregulation in antigen presentation often occurs in the pathogenesis of several chronic skin conditions including HS, in which this event mainly results in skin lesions, *via* the IL-12/IL-23 signalling pathway. Both IL-12 and IL-23 receptors share the IL-12Rβ1 subunit encoded by the *IL-12Rb1* (Interleukin 12 Receptor Subunit Beta 1) gene; previous genetic studies have established an association between *IL-12Rb1* gene and autoimmune/inflammatory disorders in which the presence of missense SNP alleles determined an altered function of the encoded subunit, potentially resulting in aberrant antigen presentation, thus making this locus particularly attractive as a causative candidate gene for HS as well. Giatrakos et al. conducted a study on 139 HS patients and 113 healthy controls, indicating that the SNPs identified in the *IL-12Rb1* gene do not convey genetic predisposition; however, two common haplotypes—h1 and h2—have been recognized: carriers of h2 showed an increased risk of disease severity, whereas individuals with the h1 haplotype presented a late-onset disease [Giatrakos et al., 2013].

Antimicrobial peptides (AMPs) constitute a class of crucial mediators that are known to take part to inflammatory responses occurring in the skin. Specifically, these molecules are expressed by several skin cells, including keratinocytes, and represent key components of the

innate immunity response capable of counteracting invading pathogens and commensals. The study of these peptides appears as particularly intriguing in the context of HS; indeed, a deregulated growth of the skin's microflora together with pathogen colonization of the skin of patients constitute major issues supported by the use of antibiotics as a first line therapeutic approach [Ingram et al., 2015; Orenstein et al., 2020]. Considering the role of microbial infections in HS, one would expect a decrease in the expression of AMPs to explain the increased susceptibility of patients to develop bacterial infections. Nevertheless, a study conducted by Giamarellos-Bourboulis et al. yielded an unexpected result. Human β-defensins (hBD)-2 and hBD-3 - encoded by the *DEFB4* and *DEFB103* genes respectively – are localized in a β-defensin cluster presenting copy number variations and they are well established peptides whose expression is associated with the severity of skin infections by *Staphylococcus aureus* [Zanger et al., 2010; Giamarellos-Bourboulis et al., 2016]. The authors demonstrated that high copy numbers of the β-Defensin Cluster conferred genetic susceptibility to HS, interfering directly with the clinical phenotype; moreover, the same authors confirmed, in two independent cohorts, that the presence of more than six defensins cluster copy number had an increased risk of developing HS, whereas the presence of fewer than six copies was linked to an earlier onset of the disease, fewer areas affected and a less frequent presentation of permanent purulence of skin lesions [Giamarellos-Bourboulis et al., 2016].

Myeloid differentiation primary response protein MyD88 gene (*MYD88*) encodes for a cytosolic adaptor protein that exerts fundamental functions both in innate and adaptive immune response by acting as a signal transducer in the Toll-like receptors and IL-1 signaling pathways. Defects related to this gene are commonly associated to an increased susceptibility to develop pyogenic bacterial infections. By studying a cohort of 101 HS patients, Agut-Busquet and colleagues observed that a polymorphism in the *MYD88* gene (GG genotype of rs6853) was associated to an increased risk in developing a severe form of the disease [Agut-Busquet et al., 2018].

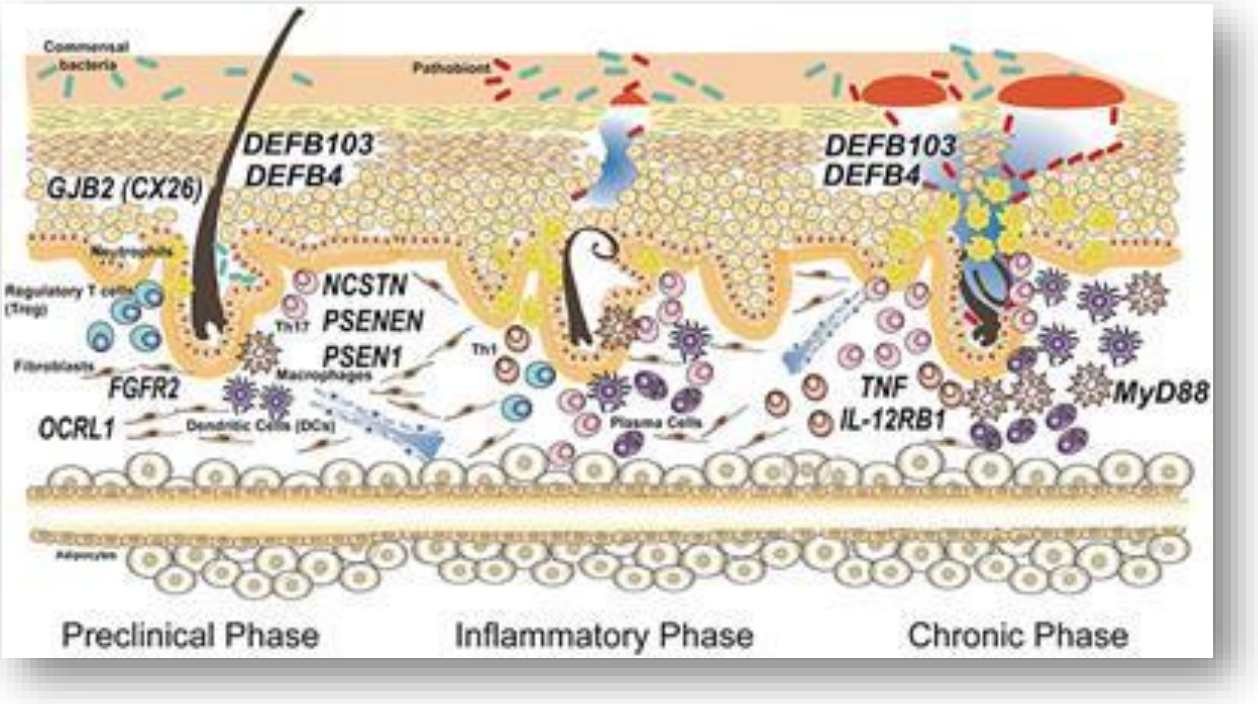

Figure 6: Genes associated with susceptibility and progression of sporadic HS. Common polymorphisms in genes encoding for proteins involved in the immune response have been associated to severity of the disease and influence the inflammatory and chronic phases (Tricarico et al., 2019).

The impact of genetics in the susceptibility and clinical progression of different forms of HS, should not be limited to the evaluation of single mutations affecting the functionality of proteins potentially associated with the disease. Indeed, other genetic variations, linked for example to transcriptomic signatures or epigenetic changes, must be considered to provide new insights into which genes (and therefore proteins) are possibly involved in HS and the broad spectrum of the disease phenotype.

Coates et al. performed the analysis of the skin transcriptome in HS uncovering gene signatures that collectively indicated that the pathogenesis of HS might be driven by changes in the expression of AMPs, altered sweat gland function and shared similar biologic pathways activated in chronic wounds. Indeed, the top differentially expressed genes between lesional skin and unaffected regions of HS samples encompassed: i) increased expression of various AMPs, of which S100A7A and DEFB4 were the most significantly up-regulated in HS lesional skin; ii) increased expression profile of epidermal differentiation complex (EDC) cluster of

genes that encode for proteins involved in the terminal differentiation of keratinocytes, potentially suggesting that the presence of high levels of terminally differentiated keratinocytes in HS lesions may lead to a reduced regenerative capacity; iii) decreased expression of genes associated with sweat-gland function, district known to be directly involved in the regulation of sweat generation, epidermal homeostasis, wound-healing, re-epithelialization and cutaneous immunity, therefore indicating an overall loss of normal skin architecture and function due to a decreased number of sweat glands [Coates et al., 2019]. Furthermore, whole-transcriptome analysis with total RNA sequencing (RNA-Seq) indicated that HS lesional skin showed a unique heterogeneous and complex inflammatory signature enriched in genes involved in complement activation, B cell signalling, phagocytosis-associated pathways, neutrophil recruitment, macrophage activation, wound healing, chronic inflammation, TNF-α secretion, lipopolysaccharide (LPS) signalling, IL-12 production, and T-cell recruitment. Noteworthy, also unaffected regions of HS presented defects in immune regulatory pathways when compared to the skin transcriptome of healthy controls, highlighting a significant reduction in immune regulatory pathways and a concurrent activation of specific proinflammatory networks [Lowe et al., 2020]. Single-cell RNA-seq (scRNA-seq) technique has been extensively assessed to unravel the cellular composition of HS, confirming that myeloid cells and B cells are the major contributors to the HS transcriptome profile as skin lesions progress, further indicating that myeloid cells appear to adopt a strong inflammatory phenotype driven by the IL-1 pathway, while an increase of B cells persists, further differentiating into memory B cells, plasma cells, and plasmablasts [Lowe et al., 2020]. In a study conducted by Gudjonsson et al., the authors observed extensive interactions amongst all the principle cellular types in HS, such as interactions between B cells and plasma cells with stromal tissue cells as well as other immune cell components. In addition, endothelial and keratinocyte populations resulted to present extensive interactions with members of the Notch signalling pathway. Both IL17A- and interferon gamma (IFN-γ)-expressing T cells were registered in HS skin [Gudjonsson et al., 2020].

The genes that have so far been identified as frequently altered in different cohorts of HS cases could contribute to the assessment of genetic analysis in which a gene panel is used for the most common variants. However, considering the highly complex and heterogeneous genetic background in HS cases, the analysis of gene panels based on previously identified susceptibility genes for HS, usually fails in determining novel potential causative variants in the most patients. Indeed, cases with a clear HS phenotype but presenting no association with a known disease gene, might present novel gene associations or express a combination of

variants in several genes (complex genetic trait). In this critical context, technologies such as whole exome sequencing (WES), constitute a revolutionary approach that allows the concurrent analysis of multiple genomic coding regions. This experimental approach allows broader screening in patients with specific disease patterns, and potentially leads to the identification of causal variants underlying disease pathogenesis [Kosukcu et al., 2021; Luo et al., 2021; Li et al., 2017].

1.5.2 Aberrant immune system response

The development of aberrant immune responses in HS is thought to be tightly associated to the occurrence of mutations in susceptibility genes associated to innate and adaptive immune responses. Moreover, enriched transcriptomic signatures of HS lesional skin showed an unregulated production of antimicrobial peptides (AMPs), altered sweat gland function, unbalanced neutrophil recruitment and the involvement of several impaired pathways such as phagocyte-associated pathways, macrophage activation, TNF-α secretion, B-cell signalling and T-cell activation [Lowe et al., 2020; Gudjonsson et al., 2020]. The major pathogenic feature in HS is a deregulated immune activation that promote a chronic inflammatory state, with an NLRP3 inflammasome-driven predominance of interleukin (IL)-1β and a clear contribution of T helper (Th)-17 and -1 lymphocytes [Sabat et al., 2020].

Concerning cutaneous immune responses, early pathogenic events in HS are thought to be triggered by damage-associated molecular patterns (DAMPs), induced by enhanced mechanical friction in intertriginous areas and by HS predisposing factors; DAMPs, once released, promote the transfer of microbial components in the skin, thus inducing the activation of resident immune cells, - mainly macrophages -, with the production of pro-inflammatory cytokines like TNF-α and IL-1β and chemokines that in turn favour perivascular and perifollicular immune cell infiltration [Sabat et al., 2020]. In this context, a further exacerbation of the inflammatory response is mainly caused by increased bacterial sensing exerted by local macrophages and dendritic cells (DCs) due their increased expression of Toll-like receptor (TLR) 2 [Hunger et al., 2008], and by the activation of the inflammasome platform as a result of DAMPs recognition, which promotes the cleavage/activation and secretion of IL-1β [Witte-Handel et al., 2019]. This pathogenic scenario has been confirmed by the detection of high expression of

both TLR2 and inflammasome complex in HS lesional skin [Sabat et al., 2020]. As a direct consequence, HS developing lesions are characterised by massive perifollicular immune cell infiltrates including neutrophils, monocytes - which in the skin can differentiate into macrophages or dendritic cells -, B/plasma cells as well as different subgroups of effector/memory T cells, that are generated in the skin-area-associated draining lymph nodes [Sabat et al., 2020]. This massive immune cell infiltrate also includes leukotriene B4 (LTB4), the complement component C5a and lipocalin (LCN)2, that are important phagocytes and producers of pro-inflammatory cytokines; these cells also form neutrophil extracellular traps (NETs), which can inhibit bacterial growth but also favour autoimmune features.

Interferon-γ (INF- γ) is the principal cytokine produced by Th-1 lymphocytes and possesses several crucial proprieties including further activation of endothelial cells that additionally favour the infiltration of immune cells into the injured area, induction of the Th-1 attracting chemokines production and activation of tissue-resident macrophages (Schroder et al., 2003; Sabat et al., 2020). IL-17, the main cytokine secreted by Th-17 lymphocytes, is well known to exerts several functions such as: stimulate the production of chemokines that prompt the recruitment of neutrophils; induce the synthesis of cytokines that primarily sustain IL-17-mediated inflammatory responses and stimulate the production of AMPs [Albanesi et al., 1999]. IL-26 is another Th17 cell cytokine upregulated in HS lesions [Scala et al., 2019]; while its receptor-dependent cytokine properties are debated, IL-26 directly kills bacteria [Scala et al., 2019]. Moreover, IL-26 acts as a carrier of DNA released from damaged cells to intracellular DNA-binding pattern recognition receptors (PRRs), with the consequent induction of macrophages-related inflammatory response [Wolk et al., 2020].

While Th1 and Th17 cells and their main mediators [interferon IFN-γ (Th1), IL-17 and IL-26 (Th17)] become abundant in HS skin, Th22 cells and IL-22 are not. Indeed, the levels of IL-22 found in HS skin are low and this might be due both to the lack of Th-22 infiltrates and/or to the high expression of IL-22 inhibitors. Like IL-17, IL-22 that acts exclusively on keratinocytes, is one of the major inductors of AMPs synthesis, and low levels of IL-22 might at least partially explain the insufficient blockade of bacterial propagation in the inflamed skin. Furthermore, IL-22 is also involved in counteracting tissue damage and metabolic alterations during inflammatory processes [Sabat et al., 2020] and a decreased expression of this cytokine might partially justify tissue destruction and the onset of systemic metabolic alterations observed in some HS cases.

Individual proinflammatory cytokines are also strong inducers of extracellular matrix-degrading enzymes, the matrix metalloproteinases (MMPs) [Witte-Handel et al., 2019]. At this

stage, these enzymes may be involved in the thinning of the basement membrane surrounding the hair follicle unit, as detected in perilesional HS skin, thus increasing the fragility of the inflamed and dilated hair follicle [Danby et al., 2013].
The follicular rupture and the consequent release of its content (including bacteria, DAMPs, keratin fibres and sebum components) into the surrounding tissue massively boosts inflammation; this latter eventually leads to clinically visible dermal nodules and abscesses. The chronic inflammatory state leads to destruction of skin architecture, recurring development of abscesses, wounding, and subsequent fibrotic scarring with adjacent areas also containing areas of interfollicular inflammation with acanthosis [van der Zee et al., 2012].
In this pathogenic step, HS lesions also show an increased expression of IL-36α, IL-36β and IL-36γ, which are mainly localized to keratinocytes [Hessam et al., 2018] and play a key role in the induction of neutrophil-attracting chemokines, specific cytokines, and AMPs [Foster et al., 2014].

Due to the enhanced and persistent inflammatory state in HS skin, not surprisingly different cytokines and mediators might reach the bloodstream and contribute to the onset of systemic inflammation, justifying the occurrence of concomitant comorbidities [Sabat et al., 2020]. For example, high IL-1β and IL-6 circulating levels can promote the production of the acute-phase protein serum amyloid A that might lead to severe outcomes such as amyloid A amyloidosis and atherosclerosis [Shridas and Tannock, 2019], whereas elevated circulating levels of IL-17 and TNF-α might be linked to the onset of HS-associated Crohn's disease, spondyloarthropathy or spondylarthritis [Sabat et al., 2020; Nomura, 2020].

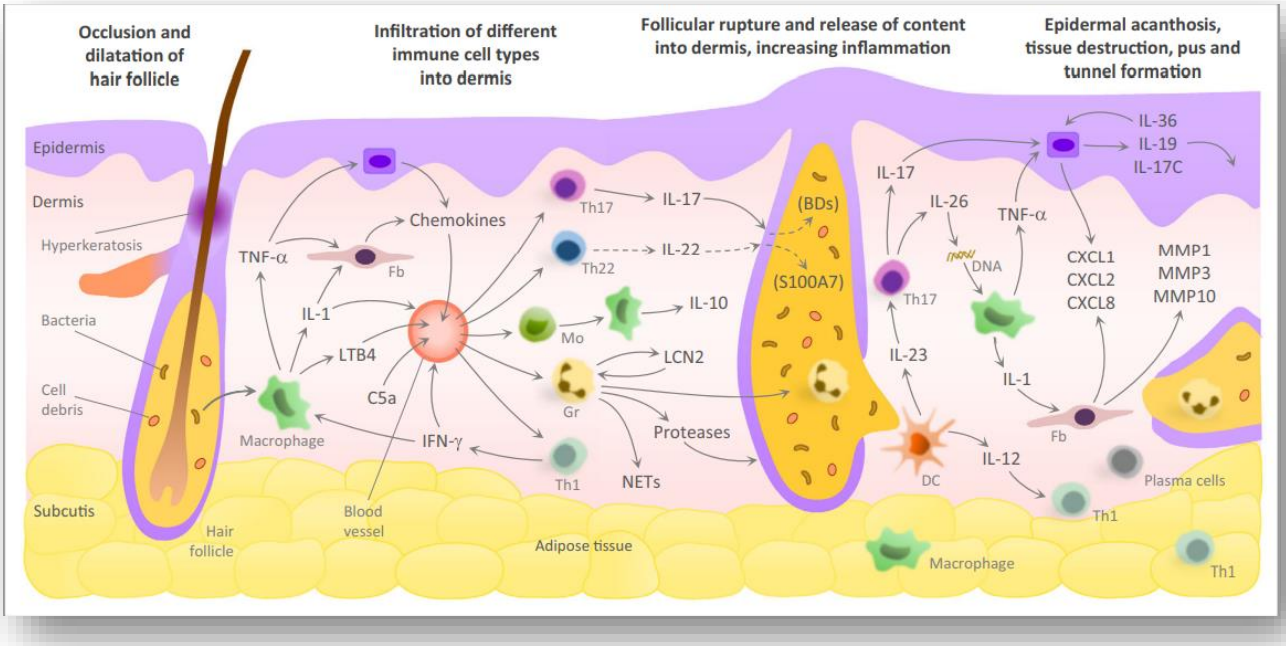

Figure 7: Pathogenetic events in HS (Wolk et al., 2020)

1.5.3 Cutaneous microbiome

Skin "microbiota" can be regarded as the whole and complex community of bacteria, viruses, and fungi which inhabit the skin surface, the hair follicles, and the dermis, while "microbiome" is considered as the set of microorganisms, their genomes, and the surrounding environmental habitat). The impact of these microbes on pathogenesis of HS represents an important aim for researchers [Marchesi et al.,2015].

Skin topography influences microbial colonization and composition; in the apocrine gland-rich skin, alteration of the homeostatic symbiosis between specific microbiota and cutaneous immune system might lead to IL-17-driven inflammation in HS [Jenei et al., 2019]. It has been suggested that HS is associated with dysbiosis, intended as an imbalance of the skin "healthy" resident microbiome, although its exact role in HS pathogenesis remains elusive and is yet to be fully clarified [Schell et al., 2021].

Wark and Cains reported that *Staphylococcus aureus*, coagulase-negative staphylococci, and Enterobacteriaceae species have been commonly cultured from HS skin, as well as *Porphyromonas* and *Prevotella*, both anaerobic bacteria, were relatively increased in HS skin, as demonstrated by 16S rRNA sequencing studies [Wark et al., 2020]. Ring and colleagues,

using NGS approach to explore the follicular skin microbiome of HS patients and healthy controls, identified 5 microbiome types and demonstrated that microbiome differed significantly in HS (lesional and unaffected regions) patients compared to that in healthy controls [Ring et al., 2017]. They found that *Corynebacterium* species (type I) or *Porphyromonas* and *Peptoniphilus* species (type IV, not revealed in healthy controls) were predominant in HS lesional skin; in contrast, *Acinetobacter* and *Moraxella* species (type II) were prevalent in HS unaffected skin, whereas *Cutibacterium acnes* showed a significantly higher relative abundance in healthy controls compared with HS skin [Ring et al., 2017]. More recently, Ring et al., using NGS to investigate the specific microbiome of tunnels, demonstrated that *Porphyromonas spp*. and *Prevotella spp*. (and Corynebacterium spp.) were the most frequent genera present in HS tunnels [Ring et al., 2019]. Furthermore, the authors, in accordance with previous findings [Ring et al., 2017], did not reveal *Propionibacterium spp*. in the most abundant genera found in HS tunnels, thus hypothesizing that this deficiency might promote a skin dysbiosis in HS [Ring et al., 2019].

Dysbiosis may contribute to HS pathogenesis through several mechanisms; skin microbes could i) directly produce and control AMPs production by keratinocytes, ii) activate C3a and C5a pathways, (iii) control the expression level of IL-1β, and iv) increase the production of pro-inflammatory cytokines such as IL-17 and IFN-γ by dermal T cells [Belkaid et al., 2014]. Notably, *Porphyromonas* and *Prevotella* may contribute to HS pathogenesis, promoting an exaggerated AMP secretion, and it has been showed that specifically *Prevotella* could favour the production of IL-23 and IL-1β [Larsen et al., 2017].

The risk of superinfections is more prevalent in severe HS patients in which bacteria may contribute to maintain chronic inflammation [Benzecry et al., 2020], but further microbiological studies are needed to clarify whether dysbiosis represents a possible trigger of HS pathogenesis or is a consequence of the underlying inflammatory process [Wark et al., 2020].

1.5.4 Hormonal influences

Important clues about the potential role of sex hormones (progestogens, androgens and oestrogens) in the pathogenesis of HS include i) gender (female) prevalence in HS affected individuals, ii) age of disease onset, iii) premenstrual HS flares, iv) worsening of clinical symptoms during pregnancy and post-menopause and v) efficacy of antiandrogen therapy

[Mortimer et al., 1986; Riis et al., 2016; Karagiannidis et al., 2016]. The appearance of similar phenotypic features between HS and acne vulgaris, a well-established androgen-dependent disorder, suggested that at least partially overlapping pathogenic patterns involving androgen dysfunction could occur also in HS.

HS usually occurs between 11 and 30 years of age, range in which cutaneous androgen enzyme activity is at its maximum. HS female patients often report to suffer from flares in the pre-menstrual period (with an occurrence of 44–63%) when the level of progesterone is high and are generally more prone to develop androgen-related consequences such as irregular menstruations, acne and hirsutism (Mortimer et al., 1986). Moreover, the prevalence of polycystic ovary syndrome (PCOS), a hyper-androgenic disorder, among HS cases is 9% against 2,9% registered in non-affected females [Garg et al., 2018]. *In vivo* studies in mouse models showed that androgens induced an increased pro-inflammatory response driven by augmented levels of TLR-mediated monocyte expression of TNF-α, thus also strongly suggesting a possible correlation between androgenic imbalance and inflammatory phenotypes [Lai et al., 2009]. More recently, Collier and colleagues confirmed HS worsening with menses in 62.4% of 282 women, mainly during the week preceding their onset (138 out of 175 women) [Collier et al., 2020].

However, the fact that i) the levels of plasma testosterone, dehydroepiandrosterone and apocrine gland androgen converting enzymes are no different in HS patients if compared to controls as demonstrated by Barth and Kealey [Barth and Kaeley, 1991], ii) the disease commonly manifests many years after puberty [Seyed Jafari et al., 2020], and iii) anti-androgen therapies combined with oestrogens do not result to be more beneficial than the administration of sole oestrogens [Mortimer et al., 1986], must be taken seriously to assert a possible androgenic involvement in HS. Rather recently, through a transcriptomic analysis from 17 skin biopsies of HS patients and 13 patient-matched controls, it has been showed an increased androgen receptor (AR) transcriptional activity in HS lesions and an upregulation of genes under the transcriptional control of three key stem cell transcription factors [Gaunter et al., 2018]. These findings led to hypothesize that hormonal dysregulation of epidermal stem cells could play a key role in keratinocyte proliferation and differentiation, and consequently in follicular plugging [Gaunter et al., 2018]. Furthermore, a whole transcriptome profiling from HS lesional skin revealed that the gene upregulation profile of female apocrine glands was related to the androgen signaling pathway, supporting the hypothesis of an androgen regulation of apocrine glands in female lesional skin [Zouboulis et al., 2020].

Concerning the impact of pregnancy on HS, an amelioration of HS during pregnancy and a deterioration post-partum have been reported [Riis et al., 2016]. According to Vossen et al., this amelioration has been found more frequently in those women who reported perimenstrual deterioration of HS symptoms compared to those whose symptoms improved or were unaffected during menses [Vossen et al., 2017]. Pregnancy could influence women with HS in several ways: (i) progesterone, whose serum concentrations are increased in pregnancy, is capable of inhibiting Th17 cell differentiation, ii) IL-1 receptor antagonists and soluble TNF-α receptor increase in late pregnancy, neutralizing the effects of both IL-1 and TNF-α respectively, and iii) on the other hand, metabolic dysregulation (and weight gain) during pregnancy might worsen HS symptoms [Perng et al., 2017].
Despite all these findings, the overall impact of hormones on the pathogenesis of HS remains to be clarified although the mechanisms related to hormonal imbalances are appealing and require further investigations.

1.5.5 Lifestyle factors

Obesity

To date, the impact of environmental factors in the onset, clinical course and severity of HS has been well defined.
The association between HS and obesity has been well established, with several studies demonstrating greater disease severity with increasing body mass index (BMI) [Shalom et al., 2015; Bettoli et al., 2016]; it has been assumed that obesity play a strong pathogenic effect on HS severity, not only *via* an increased skin fold mechanical friction that in turn promotes follicular occlusion and induces rupture of dilated follicles in HS patients, but also through a systemic low-grade inflammation promoted by adipokine unbalance. In fact, adipokine balance in HS is shifted towards increased secretion - by adipocytes and macrophages of white adipose tissue – and expression of the so-called “bad adipokines” like resistin, chemerin and leptin, contributing to the augmentation of inflammatory response in the skin of HS patients [Malara et al., 2018]. Furthermore, they play a role, together with the concomitant release of TNF-α, IL-1β, and IL-6, in insulin resistance, alteration of glucose and lipid metabolism and vascular and cellular dysfunction [Wolk et al., 2016].

HS patients are at increased risk of developing other disorders, such as high cholesterol, high blood pressure, and diabetes mellitus with a prevalence ranging from 7.1% to 24.8% [Garg et al., 2018]; furthermore, a rather recent studies found a significant association between HS and metabolic syndrome (MetS) [Phan et al., 2019]. MetS includes a cluster of at least three of the following five risk factors: obesity, hypertension, hyperglycaemia, dyslipidaemia, and high-density lipoprotein cholesterol. Sabat et al., showed that MetS, likewise to obesity, was significantly more common in HS patients when compared to the control group (40.0% vs. 13.0%) [Sabat et al., 2020]. Nevertheless, the exact relationship between HS severity and HS in association with MetS remains to be elucidated.

Based on this evidence, clinicians should always recommend losing weight. Western diet seems to play a pathogenetic role in the initial plugging of the follicular duct; it has been demonstrated that dairy products and red meat induce elevated levels of insulin-like growth factor (IGF)-1 and branched chain amino acids (including leucine, isoleucine, and valine). Consequently, IGF-1 stimulation makes the androgen receptor available for its ligands [Danby et al., 2015] and favours the expression of enzyme 5-α-reductase in the skin [Saric-Bosanac et al., 2020], thus stimulating androgen-driven infundibular occlusion and subsequent follicle rupture [Danby et al., 2015]. Moreover, dairy products and red meat lead to the activation of mammalian target of rapamycin (mTOR), whose expression has been found increased in HS lesions compared to HS unaffected regions [Monfrecola et al., 2016]. Notably, mTOR signaling has been implicated in promoting adipogenesis and lipogenesis, as well as in triacylglycerols accumulation in sebaceous glands [Monfrecola et al., 2016; Marasca et al., 2019].

Smoking

The exact mechanisms by which smoking contributes to HS pathogenesis remain unclear, although it has been estimated that ~90% of HS patients are smokers [Sartorius et al., 2009] and that smokers (current or former), in comparison with non-smokers, are at increased risk of having more than two body areas affected [Dessioniti et al., 2017; Matusiak et al., 2009]. Furthermore, there is increasing evidence suggesting that smoking status may also influence treatment response; Kromann et al., observed that 66% of HS patients (33 out of 50 subjctes) who reported disease remission were non-smokers in contrast to 34% of active smokers, suggesting that non-smoking status could be associated to a greater rate of HS self-reported remission [Kromann et al., 2014]. Similarly, Denny et al., found that non/former smokers were

more prone to have HS improvement in response to first-line medical treatment compared with current smokers [Denny et al., 2016].

From a biologic point of view, nicotine has been related to pathogenic events in HS such as epidermal hyperplasia, follicular plugging, neutrophil chemotaxis, cytokine production by keratinocytes and monocytic cells, biofilm formation and down regulation of AMPs. Furthermore, it has been shown that agonists contained in cigarette smoke such as benzopyrene induce an increase of IL-17 expression and a concomitant decrease of regulatory T cells (Tregs) *via* activation of the aryl hydrocarbon receptor (AhR) signaling, thus aggravating the ratio Th17/Treg [Melnik et al., 2018].

1.6. TREATMENTS

Independent of the clinical course and severity of HS, all patients should follow basic therapeutic measures, including self-education about the disease, maintaining high hygiene standards, cessation of smoking, and losing weight. [Saunte et al., 2017].

Topical application of clindamycin twice a day on the affected areas represents the mainstay treatment in the milder localized form of HS, while systemic antibiotic therapies (e.g., tetracyclines, clindamycin, rifampicin) in different regimens are considered first-line therapy according to guidelines and expert opinions, often required to control the disease and reduce the flares in non-responders to topical therapy and moderate HS [Zouboulis et al., 2015].

Ertapenem, an intravenous broad-spectrum antibiotic, is highly effective and can be used as an adjunctive treatment to surgery [Join-Lambert et al., 2016], although doesn't possess anti-inflammatory proprieties and is reserved as third-line therapy for a single six-week course as rescue therapy or during surgical management planning [Mendes-Bastos et al., 2018].

No unrestricted recommendation is given for the use of conventional immunosuppressant or immunomodulatory drugs including prednisolone [Fearfield et al., 1999], dapsone [Yazdanyar et al., 2011], retinoids [Hogan et al., 1988], and ciclosporin [Rose et al., 2006], as there is little or conflicting evidence for their efficacy [Alikhan et al., 2019].

Adalimumab, a TNF-α-antagonist, is the only biologic agent approved by EMA (European Medicines Agency) and FDA (Food and Drug Administration) for the treatment of moderate-to-severe forms of HS [Kimball et al., 2016]. It has been demonstrated, in clinical trials and real-life observational studies, a significant improvement (with adalimumab) ranging from

41.8% to 77% of treated patients [Markota et al., 2022]; the evidence of a "window of opportunity" supports the early use of adalimumab in HS to ensure better clinical response [Marzano et al., 2021].

Surgical treatments (e.g., deroofing, incision and draining, local excision of the lesions) are considered good therapeutic options in the most severe stage of HS with extensive and long-standing sinus tracts/fistulae formation or in treatment-refractory disease [Kraft et al., 2022].

To date, several biologics and small molecules are under investigation for moderate-to-severe HS including, among others, inhibitors of IL-1α, IL-17, IL-23, IL-36, complement 5a (C5a) /C5a receptor blocking agents and janus kinase (JAK) signalling inhibitors [Scala et al., 2021] (**Figure 8**).

Future treatments should be guided by personalized therapies, biomarkers, and pharmacogenomics to maximise clinical outcomes and limit adverse events according to specific patient characteristics at both genetic and phenotypic levels [Scala et al., 2021].

Table 1. Therapeutic approach of HS.

Hurley Staging (HS Severity)	Clinical Features	Recommended Treatments	Surgery
Stage I (mild)	Single or multiple abscesses without sinus tracts and scarring	Patient education: maintaining hygiene standards, cessation of smoking, losing weight * Topical clindamycin, antisepticsIntralesional corticosteroids	Incision and drainage Deroofing Laser
Stage II (moderate)	Recurrent abscesses with sinus tracts and scarring	Systemic antibiotics: tetracycline, clindamycin + rifampicin, rifampicin + moxifloxacin + metronidazole, ertapenem Immune modulators: prednisolone, retinoids, dapsone, cyclosporine	Local intervention Deroofing Laser
Stage III (severe)	Diffuse or multiple interconnected sinus tracts and abscesses across the entire area	Biologics: adalimumab, bimekizumab (phase III)/secukinumab (phase III), others	Extensive radical surgical excision

Figure 8: Therapeutic approach of HS (Scala et al., 2021).

1.7. SYNDROMIC FORMS OF HS

In a minority of patients, HS may occur in the context of rare syndromes with other immune-mediated inflammatory diseases or inherited conditions, presenting as "syndromic" HS [Gasparic et al., 2017]. The definition of syndromic HS is still evolving, as there is a wide range of monogenic and polygenic conditions increasingly associated with HS-lesional response pattern, thus complicating the diagnosis and classification of these complex conditions.

Three subtypes of syndromic HS have been suggested by Gasparic et al., 2017 (**Figure 9**):

i) syndromic HS associated with a genetic condition and related diagnostic testing - Dowling-Degos disease (DDD), Down syndrome (DS), keratitis-ichthyosis-deafness syndrome (KIDS), and pyogenic arthritis, pyoderma gangrenosum, acne, and suppurative hidradenitis (PAPASH);

ii) syndromic HS associated with follicular keratinization disorders or structural disorders-follicular occlusion syndrome (FOS), DDD, DS, and KIDS;

iii) syndromic HS associated with autoinflammatory disease both monogenic [familial Mediterranean fever (FMF)] and polygenic (**Figure 10**)

Table 1 Spectrum of conditions associated with syndromic hidradenitis suppurativa

Classification	Syndrome	Involved genes or chromosomal alterations
Group 1	Keratisis-ichthyosis-deafness syndrome (KIDS)	*GJB2*
	Down syndrome	Trisomy chromosome 21
	Dowling Degos disease	*KRT5, POFUT1, POGLUT1, PSENEN*
Group 2	Follicular occlusion syndrome	Yet unknown
Group 3	Pyoderma gangrenosum, acne, suppurative hidradenitis (PASH)	*MEFV, NOD2, NLRP3, IL1RN, PSTPIP1, PSMB8, NCSTN*
	Pyogenic arthritis, pyoderma gangrenosum, acne, suppurative hidradenitis (PAPASH)	*PSTPIP1*
	Psoriatic arthritis, pyoderma gangrenosum, acne, suppurative hidradenitis (PsaPASH)	Yet unknown
	Pyoderma gangrenosum, acne, suppurative hidradenitis, ankylosing spondylitis (PASS)	Yet unknown
	Familial Mediterranean fever (FMF)	*MEFV*
	Synovitis, acne, pustulosis, hyperostosis, osteitis (SAPHO)	*LPIN2, NOD2, PSTPIP2, IL1RN*

Figure 9: Spectrum of conditions associated with syndromic HS and related genes involved (Garcovich et al., 2021).

1.7.1 Syndromic HS in the spectrum of autoinflammatory disorders

Syndromic HS and familial Mediterranean fever

FMF is the prototype of monogenic autoinflammatory disease with a pattern for autosomal recessive inheritance, affecting worldwide more than 120,000 people [Ben-Chetrit et al. 2009], but occurring primarily among Jews, Turks, Armenians, and Arabs, in as many as 1/500 persons [Daniels et al. 1995].

This disease is caused by gain-of-function pathogenic variants of *MEFV* (MEditerraneanFeVer) gene, that encodes the protein pyrin, mainly expressed in neutrophils and monocytes. Pyrin is a member of the pyrin-domain (PYD)–containing proteins; the binding of pyrin to ASC (Apoptosis-Associated Speck-like protein containing a C-terminal caspase recruitment domain) leads to activation of ASC, with consequent recruitment and activation of procaspase-1 that, once in its active form, is crucial for the proteolytic cleavage and activation of IL-1β that acts as the major cytokine in the inflammatory process of FMF [Padeh and Berkun 2016]. Since its discovery in 1997, more than 350 sequence variants have been described for the *MEFV* gene, but an accurate genotype-phenotype correlation has not yet been reached due to the existence of complex alleles, modifier loci, genetic heterogeneity and epigenetic determinants [Ebadi et al., 2017].

Epidemiologic evidence from a case-control study documented an increased prevalence of FMF (0.7% vs 0.1% in controls) in a cohort of 4417 patients with HS, suggesting a close association between these two conditions.

Patients with HS-FMF carried also heterozygous pathogenic variants in the *MEFV* gene; a rather recent case-control study confirmed this finding, reporting an increased prevalence of *MEFV* mutations in patients with a complex HS clinical phenotype [Vural et al., 2019].

Such clinical and genetic association reinforces the importance of an autoinflammatory component in the pathogenesis of moderate-severe HS.

Syndromic HS and related disease spectrum

The presence of HS is distinctive for the pyoderma gangrenosum (PG), acne, and suppurative hidradenitis (PASH); PASH with pyogenic arthritis (PAPASH); psoriatic arthritis, PG, acne, HS (PsAPASH) and PG, acne, HS and ankylosing spondylitis (PASS). PASH, PAPASH,

PsAPASH, and PASS syndromes, can be considered as a continuous clinical spectrum of auto-inflammatory disorders with prevalent cutaneous/joint involvement [Cugno et al., 2017].

PASH syndrome was initially considered a monogenic skin disease, but new scientific evidence is demonstrating its polygenic autoinflammatory nature [Duchatelet et al., 2015]. In the first two reported PASH probands, it has been showed the presence of alleles carrying CCTG microsatellite repeat in the *PSTPIP1* (Proline-Serine-Threonine Phosphatase Interacting Protein 1) promoter, but gene expression does not appear to be modulated by the (CCTG)n motif [Braun-Falco et al., 2012].

A mutation in the *NCSTN* gene has been found in one PASH patient; this genetic change referred to 8-nucleotide deletion (NM_015331.2: c.344_351del) leading to a premature stop codon with a loss of function of the encoded protein. As mentioned above, pathogenic variants in *NCSTN* are mainly related to familial form of HS and this mutation didn't allow to distinguish it from the previously reported HS mutations, thus supporting a common genetic background between isolated HS and PASH syndrome [Pink et al., 2011].

Subsequently, targeted sequencing approach has been performed in 4 PASH patients to identify potential disease-causing mutations in ten candidate genes already known to be involved in inflammation pathway, describing potential causative variants in two target genes: *NOD2* (NM_022162.3: c.2104C>T; p.Arg702Trp and c.2722G>C; p.Gly908Arg) and *PSMB8* (Proteasome 20S Subunit Beta 8) (NM_148919.4:c.22G>A; p.Gly8Arg) [Marzano AV et al., 2014]. In the same study, the expression of cytokines, chemokines and other effector molecules has been analysed both in HS lesional skin and in serum of these patients, founding a cutaneous overexpression of IL-1β and its receptors probably due to dysregulation of inflammasome; furthermore, in skin biopsies from ulcerative lesions of PG, an overexpression of TNF-α, IL-17 and their receptors has been reported. Notably, the serum levels of these cytokines were within the normal range, suggesting a "confined" skin inflammation. Finally, several chemokines such as IL-8, CX-C motif ligand (CXCL) 1/2/3, CXCL16 and RANTES were also overexpressed [Marzano AV et al., 2014].

Marzano et al. first described PAPASH syndrome, reporting the case of a 16-year-old female with pyogenic arthritis, PG, acne and HS, in whom genetic analyses evaluating exons 10 and 11 of the *PSTPIP1* gene showed a missense mutation (c.831G > T; p.Glu277Asp) [Marzano et al., 2013]. In addition, a missense variant in *IL1RN* (Interleukin 1 Receptor Antagonist) has been also identified [Marzano et al., 2013].

The SAPHO syndrome, first described in 1987, is commonly classified as a reactive disorder, characterized by sterile, inflammatory involvement of the skin and joint tissues, and driven by the IL-1β pathway. The onset of the disease can occur at any age, but rarely in the older age group. So far, no specific mutations have been found, even though the familial character of SAPHO syndrome highlights the occurrence of potential genetic factors. The involvement of putative autoinflammatory genes such as *NOD2* and *PSTPIP2* (Proline-Serine-Threonine Phosphatase Interacting Protein 2), - an important paralog of *PSTPIP1* gene -, is still controversial [Hurtado-Nedelec et al., 2009].

HS may present as a common cutaneous manifestation of SAPHO, as described in several case reports [Vekic et al., 2018; Genovese et al., 2019]. Rather recently, a heterozygous frameshift mutation of *NCSTN* has been found in a patient affected by the SAPHO syndrome, suggesting a potential genetic link between the two conditions [Li et al., 2018].

Przepiera-Będzak et al., reported that *Cutibacterium acnes* antigen acts as a trigger factor that initiates the inflammatory process, although it has been only occasionally found in bacterial cultures [Assman et al., 2009; Colina et al., 2014]. An interesting association has been established between copy number variations (CNV) of the following genes: *CSF2RA* (Colony Stimulating Factor 2 Receptor Subunit Alpha), *NOD2*, *MEGF6* (Multiple EGF Like Domains 6), *ADAM5* (ADAM Metallopeptidase Domain 5) and the predisposition of SAPHO syndrome [Guo et al., 2019].

1.7.2 Syndromic HS in the spectrum of keratinization and follicular occlusion disorders and other genetic syndromes

First, follicular occlusion (FO) triad has been defined as the coexistence of acne, HS and dissecting cellulitis of the scalp (DCS). Pilonidal cyst has been subsequently added, leading to the definition of the FO tetrad, a symptom complex consisting of these four conditions having a similar pathophysiology. However, both entities may be referred to as the follicular occlusion syndrome (FOS) [Vasanth et al., 2014]. The exact pathogenesis of this group of disease is still unknown but available evidence suggests that they share the same pathophysiological background with a follicular occlusion in apocrine sweat gland-bearing areas as primum movens [Vasanth et al., 2014].

Follicular occlusion and consequently HS disease have also been observed in other genetic syndromes, such as KIDS and DS. Patients affected with DS present an increased prevalence of follicular skin disorders, including folliculitis (21%), keratosis pilaris (17.3%), and HS [Firsowicz et al., 2019]; the prevalence of HS in patients with DS has been estimated to be 2.1% to 14.6% in cross-sectional studies and it is associated with an earlier age of onset than in the general HS patient population [Poizeau et al., 2019]. A possible pathogenetic explanation could lie in the increased expression of amyloid precursor protein due to the trisomy of chromosome 21 that promote an impairment of Notch signaling pathway, one of the main pathways involved in HS pathogenesis [Blok et al., 2014].

Syndromic HS in combination with FOS has been also reported in KIDS, a rare congenital disorder, clinically characterized by keratitis, erythrokeratoderma, and neurosensory deafness, caused by mutations in *GJB2* (Gap Junction Protein Beta 2) gene, that encodes a member of the gap junction protein family with a key role in the growth, maturation, and stability of the epidermis [Garcia-Vega et al., 2019; Sloan-Heggen et al., 2016]. The correlation between HS and GJB2 is still to be clarified, but it has been speculated that HS could be the result of the hyperproliferative tendency of epidermis in KID patients to promote follicular plugging and cyst formation, promoting an exaggerated inflammatory response in resident immune cells [Sloan-Heggen et al., 2016].

Pathogenic variants in *NCSTN* gene have been also described in HS-associated with Dowling–Degos disease (DDD) [Garcovich et al., 2020]; this latter is a rare autosomal dominant disorder of hyperpigmentation caused by loss-of-function pathogenic variants in the non-helical head domain of the keratin 5 (*KRT5*) gene [Betz et al., 2006]. Other causative genetic changes include mutations in *POFUT1* (Protein O-Fucosyltransferase 1), *POGLUT1* (Protein O-Glucosyltransferase 1), and *PSENEN*, and all these genes may confer disease susceptibility by affecting Notch signalling pathways [Ralser et al., 2017; Basmanav et al., 2014]. Notably, *POFUT1* and *POGLUT1* mutations have been associated to a clinical subtype of DDD that commonly involves non-flexural sites, whereas *PSENEN* and *NCSTN* pathogenic variants have been specifically attributed to a form of DDD associated with HS [Ralser et al., 2017].

HS-like lesions have been also observed in nevoid acne and Nevus Comedonicus (NC), comprising a group of dilated hair follicle openings filled with black keratinous plugs, that was initially described as “localized acne” [Pavithra et al., 2011]. In some cases of NC, a somatic

missense mutation in *FGFR2* (Fibroblast Growth Factor-Receptor gene 2) gene has been identified [Higgins et al., 2017]; this gene is mainly expressed in keratinocytes, sebaceous glands and hair follicles, and is known to mediate specific epidermal-dermal signaling pathways that regulate cell proliferation, differentiation, apoptosis, migration, the formation of skin appendages, the proliferation and lipogenesis of sebaceous glands, and to maintain the homeostasis of the pilosebaceous unit [Melnik, 2009]. The variant c.492G>C, pLys164Asn in *FGFR2* gene, reported in a HS case, is thought to possess a pathological consequence linked to the resulting impairment of the tyrosine-kinase activity of the fibroblast growth factor receptor [Higgins et al., 2017]. Even though to date no functional studies are available on this mutation, an important aspect to consider is the involvement of *FGFR2* in the activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and Raf/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2) signaling pathways; indeed, the activation of PI3K/Akt axis, caused by mutations in the γ-secretase complex, is defined as HS-related and leads to aberrant keratinocyte proliferation and differentiation [Xiao et al., 2016].

Mutations in HS patients have been found in *OCRL1* gene encoding for inositol polyphosphate 5-phosphatase (OCRL1), a lipid phosphatase that catalyses the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) which is critically involved in membrane trafficking, membrane/cytoskeletal interface, polarisation of epithelial cells and cell signalling [Cui et al., 2010; Czech, 2000]. Mutations in OCRL1 gene are known to be associated to a X-linked disorder characterised by proximal tubule dysfunction, namely the Dent Disease (DD2). In this context, Marzuillo et al. conducted a study on 5 DD2 patients carrying mutations in OCRL1 gene, 4 of which were histologically diagnosed with HS during follow-up, hence suggesting a novel possible association between DD2 and HS [Marzuillo et al., 2018]. Specifically, OCRL1 mutations induce a decrement in OCRL1 activity ultimately resulting in a progressive accumulation of PIP2 substrate in the plasma membrane; this accumulation of in fibroblasts seems to promote *Staphylococcus spp*. driven aggression, therefore suggesting that in HS cases these variants might, at least partially, explain the observed increased susceptibility to cutaneous infections [Marzuillo et al., 2018].

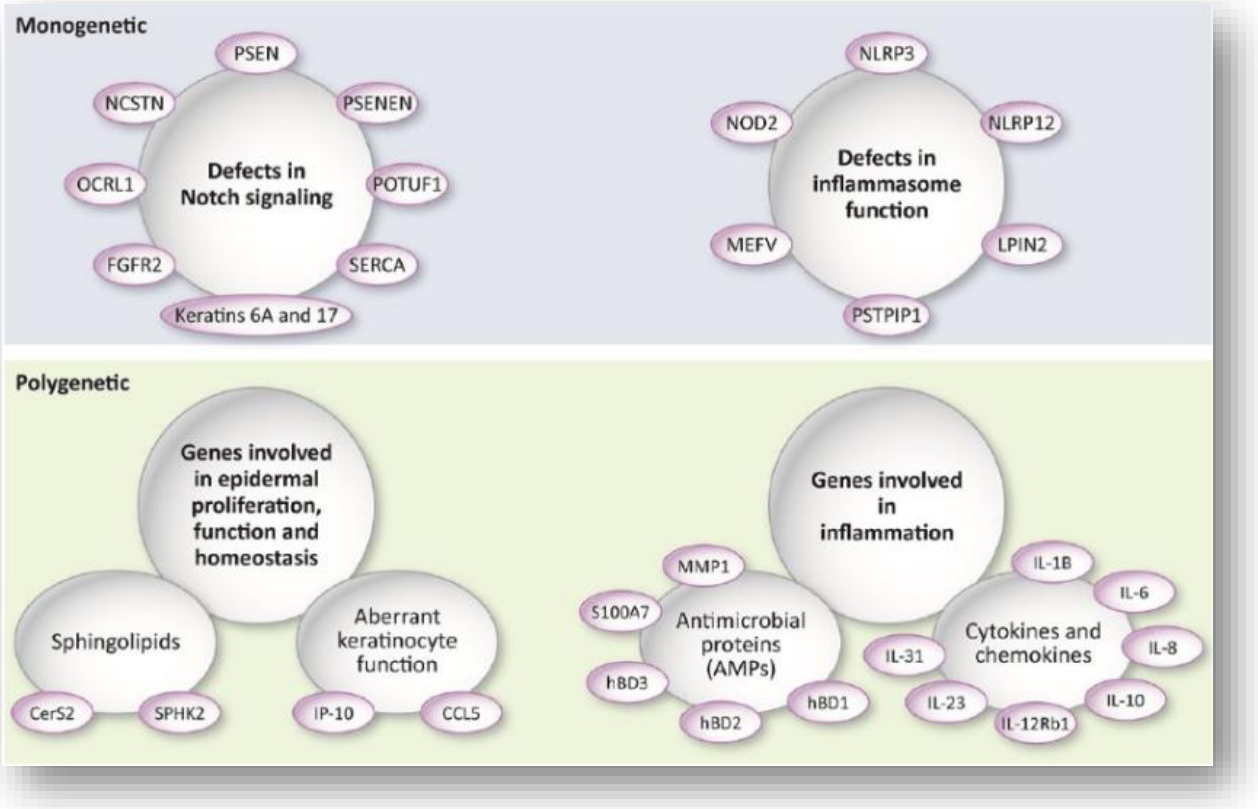

Figure 10: Genetic variations in HS and its related conditions (Jfri et al., 2019).

2. AIM

Hidradenitis suppurativa (HS) and its syndromic form are highly debilitating autoinflammatory skin diseases, whose multifactorial pathogenesis is complex and not yet fully elucidated, presenting with a close interplay between genetic, immunologic, epigenetic, and environmental/lifestyle factors. Notably, in HS and its different variants, some pathogenic variants are found to be recurrent and to mainly occur in susceptibility genes encoding for three subunits of the γ-secretase complex (*NCSTN, PSENEN, PSEN1*) and others associated with innate immunity response as well keratinocytes differentiation and proliferation. Nevertheless, a great genetic heterogeneity persists and often the mutations themselves are not informative of the biological signaling pathways commonly disrupted in these patients as well as having no clear and definite correlation with the clinical phenotype.

The assessment of patients-based genetic studies and the increasing availability of -omic approaches in both clinical and research settings, further supported by in vitro and in vivo functional assays, is helping to unravel pathogenic scenario underlying these conditions as well as the causative role of certain variants driving HS susceptibility and disease onset, thus proving to be powerful tools for the identification of novel pathogenic mechanisms potentially involved in the onset, progression and severity of HS and its syndromic forms.

Through new Whole Exome Sequencing (NGS) approaches and Variant Enrichment Analysis (VEA) workflow - a tool applicable for whole exome sequencing data – this project aims to describe genetic signature of syndromic HS patients associated with a specific clinical setting and to identify novel common signaling pathways involved in these patients, with the ultimate goal of developing personalized and targeted treatments.

3. MATERIALS AND METHODS

PART I: BIOLOGICAL SIGNALING PATHWAYS INVOLVED IN 5 PATIENTS WITH SYNDROMIC HIDRADENITIS SUPPURATIVA

3.1 PATIENTS

Thanks to the Biomolecular Analyses for Tailored Medicine in AcneiNversa (BATMAN) project, funded by ERA PerMed and by a grant partially funded by Italian Ministry of Health, Current research IRCCS (R.C 2011–2019, N.26), five patients presented with syndromic HS (PASH and PAPASH) have been recruited. These patients have been followed-up at the Dermatology Unit of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milan (Italy) from January 2011 to May 2019. All the enrolled probands signed a written informed consent previously approved by the Area B Milan Ethics Committee (R.C 2011–2019, N.26) and received pre-test genetic counselling in accordance with guidelines.

3.2 GENETIC ANALYSES

3.2.1 Genomic DNA extraction from saliva

Genomic DNAs from enrolled patients have been extracted from saliva using an all-in-one kit for the collection, stabilisation, and transportation of DNA (Oragene DNA OG 500, DNAGenotek, Ottawa, CA). DNA extraction was then performed through an ethanol precipitation using the prepIT-L2P2 (Oragene and ORAcollect® collection kit, Genotek, Canada) kit following the manufactures' protocol.

3.2.2 Whole exome sequencing (WES) and data analysis

DNA quantity and quality check has been assessed with Qubit assay (Invitrogen, Oregon USA) and agarose gel; subsequently all samples have been processed with a whole exome sequencing (WES) approach - providing expected coverage of 100X - outsourced by Macrogen (Seoul, Korea). Briefly, DNA exome sequencing reactions have been performed through Illumina^{R} HiSeq2500 System, after the library preparation (SureSelect Human all exons V6 kit).

The global coverage has been re-calculated through Picard tools and retrieved an average of 93.9%, 36.0% and 9.3% for 10, 50, 100x coverage, respectively. Adapters have been trimmed using the Trim Galore [Braham Bioinformatics. Trim Galore. https: //www.bioinformatics.babraham.ac.uk/projects/trim_galore/] searching and removing Illumina adapters, reads with length below 15 base pairs, and low-quality ends from reads with Phred33 score below 20 (http://www.bioinformatics.babraham.ac. uk/projects/trim_galore/).

The FASTQ file has been aligned using Burrows-Wheeler Aligner (BWA) Software Package [https://doi.org/10.1093/bioinformatics/btp698], specifically the bwa-mem tool, with the Human Genome version 38 (GRCh.38) as genome reference. Picard tools (https://broadinstitute.github.io/picard/) has been used to mark and remove duplicates reads; GATK v. 4.1.2.0 (https://software.broadinstitute.org/gatk/) allowed base recalibration and variant calling, excluding all variants with low mapping and genotyping quality (MQ < 40 and GQ < 20, respectively). Quality control and assurance have been visualized by fastQC. ANNOVAR software [Wang et al., 2010] has been used for variant annotation based on databases relative to the GRCh.38 genome reference (dbSNP 151, CADD; GERP++, SIFT, PolyPhen2, FATHMM, COSMIC70, ClinVar, 1000 Genomes Project, ExAC 03, genomicsSuperDups, wgRNA, GWAS Catalog, and Interpro).

A novel bioinformatic pipeline has been customized, through R Software Environment, to analyse the annotated exonic variants and subsequently to unravel the involvement of biological signaling pathways in PASH and PAPASH phenotypes. In brief, the investigation focused on variants located in the coding regions (ExonVar). Instead of searching only known or novel potential mutations, the framework outputted the distribution, density, PolyPhen, SIFT, FATHMM description of ExonVar, including single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs) for each patient. Subsequently, the pathway prediction has been performed employing two strategies: (i) raking common genes with the higher score in the three proteins functional algorithm prediction, (ii) including all genes carrying, at least one, ExonVar in a pathway enrichment analysis (PEA) using the Reactome database (https://reactome.org/) from ReactomePA R package. Pathways with adjusted p-value < 0.05 have been included for further analyses. Next, pathways shared by all five patients, as well as exclusive pathways, have been visualized through Venn Diagram. To utilize the overall ExonVar impact, the median HumDiv PolyPhen, SIFT and FATHHM score has been established for each shared pathway by calculating the mean of nonsynonymous (ns) ExonVar values retrieved with PolyPhen analysis.

PART II: A PRELIMINARY GENOTYPE-PHENOTYPE CORRELATION IN 10 UNRELATED PATIENTS WITH SYNDROMIC HS

3.3 PATIENTS

Ten unrelated syndromic HS patients (PASH, PAPASH, PASH/SAPHO overlapping syndrome) - five of whom have been previously included in the first part of this project – have been recruited and followed-up at the Dermatology Unit of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milan (Italy) from January 2011 until July 2021. All the enrolled subjects signed a written informed consent previously approved by the Area B Milan Ethics Committee (protocol No. 487_2020) and received pre-test genetic counselling.

3.4 GENETIC ANALYSES

3.4.1 Genomic DNA extraction from saliva

Genomic DNA from enrolled individuals was extracted from saliva following the same procedure reported in the section 3.2.1.

3.4.2 Whole exome sequencing (WES) and data analysis

Whole exome sequencing library preparation and sequencing have been performed as mentioned in the section 3.2.2.
Following this, the sequencing coverage has been re-calculated and resulted in an average of 93.9%, 36.0%, and 9.3% for 10, 50, and 100× coverage, respectively. Adapter trimming, short and low-quality reads has been filtered out using the Trim Galore v.0.39, using default parameters (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) [Braham Bioinformatics. Trim Galore.
https: //www.bioinformatics.babraham.ac.uk/projects/trim_galore/]. The read alignment has been carried out using Burrows-Wheeler Aligner (BWA) Software Package, using the Human Genome version 38 (GRCh.38) as genome reference. Duplicate removal and base recalibration have been performed by Picard tools (https://broadinstitute.github.io/picard/) [Picard tools – By

Broad Institute. https://broadinstitute.github.io/picard/.] and GATK v.4.1.2.0 (https://software.broadinstitute.org/gatk/) [GATK. https://gatk.broadinstitute.org/hc/en-us.], respectively. Variant calling and filtration have been also made using GATK v.4.1.2.0. ANNOVAR software for gene-based and filter-based variant annotations (dbSNP 151, CADD; GERP++, SIFT, PolyPhen2, FATHMM, COSMIC70, ClinVar, 1000 Genomes Project, ExAC 03, genomicsSuperDups, wgRNA, GWAS Catalog, and Interpro), relative to the GRCh.38 genome reference, has been used. A homemade custom pipeline, using R Software, has been created to analyse the annotated exonic content. The research focused on variants in coding regions (ExonVar), including those related to exon splicing; the distribution, density, and description of ExonVar, including single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs) for each patient have been retrieved.

An impact mutational score has been developed to define a variant with potential impact on the protein by assembling multiple algorithmic prediction results, including SIFT, PolyPhen2, FATHMM. A value of 1 has been ascribed in the impact mutational score each time the algorithm predicted a damage impact; subsequently, CADD values 15 has been added in the score. Variants carrying an assembly ≥5 or CADD >15 have been considered a potential impact variation (ImpactVar). For the sequencing quality assessment (regarding the variants of interest), the QUAL parameter standing for a Phred-scaled score for the base assertion had been made; the AD parameter, in terms of read depth, has been also calculated.

Two approaches have been employed: first, variations in three genes (*NCSTN*, *PSENEN*, and *PSEN1*) belonging to the gamma-secretase complex have been searched and secondly, homozygous rare ExonVar have been analysed.

3.4.3 Sanger sequencing for the confirmation of three selected variants identified by WES

Sanger sequencing validation has not been performed in all cases since the coverage depth of WES analyses made it redundant. However, as previously suggested by Arteche-Lopez et al. [Arteche-López et al., 2021], Sanger sequencing has been performed as an internal quality control for three variants of two autoinflammatory genes, confirming the WES results.

Genomic DNA samples have been amplified using the KAPA2G Fast HotStart Ready Mix (KAPA Biosystems) using primers for nicastrin (*NCSTN*) gene (*NCSTN*_9to10forward primer: 5'-GCTGACTAGCAGTTGAGGTGAC-3', *NCSTN*_9to10reverse primer 5'-TCCAATCCTGTCTCTTACCCTG-3'), (*NCSTN*_5forward primer: 5'-TTGAGTCACCACCTCCTTTTG–3',*NCSTN*_5reverse primer: 5'-GCCTCAAGTGATCCTCCTGTC-3'), and *NOD2* gene (*NOD2*_forward primer: 5'-ACT GAG CCT TTG TTG ATG AGC-3', *NOD2*_reverse primer 5'-TCA GAT CCT TCA CAT GCA GA-3') (Eurofins Genomics, Germany).

The following protocol and PCR program has been employed:

- KAPA2G Fast HotStart Ready Mix: 6,25 µl
- Gene_forward primer (10 µM): 0,625 µl
- Gene_reverse primer (10 µM): 0,625 µl
- H_20: 4,25 µl
- Genomic DNA (50 ng): 0,75 µl

for a final volume of 12,5 µl.

Step	**Temperature**	**Time (min.)**	**Number of cycles**
Initial denaturation	95 °C	3:00	1
Denaturation	95 °C	0:10	40
Annealing	55 °C	0:10	
Extension	72°C	0:05	
Final extension	72°C 4°C	1:00 Hold	1

Sanger sequencing of DNA amplification products was performed by Eurofins Genomics (Eurofins, Germany) while the obtained chromatograms have been analysed using the CodonCode Aligner Software (Version 8.0.2, LJ-COR Inc.).

PART III: VARIANT ENRICHMENT ANALYSIS TO EXPLORE PATHWAYS FUNCTIONALITY IN TWELVE SYNDROMIC HS PATIENTS THROUGH WES ANALYSIS

3.5 PATIENTS

Twelve unrelated syndromic HS patients (PASH, PAPASH, PASH/SAPHO overlapping syndrome) - ten of whom have been previously included in the second part of this project – have been recruited and followed-up at the Dermatology Unit of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milan (Italy) from January 2011 until December 2021. All the enrolled subjects signed a written informed consent previously approved by the Area B Milan Ethics Committee (protocol No. 487_2020) and received pre-test genetic counselling.

3.6 GENETIC ANALYSES

3.6.1 Genomic DNA extraction from saliva

Genomic DNA from enrolled individuals was extracted from saliva following the same procedure reported in the section 3.2.1.

3.6.2 Whole exome sequencing (WES) and data analysis

Genomic DNAs was extracted from saliva samples using the Oragene DNA OG 500, DNAGenotek (Ottawa, ON, Canada) kit following the manufacturer's instructions. Agarose gel (2%) and Qubit instrument (Invitrogen®, OR USA) have been used to evaluate DNA quantity and quality prior to sequencing. Exome sequencing has been performed in outsourcing by Macrogen Europe (Amsterdam, The Netherlands). The Exome Sequencing Analysis, with a declared 150× coverage, used the Illumina® SureSelect Human V7 Kit Library preparation and sequencing reaction, in an Illumina® HiSeq 2500 System, generating pair-end reads of 150 base pairs.

The exome annotation and data analysis workflow started from quality control of the fastq.gz input, using fastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), by which an

overall summary of the sequencing performance can be assessed (in terms of total sequences, sequence length, GC proportions, sequence quality score, and adapter content); after that, library adapters and reads (single or pair-ended) with lengths below 25 base pairs and low Phred score (Q < 20) have been removed using the Trim Galore application (http://www.bioinformatics.babraham.ac.uk/projects/ trim_galore/) [Braham Bioinformatics. Trim Galore. https: //www.bioinformatics.babraham.ac.uk/projects/trim_galore/].

The polished.fastq.gz files with the raw reads after QC have been aligned using the Burrows-Wheeler Aligner software package [Li et al., 2010], mapping them using the most recent Reference Human Genome version (GRCh.38).

Picard tools (https://broadinstitute.github.io/picard/) have been used for marking and removing duplicate reads, while GATK (https://software. broadinstitute.org/gatk/) for base recalibration. During the variant calling step, Strelka2 has been used for variant calling [Kim et al., 2018], while GATK and vcftools [Danecek et al., 2011] have been used to exclude low-quality variants.

Variant annotation has been performed using ANNOVAR [Wang et al., 2010] software, using databases related to the GRCh.38 genome reference. R Software [https://www.r-project.org/] has been used to manipulate the ANNOVAR results for the purposes of descriptive and inferential analysis. The analysis consisted of two main parts: individual analysis, with the aim of summarizing the descriptive data for each sequenced sample, and group data analysis, which aims to draw comparisons between groups of patients. Additionally, for both the individual and group categories, the so-called "Variant Enrichment Analysis" (VEA) has been performed.

3.6.2 Variant Enrichment Analysis (VEA)

Variant Enrichment Analysis (VEA) is based on Pathway Enrichment Analysis for expression data [Yu et al., 2016]. First, statistical differences between the number of genetic variants in each pathway and that in a reference dataset - GnomAD Exome v3.0 [Karczewski et al., 2020] have been searched. Then, R package 'ReactomePA' and 'reactome.db' have been used to fetch pathway information of each gene containing at least one variant in the dataset [Yu et al., 2016]. As the occurrence of some genetic variants could be related to specific population genetic background, only the Non-Finnish European (nfe) common variant information in the reference dataset has been considered, since the case population included in this study is from Italy. Once achieved the number of common variants per pathway from the case population and reference dataset, Fisher's Exact test with False Discovery Rate (FDR) has been used to identify statistical

differences in the proportion of variants. Adjusted p-values < 0.05 have been considered as significant in this analysis. All analyses have been performed using the R software package (https://www.r-project.org.). The initial list of Enriched Pathways has been filtered through a Venn diagram to set apart exclusive enriched pathways (eEP) for each studied group (PASH, PAPASH, and overlapping PASH/SAPHO). Then, the eEP based on odds ratio (OR) higher than 1.5 and by the number of important variations (ImportantVar) presented in each eEP have been ranked. ImportantVar is defined as genetic variants with a positive damage prediction score, based on multiple algorithm prediction (CADD, SIFT, PolyPhen2, FATHMM), and variants with functional impact, such as non-synonymous, stop codon, and start codon variations. To determine whether one of the eEP was expressed on normal or inflammatory lesional skin, and thus, involved in the pathogenesis of syndromic forms of HS, gene and protein expression have been considered. Since skin biopsies from the enrolled patients were not available, public databases of gene and protein expression have been considered to determine the ratio of protein and mRNA expression on normal or inflammatory lesional skin. Normal skin expression (protein and mRNA) has been recovered from the Human Protein Atlas database [Thul et al., 2018]. The expression probability ratio (EPR) for a given pathway in skin has been calculated dividing the number of genes/proteins expressed per total genes/proteins for the given pathway. Data expression for lesional skin has been restricted only to mRNA expression, as referenced from studies performed by Hoffman et al. (2018) [Hoffman et al., 2018] and Penno et al. (2020) [Penno et al., 2020] on skin biopsies of HS patients. The differentially expressed genes (DEGs) between lesional and perilesional skin have been used as input to perform enrichment pathway analysis. Finally, a Venn diagram has been employed, including the enriched pathways achieved by DEGs list and the exclusive enriched pathway (eEP) of each patient group.

4. RESULTS AND DISCUSSION

PART I: BIOLOGICAL SIGNALING PATHWAYS INVOLVED IN 5 PATIENTS WITH SYNDROMIC HIDRADENITIS SUPPURATIVA

4.1 CLINICAL FEATURES OF SYNDROMIC HS PATIENTS (PASH/PAPASH)

The first part of this project included 5 syndromic HS patients, 4 of whom diagnosed with PASH and the other one affected by PAPASH syndrome. Mean age of HS onset was 20.2 (range, 15–32) years, with mean diagnostic delay of 3 years. The most frequently involved regions were perianal area (n = 3) and axillae (n = 3), followed by inguinal region (n = 2), genitalia (n = 2), neck (n = 1), back (n = 1), gluteal region (n = 1) and submammary folds (n = 1). Genetic counselling revealed a negative family history for HS in all patients. Among the risk factors, sweating was the most common (n = 4), followed by obesity (n = 2) and seasonal worsening (n = 2), while smoking has been reported only by a patient. All patients had a severe form of HS (Hurley stage III), with a mean International Hidradenitis Suppurativa Severity Score System (IHS4) [Zouboulis et al., 2020] of 16.8 (range, 12–34).

Mean age at onset of pyoderma gangrenosum (PG) was 23.8 (range, 18–33) years. Ulcerative lesions were present in all cases and associated with vegetating features in one patient; the most frequently involved sites were lower limbs (n = 3), followed by presternal region, (n = 1), back (n = 1) and perianal region (n = 1). Neither family history of PG nor extracutaneous involvement have been recorded.

Finally, mean age at onset of acne was 17.8 (13–33) years. Acne lesions involved the face in all cases and the trunk in two of these patients. The most relevant comorbidities were inflammatory bowel disease (IBD) (n = 1) and psoriasis (n = 1). History of arthritis involving the wrists and ankles has been recorded only in the patient diagnosed with PAPASH, according to clinical definition.

An increase in inflammatory markers has been observed in all patients, with mean C reactive protein of 22.14 mg/dl (range, 7.5– 43.7) and erythrocyte sedimentation rate of 38.9 mm/hr (range, 12–68).

All patients underwent several cycles of systemic and topical antibiotics, without clinical efficacy, in contrast to the clinical benefit obtained with the use of biologic agents; indeed,

adalimumab (n = 2), infliximab (n = 2), and ustekinumab (n = 1) have been administered with a good control of the cutaneous picture.

An example of clinical features of syndromic HS (PASH) is represented in **Figure 11.**

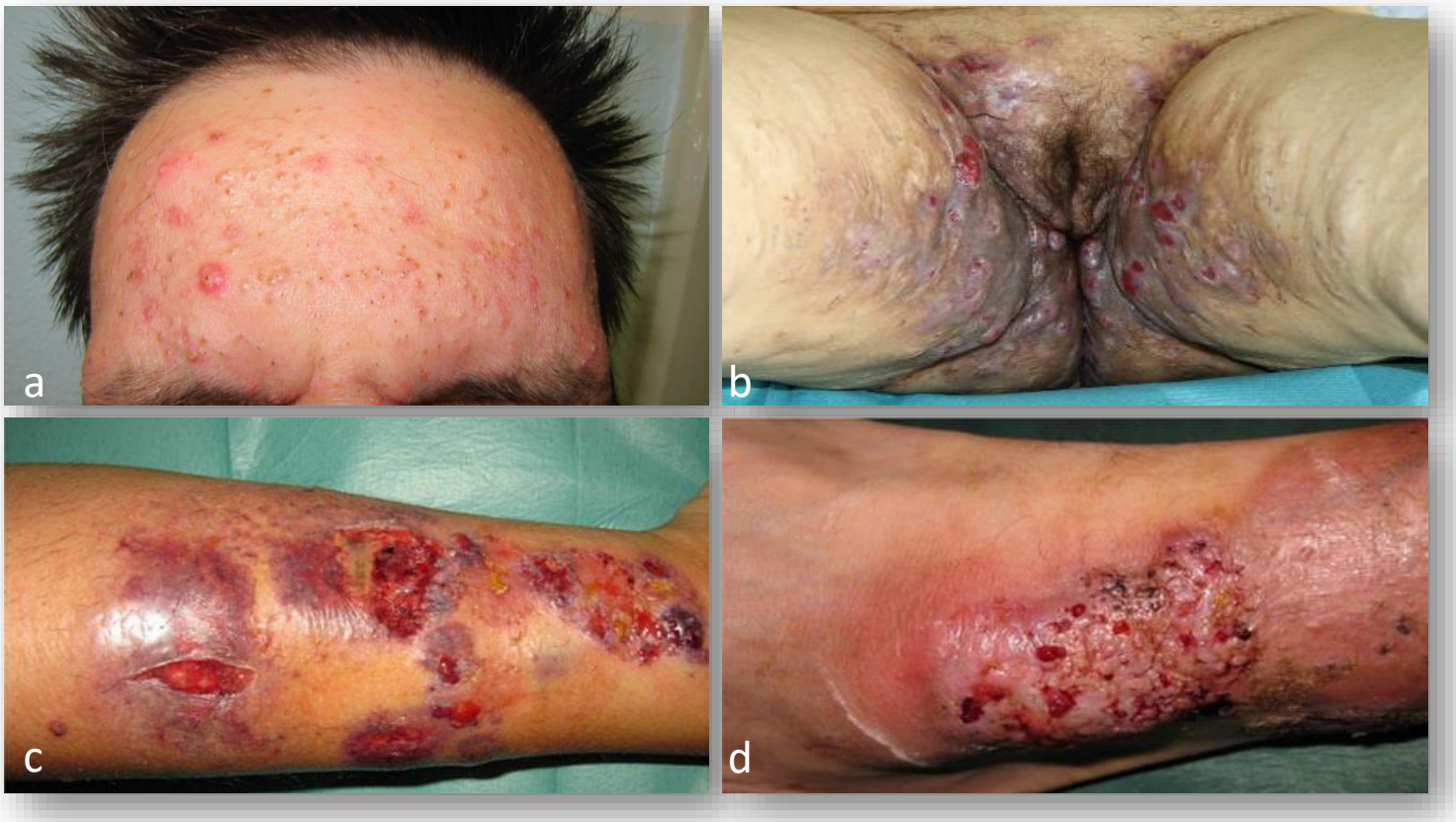

Figure 11: Pyoderma, acne and suppurative hidradenitis (PASH) syndrome. (a) Acne consisting of papules, pustules and comedones on the forehead; (b) ulcerated nodules of HS coalescing into infiltrated plaques on the genitalia; (c) ulcerative lesions of PG on the arm; (d) PG manifesting as ulcer with vegetating features. Adapted from Brandao L., et al. Altered keratinization and vitamin D metabolism may be key pathogenetic pathways in syndromic hidradenitis suppurativa: a novel whole exome sequencing approach. J Dermatol Sci. 2020 ;99(1):17-22. doi: 10.1016/j.jdermsci.2020.05.004

4.2 GENETIC ANALYSES

320,533 different variants with an average of 132,788 individual variations in each patient have been found. Most of them were distributed on chromosome 1, 19 and 2 (mean of 11,281, 8,924, 8,607, respectively). Exonic variations (ExonVar) represented 13.98% (44,815) of all mutations and were distributed along 13,170 genes; around 48% of ExonVar had a possible damaging impact on encoded proteins. The nonsynonymous (ns) ExonVar were the most frequent (47.2%), affecting 8,843 different genes (**Table 1**).

Exon function	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	All patients
Nonsynonymous SNP	10862	10787	10768	10875	10569	21175
Insertion/Deletion Frameshift	102/141	109/144	95/160	92/123	97/132	216/305
Stop Gain/Loss	104/15	102/14	100/13	95/10	81/9	231/2
Insertion/Deletion nonframeshift	174/207	151/203	170/211	168/211	158/198	342/438
Synonymous SNP	11798	11545	11446	11698	11388	21670
Unknown	213	207	245	228	272	469

Table 1: Count and classification of the exonic variants (ExonVar) found in PASH and PAPASH patients. Adapted from Brandao L., et al. Altered keratinization and vitamin D metabolism may be key pathogenetic pathways in syndromic hidradenitis suppurativa: a novel whole exome sequencing approach. J Dermatol Sci. 2020 ;99(1):17-22. doi: 10.1016/j.jdermsci.2020.05.004.

Only these genes and ns ExonVar have been considered for subsequent analysis. Patients presented 90 genes carrying mutations with deleterious and/or damage impact; within these 90 genes, 14 were in common among the 5 patients (**Table 2**) and bared 237 nsExonVar (54 and 183 in homozygosis and heterozygosis, respectively).

Common 14 genes among five syndromic HS patients
ACAT2 (Acetyl-CoA Acetyltransferase 2)
COL12A1 (Collagen Type XII Alpha 1 Chain)
CTPB2 (C-Terminal Binding Protein 2)
EPB41L4A (Erythrocyte Membrane Protein Band 4.1 Like 4A)
KCNJ12 (Potassium Inwardly Rectifying Channel Subfamily J Member 12)
KRT3 (Keratin 3)
KRT13 (Keratin 13)
KRT32 (Keratin 32)
KRT76 (Keratin 76)
LRP2 (LDL Receptor Related Protein 2)
MYH15 (Myosin Heavy Chain 15)
SLC25A5 (Solute Carrier Family 25 Member 5)
VDR (Vitamin D Receptor)
ZNF221 (Zinc Finger Protein 221)

Table 2: 14 genes carrying mutations with deleterious and/or damage impact in common among the 5 patients. Adapted from Brandao L., et al. Altered keratinization and vitamin D metabolism may be key pathogenetic pathways in syndromic hidradenitis suppurativa: a novel whole exome sequencing approach. J Dermatol Sci. 2020 ;99(1):17-22. doi: 10.1016/j.jdermsci.2020.05.004

In the pathway enrichment analysis (PEA), only 10 genes have been included, allowing to retrieve 4 pathways shared by all patients: (1) Vitamin D (calciferol) metabolism, (2) keratinization, (3) formation of the cornified envelope and (4) metabolism of steroids. The analysis has been extended to check how these four pathways were altered by all functional mutations in each patient (**Table 3**): for instance, about 45% of genes on Vitamin D pathway carried at least one ExonVar; up to 7 variations impacted the protein function, showing that this pathway is subject to mutations and its function could be reduced or impaired.

Path (ID)	ID	Gene Count[a]	Pathway Density (%)	nsExonVar Count				Median impact			
				All	PolyPhen2	SIFT	FATHMM	PolyPhen2[b]	SIFT	FATHMM	
Vitamin D -calciferol-metabolism (R-HSA-196791)	P1	5	45.45	20	5	7		7	0.996	0.005	−2.880
	P2	5	45.45	15	3	4	4	0.993	0.015	−2.880	
	P3	4	36.36	13	3	5	5	0.993	0.020	−2.880	
	P4	5	45.45	15	4	4	4	0.995	0.015	−2.880	
	P5	4	36.36	12	2	3	5	0.993	0.020	−2.840	
Keratinization (R-HSA-6805567)	P1	102	47.66	196	32	49		53	0.982	0.010	−2.373
	P2	107	50.00	241	39	48	63	0.975	0.010	−2.380	
	P3	97	45.33	192	32	49	68	0.952	0.010	−2.375	
	P4	100	46.73	224	31	43	49	0.924	0.010	−2.405	
	P5	91	42.52	170	30	36	44	0.983	0.020	−2.410	
Formation of the Cornified envelope R-HSA-6809371	P1	67	51.94	128	24	37		53	0.988	0.010	−2.373
	P2	70	54.26	179	32	38	63	0.975	0.012	−2.380	
	P3	62	48.06	132	23	38	68	0.928	0.010	−2.375	
	P4	62	48.06	151	23	30	49	0.924	0.010	−2.405	
	P5	56	43.41	102	18	24	44	0.988	0.020	−2.410	
Metabolism of steroids R-HSA-8957322	P1	46	30.67	77	12	12		18	0.970	0.010	−2.510
	P2	38	25.33	70	11	13	16	0.970	0.015	−2.785	
	P3	44	29.33	68	7	9	16	0.970	0.025	−2.625	
	P4	41	27.33	66	9	10	12	0.993	0.022	−2.785	
	P5	38	25.33	63	8	8	15	0.982	0.025	−2.840	

[a] Number of genes carrying a nsExonVar
[b] HumDiv score.

Table 3: List of the most significant pathways, retrieved by ReactomePA, associated to the variants with a functional impact on the protein function. Adapted from Brandao L., et al. Altered keratinization and vitamin D metabolism may be key pathogenetic pathways in syndromic hidradenitis suppurativa: a novel whole exome sequencing approach. J Dermatol Sci. 2020 ;99(1):17-22. doi: 10.1016/j.jdermsci.2020.05.004

Overall, these findings indicated that the mainly affected pathway in PASH and PAPASH patients was vitamin D metabolism (**Table 3**), followed by the one involved in keratinization. Vitamin D plays a pivotal role in regulating skin homeostasis by controlling epidermal as well as adnexal structures' proliferation and differentiation, particularly hair follicle [Kechichian et al., 2018]; furthermore, vitamin D contributes to the homeostasis of cutaneous immune system response, thus counteracting inflammation [Navarro-Triviño et al., 2019]. Indeed, it could be hypothesised that skin innate immunity plays an important role in the amelioration of inflammatory nodules, typical lesions of HS and its syndromic forms [Guillet et al., 2014]. It is known that vitamin D exerts immunomodulating functions by increasing both the expression of Toll-like receptor 2 and the production of antimicrobial peptides that are direct transcriptional targets of vitamin D, suppressing the production of inflammatory cytokines, and promoting the production of anti-inflammatory ones.

To date, several studies described the relationship between low vitamin D levels and an inflammatory role in HS and/or acne, conditions both present in syndromic forms of HS; moreover, its dietary supplementation can induce a reduction in the number of inflammatory lesions while no change has been observed in non-inflammatory lesions neither of acne nor of HS [Kelly et al. 2014; Lim et al., 2016; Guillet et al., 2015]. In further support of these findings, the serum dosage of vitamin D in all our patients revealed a low value of the latter, evoking the concept that a genetically impaired vitamin D pathway may contribute to the pathogenesis of skin inflammation in syndromic HS. In another of our studies, we retrospectively evaluated 250 sporadic HS patients non-supplemented with vitamin D, measuring serum levels of 25-hydroxyvitamin D (25OHD) at diagnosis before starting any specific HS treatment. The main finding of our study was the inverse correlation between 25OHD levels and HS severity; moreover, an inverse correlation between 25OHD levels and systemic inflammation was also found by measuring C-reactive protein, thus confirming that HS severity and inflammation are associated with major hypovitaminosis D [Moltrasio C et al., 2021].

Vitamin D receptor (VDR) knockout mice also showed a decrease in the expression of proteins involved in the formation of cornified envelope and keratinization including, among others,

involucrin, profilaggrin and loricrin [Xie et al., 2002]. Notably, vitamin D metabolism and keratinization pathways presented high variant density in our five patients, corroborating the recent view that HS and its syndromic forms can be regarded as a potential subtype of autoinflammatory keratinization disease (AIKD) [Nomura et al., 2020].

The keratinization process includes eight complex steps, among which the formation of cell envelope (cornified envelope), that it forms just beneath the cell membrane. It has been estimated that two hundred and fourteen genes are involved in this process, regulating keratin production, maturation, and degradation; the chromosomal localization of several genes encoding the various polypeptides involved in keratinization have been clarified: type I keratins on chromosome 17, type II on chromosome 12, transglutaminases on chromosome 14, and profilaggrin, trichohyalin, loricrin, involucrin, and the small proline-rich proteins on chromosome 1q21.4 [David Weedon AO MD FRCPA FCAP(HON), in Weedon's Skin Pathology (Third Edition), 2010], a gene-rich region known as epidermal differentiation complex (EDC) [Moltrasio C et al. 2022].
Several skin disorders particularly affect the integrity of the skin and impair epidermal differentiation, among which the paradigm is represented by psoriasis, an immune-mediated inflammatory skin disease with a multifactorial aetiology involving accelerated proliferation and abnormal differentiation of keratinocytes, now considered an autoinflammatory keratinization disease [Akiyama et al., 2018]. Hyperkeratinisation of the terminal hair follicle is now considered the *primum movens* in the pathogenetic scenario of HS; both hyperkeratinisation and hyperplasia of the infundibular region of the pilosebaceous unit promotes accumulation of keratin-rich material with follicular occlusion and subsequent dilatation, finally leading to hair follicle rupture with the consequent dispersion of the follicular contents (keratin fibres, pathogen associated molecular patterns (PAMPs), danger associated molecular patterns (DAMPs) and commensal flora) in the surrounding dermis, thus triggering and exacerbating a massive inflammation [Vekic et al., 2018].

A second approach of this study design focused on a global ExonVar overview. All genes bearing ExonVar from each patient have been filtered and included in PEA to “paint” an individual mutational pathway landscape. Venn diagram analysis allowed to identify 17 common pathways shared by all patients (**Figure 12**),

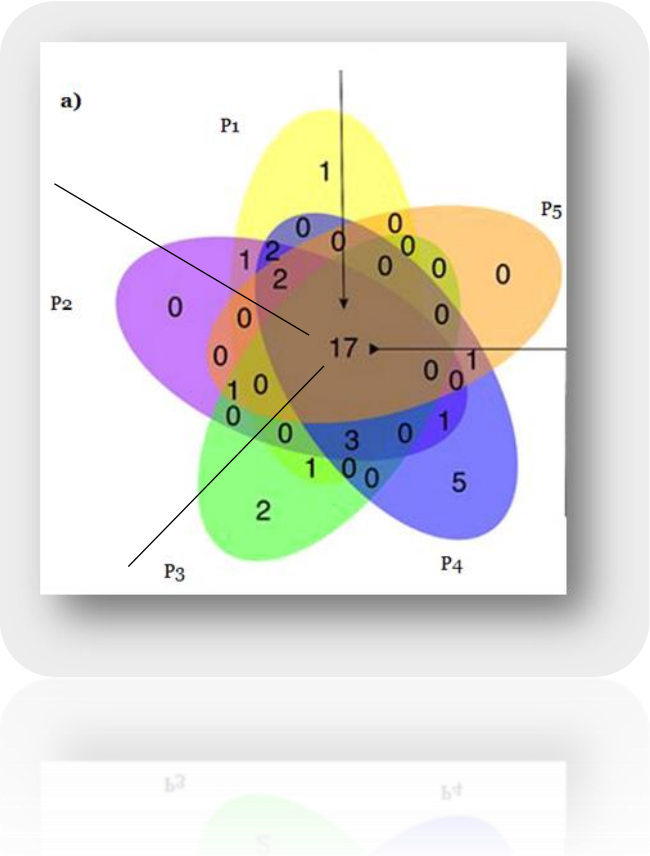

Figure 12: Venn diagram intersects 17 impaired pathways shared by all patients. Adapted from Brandao L., et al. Altered keratinization and vitamin D metabolism may be key pathogenetic pathways in syndromic hidradenitis suppurativa: a novel whole exome sequencing approach. J Dermatol Sci. 2020 ;99(1):17-22. doi: 10.1016/j.jdermsci.2020.05.004

in which 797 genes took part and 65% of those presented ExonVar (mean of 519 genes) (**Table 4**).

Patients	Gene count (%)	Nonsynonymous (%)	Stop Gain (%)
Patient 1	518 (64.99)	1.188 (10.94)	13 (12.5)
Patient 2	517 (64.87)	1.286 (11.01)	19 (12.75)
Patient 3	499 (62.61)	1.308 (11.03)	14 (13)
Patient 4	530 (66.55)	1.243 (10.92)	15 (13.68)
Patient 5	522 (65.5)	1.289 (11.24)	11 (16.05)

Table 4: Exome Analysis; **b)** percentage of gene count, nonsynonymous and stop gain variants associated to the common pathways found in syndromic HS patients. Adapted from Brandao L., et al. Altered keratinization and vitamin D metabolism may be key pathogenetic pathways in syndromic hidradenitis suppurativa: a novel whole exome sequencing approach. J Dermatol Sci. 2020 ;99(1):17-22. doi: 10.1016/j.jdermsci.2020.05.004.

Around 11% of all nsExonVar impacted these 17 pathways with mean value higher than 0.94 in HumDiv PolyPhen score. Additionally, more than 10% of all other mutations presented a functional variant impact. As listed in **Table 5**, the first 11 pathways were all related to biological processes involving the skin such as collagen and extracellular matrix (ECM) remodeling.

Pathway Name	Pathway ID (R-HSA)
Collagen degradation	1. R-HSA-1442490
Degradation of the extracellular matrix	2. R-HSA-1474228
Extracellular matrix organization	3. R-HSA-1474244
Collagen formation	4. R-HSA-1474290
Collagen biosynthesis and modifying enzymes	5. R-HSA-1650814
Assembly of collagen fibrils and other multimeric structures	6. R-HSA-2022090
Integrin cell surface interactions	7. R-HSA-216083
Laminin interactions	8. R-HSA-3000157
Non-integrin membrane-ECM interactions	9. R-HSA-3000171
ECM proteoglycans	10. R-HSA-3000178
Collagen chain trimerization	11. R-HSA-8948216
Olfactory Signaling Pathway	12. R-HSA-381753
Diseases associated with O-glycosylation of proteins	13. R-HSA-3906995
Defective B3GALTL causes Peters-plus syndrome (PpS)	14. R-HSA-5083635
Defective GALNT12 causes colorectal cancer 1 (CRCS1)	15. R-HSA-5083636
O-linked glycosylation	16. R-HSA-5173105
O-glycosylation of TSR domain-containing proteins	17. R.HSA-5173214

Table 5: Biological signaling pathways altered in syndromic HS patients. Adapted from Brandao L., et al. Altered keratinization and vitamin D metabolism may be key pathogenetic pathways in syndromic hidradenitis suppurativa: a novel whole exome sequencing approach. J Dermatol Sci. 2020 ;99(1):17-22. doi: 10.1016/j.jdermsci.2020.05.004

Matrix metalloproteinases (MMPs) are a zinc-dependent extracellular proteases that break down and remodel the ECM [Lukes et al., 1999]. It has been postulated that MMPs could actively participate in HS pathogenesis by releasing biological active peptides and inflammatory factors from the extracellular matrix (ECM) [Sanchez et al., 2019].

Moreover, MMPs have been shown to play a role in the recruitment of neutrophils to sites of inflammation, facilitating extravascular migration of neutrophils through the ECM (by

degrading the matrix itself) [Heissig et al. 2010]. High lesional and serum MMP-8 levels have been showed in HS patients, as well as an increased expression of matrix degrading enzymes (MMP 1,3,9 and 10) in HS lesional skin, whose upregulation was aligned by down-regulation of tissue inhibitor of matrix metalloproteinases (TIMP). This resulted in strongly increased MMP/TIMP4 ratios in HS, indicating a strong activity of these enzymes in HS [Witte-Händel et al., 2019]. In a recent study, conducted by Kidacki et al., [Kidacki et al., 2019], has been demonstrated that MMP-1, -3, -8 and -9 levels were increased in the "invasive proliferative gelatinous mass" (IPGM) contained in the sinus tracts – typical lesions of HS and its syndromic forms -. Notably, MMPs inhibitors, TIMP1 and TIMP2, were also significantly increased in IPGM, likely in response to increased MMPs levels.

MMPs are also known to play a critical role in the conversion of Interleukin (IL)-1β into its active form [Opdenakker et al., 2001], that in turn respond by enhancing MMPs expression [Sanchez et al., 2019], potentially creating a long-lasting inflammatory loop in HS lesional skin. Finally, a recent transcriptome meta-analysis revealed the up regulation of 12 types of MMPs, mainly of MMP1 and 3, confirming that these skin intrinsic components could represent key molecules in HS disease progression [de Oliveira et al., 2022].

PART II: A PRELIMINARY GENOTYPE-PHENOTYPE CORRELATION IN 10 UNRELATED PATIENTS WITH SYNDROMIC HS

4.3 CLINICAL FINDINGS OF SYNDROMIC HS PATIENTS (PASH/PAPASH/PASH-SAPHO OVERLAPPING)

Ten syndromic HS patients (6 men, 5 women) have been enrolled in this second part of the project (**Table 6**). Seven patients, three of whom presented with a form overlapping with SAPHO syndrome, were affected by PASH and three by PAPASH. Three clinical phenotypes have been identified based on the predominance of gut inflammation (IBD/bowel bypass syndrome) (Crohn's disease [n = 2], bowel bypass syndrome [n = 2]), joint inflammation (arthritis) (SAPHO syndrome [n = 3], pyogenic arthritis [n = 2]), and coexistence of gut and joint inflammation (n = 1; ulcerative colitis and pyogenic arthritis).

Mean age at HS onset was 28.1 years, with a chronic relapsing course of the disease in all cases and a mean IHS4 of 23.3. The most frequently involved sites were the axillae (n = 9), groins (n = 9), and anogenital region (n = 9), followed by submammary/intermammary folds (n = 4), nuchal region (n = 3), and scalp (n = 1).

PG was ulcerative in all cases, with vegetating aspects in four cases, with a mean age of onset of 32.4 years; the most frequently involved site was the trunk (n = 5), followed by lower limbs (n = 4), upper limbs (n = 2), peristomal site (n = 2), and perianal region in one patient. PG was localized in four cases, multilesional in four, and disseminated in two.

Acne onset anticipated the occurrence of HS/PG lesions in all patients, with a mean age of onset of 17 years.

Arthritis of the wrists and ankles, with a mean age of onset of 27.5 years, was present in the three patients with PAPASH, while the three patients with PASH/SAPHO overlapping showed sacroiliac joint, ankle, and spine involvement.

All patients did not report any familial history of HS, PG, as well as gut or joint diseases.

Topical therapy as well as systemic antibiotics was administered to all patients, while immunosuppressive therapies were added in five patients. Subsequently, biologic agents such as infliximab, adalimumab, anakinra, secukinumab and ustekinumab were dispensed to all patients to achieve a better clinical follow-up.

	Patient	Sex	Diagnosis	Comorbidities	HS			PG			Systemic treatments	
					Age at onset (years)	Involved sites	IHS4	Age at onset (years)	Involved sites	PG Score		
JOINT	1	F	PAPASH	FMF, PCOS, PA	16	axillae, groins, anogenital area, intermammary and submammary folds	20	16	trunk	multilesional		azitromycine, doxycycline, dapsone, adalimumab, anakinra, colchicine
	2	M	PAPASH	PA	14	axillae, groins, back, nuchal region, scalp	30	18	trunk	multilesional		rifampicin, doxycycline, azitromycine, lymecycline, clindamycin, adalimumab, anakinra, prednisone
	3	M	PASH/SAPHO	SAPHO	43	axillae, groins, anogenital area	27	43	lower limbs, trunk, upper limbs	disseminated		clindamycin, infliximab
	4	M	PASH/SAPHO	SAPHO	42	axillae, groins, anogenital area	19	46	lower limbs	localized		clindamycin, methotrexate, infliximab, adalimumab
	5	F	PASH/SAPHO	SAPHO	15	axillae, groins, anogenital region, nuchal region	26	15	trunk	multilesional		rifampicin, doxycycline, azitromycine, lymecycline, clindamycin, intravenous immunoglobulin, methotrexate, isotretinoin, adalimumab, anakinra, ustekinumab, secukinumab
GUT/JOINT	6	F	PAPASH	UC, PCOS, PA	28	submammary folds, groins, anogenital area	22	36	peristomal	localized		doxycycline, infliximab
GUT	7	M	PASH	Obesity, BBS, osteoporosis, PPPP	36	axillae, groins, anogenital area	24	36	lower limbs, trunk, upper limbs	disseminated		trimetoprim-sulfametoxazole, azitromycine, cyclosporine, prednisone, adalimumab, ustekinumab,
	8	M	PASH	Crohn disease, osteoporosis	18	axillae, groins, anogenital area, nuchal region	23	19	lower limbs	multilesional		azitromycine, trimetoprim-sulfametoxazole, ceftriaxone, ciprofloxacine, lymecycline, rifampicin, amoxicillin clavulanate, methylprednisolone, dapsone, cyclosporine, adalimumab, anakinra,
	9	F	PASH	Crohn disease, coeliac disease, myasthenia gravis, PCOS	18	axillae, nuchal region, submammary folds, groins, anogenital area	19	44	peristomal	localized		clindamycin, methotrexate, infliximab, adalimumab
	10	F	PASH	Obesity, BBS, myocardial infarction, type II DM, BP	51	axillae, groins, submammary folds, anogenital area	23	51	perianal region	localized		rifampicin, doxycycline, infliximab, adalimumab

Table 6: Clinical features of ten syndromic HS patients.

PA, pyogenic arthritis; UC, ulcerative colitis; BBS, bowel bypass syndrome; BP, bullous pemphigoid; DM, diabetes mellitus; FMF, familial Mediterranean fever; PAPASH, pyogenic arthritism pyoderma gangrenosum, acne and hidradenitis suppurativa; PASH, pyoderma gangrenosum, acne and hidradenitis suppurativa; PCOS, polycystic ovarian syndrome; PPPP, palmoplantar pustular psoriasis; SAPHO, synovitis, acne, hyperostosis and osteitis. Adapted from Marzano AV, et al. Whole-Exome Sequencing in 10 Unrelated Patients with Syndromic Hidradenitis Suppurativa: A Preliminary Step for a Genotype-Phenotype Correlation. Dermatology. 2022;238(5):860-869. doi: 10.1159/000521263.

4.4 GENETIC ANALYSES

4.4.1 Exome Description: global view

Overall, 343,168 different variants have been retrieved, of which 57,707 (16.81%) were ExonVar (**Table 7**), mainly localized on chromosome 1, 2, and 19 and distributed on 14,546 genes; among these, nonsynonymous (ns) ExonVar were the most frequent (27,966) with a potential damaging action on 10,356 genes (**Table 8**).

Location of individual variants	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
Downstream	929	854	944	2867	2465	2236	2137	3243	2895	2311
Exonic	23616	23262	22904	23890	24053	23679	22847	24015	23648	23843
Exonic; splicing	15	11	8	13	10	16	14	13	13	19
Intergenic	21515	23757	25611	112833	82604	74493	73367	138631	122702	84509
Intronic	77441	63256	67870	153678	135736	122904	122751	168771	158148	132237
ncRNA_exonic	2816	2733	2814	4021	3727	3677	3575	4182	4142	3861
ncRNA_exonic; splicing	4	5	2	2	1	1	5	3	5	4
ncRNA_intronic	4984	4605	4875	14719	11858	10497	10473	16226	15318	11544
ncRNA_splicing	10	12	14	21	15	17	21	17	20	28
Splicing	85	67	76	76	95	95	78	95	87	92
Upstream	2392	2041	2267	4922	4494	4239	4030	5270	5189	4630
Upstream; downstream	169	126	155	317	270	262	225	313	310	264
UTR3	4296	3594	3780	6935	6482	5955	5879	7165	6980	6420
UTR5	2628	2328	2492	3322	3213	2987	2939	3313	3359	3296
UTR5; UTR3	4	4	8	9	8	9	9	5	11	7

Table 7: Distribution of the variants according to their location in ten syndromic HS patients.

ncRNA, non-codingRNA; UTR, untranslated region; UTR3, untranslated region on 3' side; UTR5, untranslated region on 5' side. Adapted from Marzano AV, et al. Whole-Exome Sequencing in 10 Unrelated Patients with Syndromic Hidradenitis Suppurativa: A Preliminary Step for a Genotype-Phenotype Correlation. Dermatology. 2022;238(5):860-869. doi: 10.1159/000521263.

Type of exonic variants	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10
Frameshift detetion	141	144	132	154	144	148	143	154	138	145
Frameshift insertion	102	109	97	101	110	96	107	106	107	109
Nonframeshift deletion	207	203	198	213	206	192	202	212	205	217
Nonframeshift insertion	174	151	158	173	179	175	162	174	175	172
Nonsynonymous SNV	10862	10787	10569	11083	10962	10884	10529	11048	10977	11109
Stop-gain	104	102	81	104	103	93	97	93	96	96
Stop-loss	15	14	9	12	15	16	14	11	14	10
Synonymous SNV	11798	11545	11388	11806	12107	11845	11339	11959	11719	11739
.	140712	126509	133647	327284	274742	250779	248097	370928	342488	272771
Unknown	213	207	272	232	219	226	245	248	208	235

Table 8: Exonic variants (ExonVar) for each patient according to the kind of variation. SNV, single nucleotide variation. Adapted from Marzano AV, et al. Whole-Exome Sequencing in 10 Unrelated Patients with Syndromic Hidradenitis Suppurativa: A Preliminary Step for a Genotype-Phenotype Correlation. Dermatology. 2022;238(5):860-869. doi: 10.1159/000521263.

4.4.2 ExonVar Approach

ExonVar have been filtered by Minor Allele Frequency (MAF) and impactVar; a total of 10,268 ExonVar (17.76%) were rare (GnomAD v3.0 MAF ≤0.01) while 28,553 (49.38%) presented with a potential impact on protein function. Only 3,188 (5.51%) ExonVar were rare and with potential mutational impact.

Later, we focused on homozygous mutations (**Table 9**), founding 55 rare mutations with functional impact in 43 loci (comprising 45 genes).

Patient	ExonVar_homozygotic_rare	ExonVar_homozygotic_rare_mutational impact
P1	49	1
P2	57	4
P3	85	10
P4	46	3
P5	39	1
P6	62	6
P7	128	20
P8	74	3
P9	40	0
P10	53	7

Table 9: Count of homozygous rare exonic variants in each patient. Adapted from Marzano AV, et al. Whole-Exome Sequencing in 10 Unrelated Patients with Syndromic Hidradenitis Suppurativa: A Preliminary Step for a Genotype-Phenotype Correlation. Dermatology. 2022;238(5):860-869. doi: 10.1159/000521263.

4.4.3 Genetic Variants in Autoinflammation and Keratinization Genes

Genetic variants have been found in seven autoinflammatory genes, including *MEFV* (Mediterranean Fever), *PSTPIP1* (Proline-Serine-Threonine Phosphatase Interacting Protein 1), *NLRC4* (NLR Family CARD Domain Containing 4), *WDR1* (WD Repeat Domain 1), *NOD2* (Nucleotide Binding Oligomerization Domain Containing 2), and *OTULIN* (OTU Deubiquitinase With Linear Linkage Specificity), and two keratinization genes: *GJB2* (Gap Junction Protein Beta 2) and *NCSTN* (Nicastrin), although this latter can be considered both an autoinflammatory and a keratinization gene (**Table 10**).

	Patient	Sex	Diagnosis	Gene	Change in DNA	Change in protein	Clinical significance
JOINT INFLAMMATION	1	F	PAPASH	*MEFV*	c.2080A>G	p.Met694Val	Pathogenic
				MEFV	c.2177T>C	p.Val726Ala	Pathogenic
				PSTPIP1	c.831G>T	p.Glu277Asp	Pathogenic
	2	M	PAPASH	*NCSTN*	c.1140_1141del	p.Asp381Serfs*7	Not yet reported
	3	M	PASH/SAPHO	*NCSTN*	c.482delA	p.Ile162Tyrfs*57	Not yet reported
	4	M	PASH/SAPHO	*NLRC4*	c.2668T>C	p.Cys890Arg	VUS
	5	F	PASH/SAPHO	*WDR1*	c.323A>G	p.His108Arg	Not yet reported
GUT/JOINT	6	F	PAPASH	*NLRC4*	c.541C>T	p.Arg181X	VUS
GUT INFLAMMATION	7	M	PASH	*NOD2*	c.2104C>T	p.Arg702Trp	Risk factor for IBD
				MPO	c.752T>C	p.Met251Tyr	Conflicting interpretation of pathogenicity (Pathogenic AND Variant of Uncertain Significance)
				OTULIN	c.209T>C	p.Ile70Tyr	VUS
	8	M	PASH	*NOD2*	c.3017dupC	p.Leu1007Profs*2	Risk factor for IBD
	9	F	PASH	*GJB2*	c.35delG	p.(G12Vfs*2)	Pathogenic
				OTULIN	c.345G>T	(p.Q115H)	VUS
	10	F	PASH	*NOD2*	c.2722G>C	p.(G908R)	Susceptibility factor to IBD

Table 10: Genetic Variants in Autoinflammation and Keratinization Genes, based on presence of gut, joint and gut/joint inflammation in twelve syndromic HS patients.

VUS, Variant of Uncertain Significance; IBD, Inflammatory Bowel Disease.

Adapted from Marzano AV, et al. Whole-Exome Sequencing in 10 Unrelated Patients with Syndromic Hidradenitis Suppurativa: A Preliminary Step for a Genotype-Phenotype Correlation. Dermatology. 2022;238(5):860-869. doi: 10.1159/000521263

Four PASH patients with gut inflammation showed three different variants in *NOD2* gene, [NM_001293557.2: c.2104C>T, p.Arg702Trp; c.3017dupC, p.Leu1007Profs*2; c.2722G>C p.Gly908Arg], two variants in *OTULIN* [NM_138348.6: c.209T>C, p.Ile70Thr; c.345G>T, p.Gln115His] and a variant in *GJB2* (NM_004004.6: c.35delG, p.Gly12Valfs*2) gene.

NOD2 gene encodes for an intracellular pattern recognition receptor (PRR) consisting of two caspase recruitment domains (CARDs) in the C-terminal region and eleven leucine-rich repeats (LRRs) in the N-terminal tail; it belongs to the NOD1/Apaf-1 family (also known as NOD-like receptor family) and is primarily expressed in the peripheral blood leukocytes. It plays an important role in the immune system response by recognizing bacterial molecules which possess the muramyl dipeptide (MDP) moiety and activating the NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells), a protein complex that regulates DNA transcription, production of several cytokines and cell survival [McGovern et al., 2001]. *NOD2* gene variants have been associated with Chron's disease, so that this gene is now considered an IBD candidate gene [Uniken et al., 2017].

Recently, *NOD2* sequence variants have been reported to be related to NOD2-associated autoinflammatory disease (NAID), clinically characterized by infiltrated skin lesions and gastrointestinal symptoms with recurrent episodes of fever and arthritis [Yao et al., 2019].

Several studies demonstrated an association of IBD both with HS and PG, supporting the hypothesis that similar pathogenic mechanisms contribute to these diseases and paving the way to a common targeted therapy [Principi et al., 2016; Shalom et al., 2016].

Moreover, these three conditions are all linked to an altered immune tolerance that promotes a gut dysbiosis; the link between gut inflammation, innate immunity dysregulation and autoinflammatory skin diseases is further supported by the occurrence of HS in some patients with bowel bypass surgery-associated altered gut microbiota [Garcovich et al., 2019].

Otulin encodes a member of the peptidase C65 family of ubiquitin isopeptidases, known to participate and regulate NF-κB signaling pathway in the context of immunity and inflammation [Aksentijevich et al., 2017].

Loss-of-function mutations in *Otulin* have been associated, in a mouse model, with OTULIN-related autoinflammatory syndrome, characterized by painful erythematous rash with skin nodules, gastrointestinal inflammation with prolonged recurrent fevers and joint swelling [Fiil et al., 2016]. These findings support the contribution of this gene in skin and gut inflammation.

GJB2 encodes for a member of the gap junction protein family, gap junction beta 2, more commonly known as connexin 26 (Cx26) that regulates both the epidermis and hair follicle keratinization [García-Vega et al., 2019].

The most prevalent mutations of CX26 have been associated with inherited non syndromic deafness but pathogenic variants of this gene have been also related to skin diseases characterised by abnormal keratinisation and hyperproliferation; in particular, *GJB2* mutations cause Keratitis-Ichthyosis-Deafness (KID) syndrome, clinically characterized by keratitis, erythrokeratoderma, and neurosensory deafness [Binder et al., 2005]. Recently, four cases of KID occurring in association with the follicular occlusion triad—represented by HS, acne conglobata, and dissecting folliculitis of the scalp—have been reported [Maintz et al., 2005]. The correlation between HS and GJB2 is still to be clarified, but it has been speculated that HS could be the result of the hyperproliferative tendency of epidermis in KID patients to trigger follicular plugging and cyst formation, thus promoting an exaggerated inflammatory response in resident immune cells [Maintz et al., 2005].
As mentioned above, the same pathogenic variant in the *GJB2* gene causative of KID syndrome has been found in one of our PASH patients, suggesting that a genetically disrupted keratinization pathway may contribute to the pathogenesis of skin inflammation in syndromic HS.

Three PAPASH and three PASH/SAPHO overlapping patients with concomitant joint inflammation showed a variant in *PSTPIP1* (NM_003978.5: c.831G>T p.Glu277Asp) gene, two different novel frameshift variants in *NCSTN* [NM_015331.3: c.1140_1141del, p.Asp381Serfs*7; c.482delA, p.Ile162Tyrfs*57], a variant in *WDR1* (NM_017491.5: c.323A>G, p.His108Arg) and two variants in *NLRC4* (NM_021209.4: c.2668T>C, p.Cys890Arg) gene, one of whom (NM_021209.4: c.541C>T, p.Arg181X) was present in a patient with a mixed phenotype characterized by gut and joint inflammation.
The first patient with PAPASH, in addition to having a missense mutation in *PSTPIP1*gene, exhibited two pathogenic variants in *MEFV* [NM_000243.3: c.2080A>G, p.Met694Val; c.2177T>C, p.Val726Ala] gene, related to her associated condition, Familial Mediterranean Fever (FMF), the prototypical monogenic autoinflammatory disease characterized by recurrent episodes of fever with serosa inflammation manifesting with severe abdominal or chest pain, arthralgia, monoarticular arthritis and limited erythematous skin rash [Schnappauf et al., 2019]. *MEFV* encodes for the protein pyrin, mainly expressed in neutrophils and monocytes, that belongs to the group of pyrin-domain (PYD)–containing proteins; the binding of pyrin to ASC (apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain) leads to activation of ASC, resulting in the recruitment and activation of procaspase-1. Active caspase 1 is crucial for the proteolytic activation of IL-1β and IL-18. Pyrin can also bind to the

PYD domains of other proteins capable of activating NF-κB, thus acting as a key factor in the inflammatory context [Schnappauf et al., 2019].

Since its discovery in 1997, more than 300 sequence variants have been described for the *MEFV* gene but only 14 occur commonly in FMF; 80% of them are clustered within the exon 10 while the others localized on exon 2,3 and 4.

Met694Val (c.2080A>G) is the most frequently found mutation in FMF patients and is usually associated with the most severe phenotype and early onset of the disease, whereas the Val762Ala (c.2177T>C) mutation, located in exon 2 of *MEFV* gene, has been found in some patients with episodes of fever lasting 4-5 days, with seasonal changes of frequency, stress or fatigue as triggering factor, in association with abdominal pain, arthralgia, myalgia, enlarged bilateral cervical lymph nodes, hepatomegaly, splenomegaly and seizures [https://infevers.umai-montpellier.fr/web/index.php].

Notably, the frequency of *MEFV* mutations in HS patients is higher than that in healthy controls [Vural et al., 2017], and it is likely that mutations of this gene may contribute also to the pathogenesis of HS and its syndromic forms.

PSTPIP1 encodes a cytoskeletal protein that is highly expressed in hemopoietic tissues; this protein functions via its interaction with several different proteins involved in cytoskeletal organization, inflammatory processes and immunoregulatory modulation.

The mutation Glu277Asp of *PSTPIP1* gene, whose discovered mutations are involved in PAPA (Pyogenic Arthritis, Pyoderma gangrenosum and Acne) syndrome [Wise et al., 2022], has been previously reported by Marzano *et al*., in a PAPASH patient [Marzano et al., 2013] and has been confirmed in the same patient in this study. It has been hypothesized that the disease-causing mutations compromise physiologic signaling necessary for the maintenance of a proper inflammatory response. Furthermore, considering the close association between this gene and PAPA syndrome, can be assumed that *PSTPIP1* variations may be linked to joint autoinflammation.

γ-Secretase, an intramembrane aspartyl protease complex that cleaves its substrates within their transmembrane domains, is composed of four subunits: presenilin-1(PSEN-1), nicastrin (NCSTN), anterior pharynx-defective 1 (APH-1), and presenilin enhancer 2 (PEN-2) [Nie et al., 2020].

NCSTN encodes a 78 kDa, highly glycosylated, type I single pass transmembrane protein that plays a crucial role in maintaining the stability of γ-Secretase complex (GSC) and regulating intracellular protein trafficking [Zhang et al., 2005].

NCSTN with PSEN-1 and PEN-2 can establish a "secretasome" which allows for intramembranous proteolysis of the transmembrane proteins, including Notch [Yu et al., 2000], a receptor in a highly conserved signalling pathway that regulates cell self-renewal and differentiation in several tissues and cell types [Lobry et al., 2014].

Mutations of genes encoding for the γ-secretase complex have been mainly reported in familial HS [Vossen et al., 2020], although some pathogenic variants of these genes have been also reported in non-familial syndromic HS patients. In particular, it has been reported the involvement of *NCSTN* gene in one PASH patient [Duchatelet et al., 2015] and one with SAPHO presenting with HS manifestations [Li et al., 2018].

Interestingly, in our patient with non-familial PAPASH and in one with PASH/SAPHO overlap syndrome, two novel variants in *NCSTN* gene have been found, indicating that not only familial but also syndromic HS pathogenesis may be linked to *NCSTN* variants.

WDR1 is a protein coding gene that induces the actin severing and disassembly in conjunction with ADF/cofilin family members [Fujibuchi et al., 2005]. It is also involved in several processes such as cytokinesis, chemotactic cell migration and establishment of cell polarity during follicular epithelium development [https://www.genecards.org].

It has been demonstrated that mutations in *WDR1* gene cause the onset of autoinflammatory diseases and macrothrombocytopaenia; indeed, a mouse model homozygous for a hypomorphic allele of this gene exhibited spontaneous inflammatory disease and thrombocytopenia. Both features have been suggested to result from an impairment in actin disassembly; WDR1 mutants also displayed neutrophilia although the inflammatory landscape in this condition remains unclear [Kile et al., 2007]. More recently, it has been also demonstrated that aberrant actin disassembly contributes to the pyrin inflammasome formation and triggers autoinflammatory diseases driven by IL-18, in response to lipopolysaccharide (LPS), but IL-1 independent [Kim et al., 2015].

NLR family CARD domain-containing protein 4 is a protein that is encoded by the *NLRC4* gene. Its protein is highly conserved and is best correlated with the formation of the inflammasome: after interaction with NAIP (NLR family of apoptosis inhibitory protein) and its activation, NLRC4-NAIP forms a multimeric complex, known as inflammasome. NLCR4-dependent inflammasome activates caspase 1, that in turn promotes the activation of pro-inflammatory cytokines such as IL-1β and IL-18 as well as the pyroptosis inducer Gasdermin D (GSDMD) [Mariathasan et al., 2004].

Gain of function mutations in *NLCR4* gene have been related to the NLCR4 inflammasomopathies that comprise a growing group of autoinflammatory diseases spanning a

broad clinical spectrum, from cold urticaria to neonatal-onset multisystem inflammatory disease (NOMID) and the often-fatal disease AIFEC (Autoinflammation with infantile enterocolitis) [Romberg et al., 2017].

In our series, *NLRC4* variants have been found in a patient with joint inflammation and in another one with both gut and joint inflammation, suggesting that this gene could be implicated in an ever-increasing group of inflammatory conditions.

PART III: VARIANT ENRICHMENT ANALYSIS TO EXPLORE PATHWAYS FUNCTIONALITY IN TWELVE SYNDROMIC HS PATIENTS THROUGH WES ANALYSIS

4.5 CLINICAL FINDINGS OF SYNDROMIC HS PATIENTS (PASH/PAPASH/PASH-SAPHO OVERLAPPING)

Nine patients were affected by PASH, three of whom had a form overlapping with SAPHO, and three were diagnosed with PAPASH, with a chronic-relapsing state in all cases. No positive family history for PG and/or HS has been reported. Relevant comorbidities were Crohn's disease (n = 2), polycystic ovary syndrome (n = 4), pilonidal cyst (n = 1), psoriasis (n = 1), type II diabetes mellitus (n = 1) and ulcerative colitis (n = 1). Regarding the HS lesional sites (average IHS4 of 22), axillae (n = 12) was the most frequent area, followed by groin (n = 10), anogenital region (n = 8), nuchal region (n = 4), intergluteal folds (n = 4) and submammary/intermammary folds (n = 4). PG phenotype was ulcerative in all patients and in three cases, it was associated with vegetating aspects. Age at PG onset was generally concomitant with HS onset, with an average difference of 3.7 years between the two diseases. The most recurrent sites involved were the lower limbs (n = 6), trunk (n = 4), upper limbs (n = 2), peristomal site (n = 2) and perianal region (n = 1). PG was localized in four cases, multilesional in six and widespread in two. Acne, whose onset occurred before the appearance of HS or PG lesions in all patients, was present in all enrolled subjects, with the face (in all patients) and trunk (n =6) being the most-affected areas. Arthritis in the wrists and ankles has been observed in three PAPASH cases, whereas the three patients with overlapping PASH/SAPHO syndrome showed sacroiliac joint, ankle, and spine involvement.

All cases have been treated with topical therapy; systemic antibiotics have been administered to ten patients, while oral retinoids have been dispensed to one subject. Monoclonal antibodies have been administered to all patients: adalimumab (n = 6); infliximab (n = 6) and ustekinumab (n = 2). Anakinra has been also given to four individuals.

4.6 GENETIC ANALYSES

4.6.1 WES data

The WES data obtained on PAPASH, PASH and PASH/SAPHO group representing genetic variation information are showed in **figure 13**, **14** and table **11**.

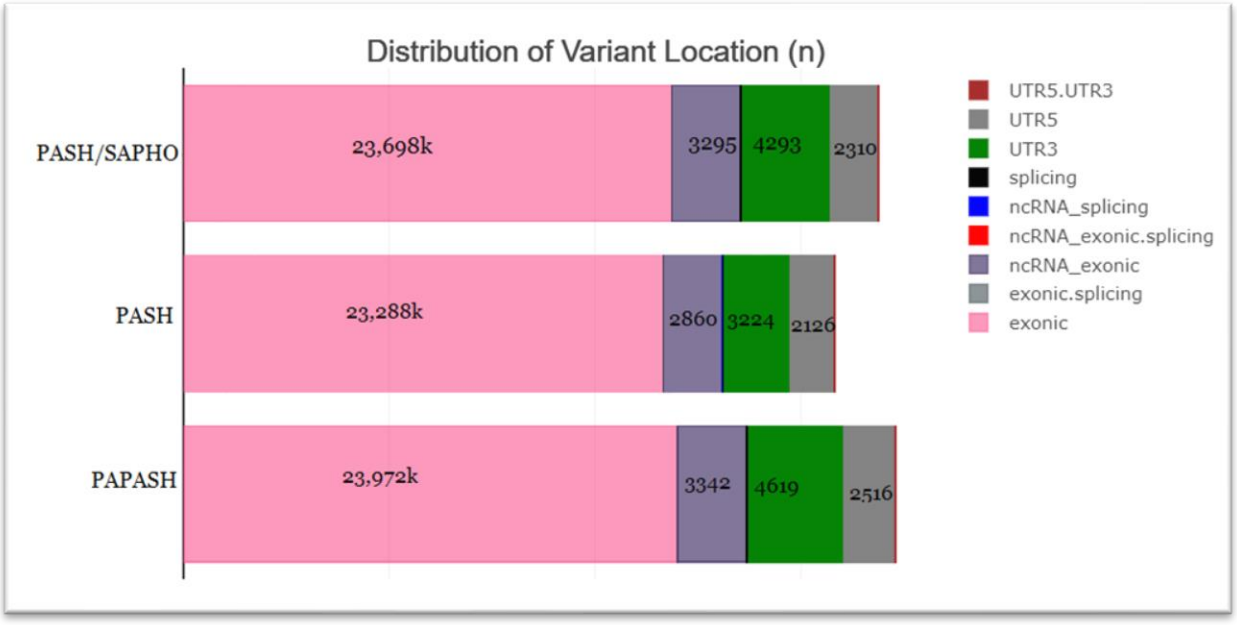

Figure 13: Distribution of variant location on groups PASH/SAPHO, PASH and PAPASH
ncRNA, non-codingRNA; UTR, untranslated region; UTR3, untranslated region on 3' side; UTR5, untranslated region on 5' side. Adapted from Brandão LAC, et al. Variant Enrichment Analysis to Explore Pathways Functionality in Complex Autoinflammatory Skin Disorders through Whole Exome Sequencing Analysis. Int J Mol Sci. 2022; 23(4):2278. doi: 10.3390/ijms23042278

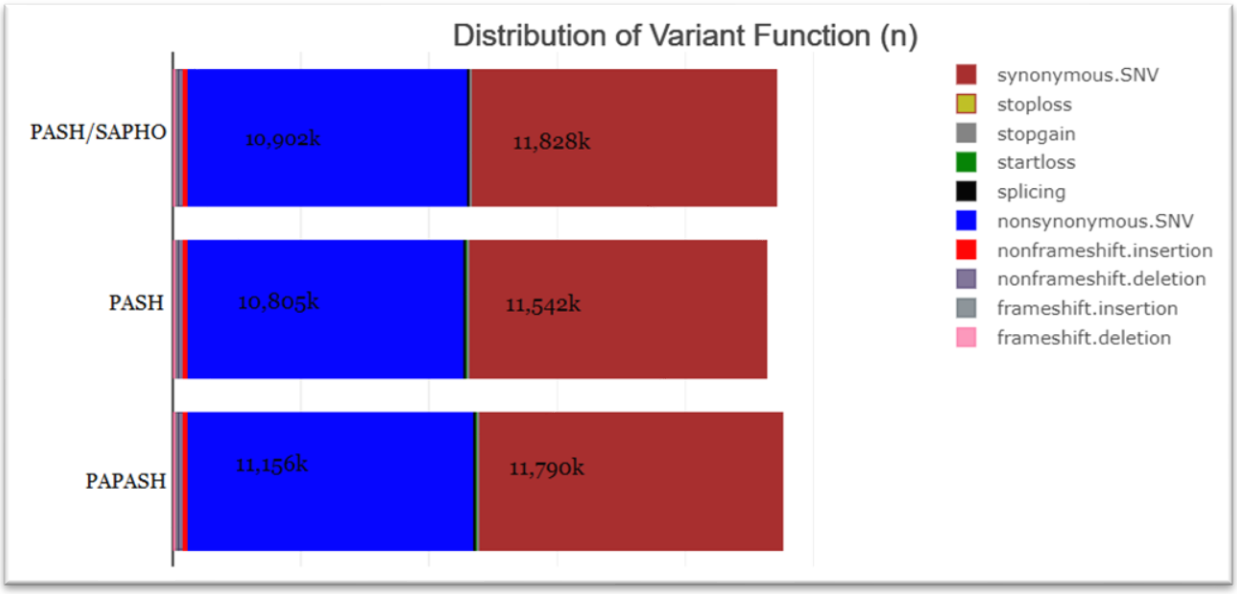

Figure 14: Distribution of variant function on groups PASH/SAPHO, PASH and PAPASH

SNV, single nucleotide variation. Adapted from Brandão LAC, et al. Variant Enrichment Analysis to Explore Pathways Functionality in Complex Autoinflammatory Skin Disorders through Whole Exome Sequencing Analysis. Int J Mol Sci. 2022; 23(4):2278. doi: 10.3390/ijms23042278

	Included	Exonic	Functional	Impact	Rare
PASH/SAPHO	17142	12185	263	2718	2825
PASH	16222	11690	269	2704	1870
PAPASH	17350	12296	285	2760	2681

Table 11: Distribution of genes carrying variants for each group: PASH/SAPHO, PASH and PAPASH. Adapted from Brandão LAC, et al. Variant Enrichment Analysis to Explore Pathways Functionality in Complex Autoinflammatory Skin Disorders through Whole Exome Sequencing Analysis. Int J Mol Sci. 2022; 23(4):2278. doi: 10.3390/ijms23042278

4.6.2 Exclusive Enriched Pathways (EPP)

To determine the enriched pathways, only the common variants shared by all individuals in each patient group have been considered. Through VEA, 33, 40 and 28 enriched pathways (EP) have been found in PAPASH, PASH and PASH/SAPHO patient groups, respectively, while 15, 25 and 12 specific exclusive EP (ePP) have been observed in the same groups, respectively (**Figure 15**).

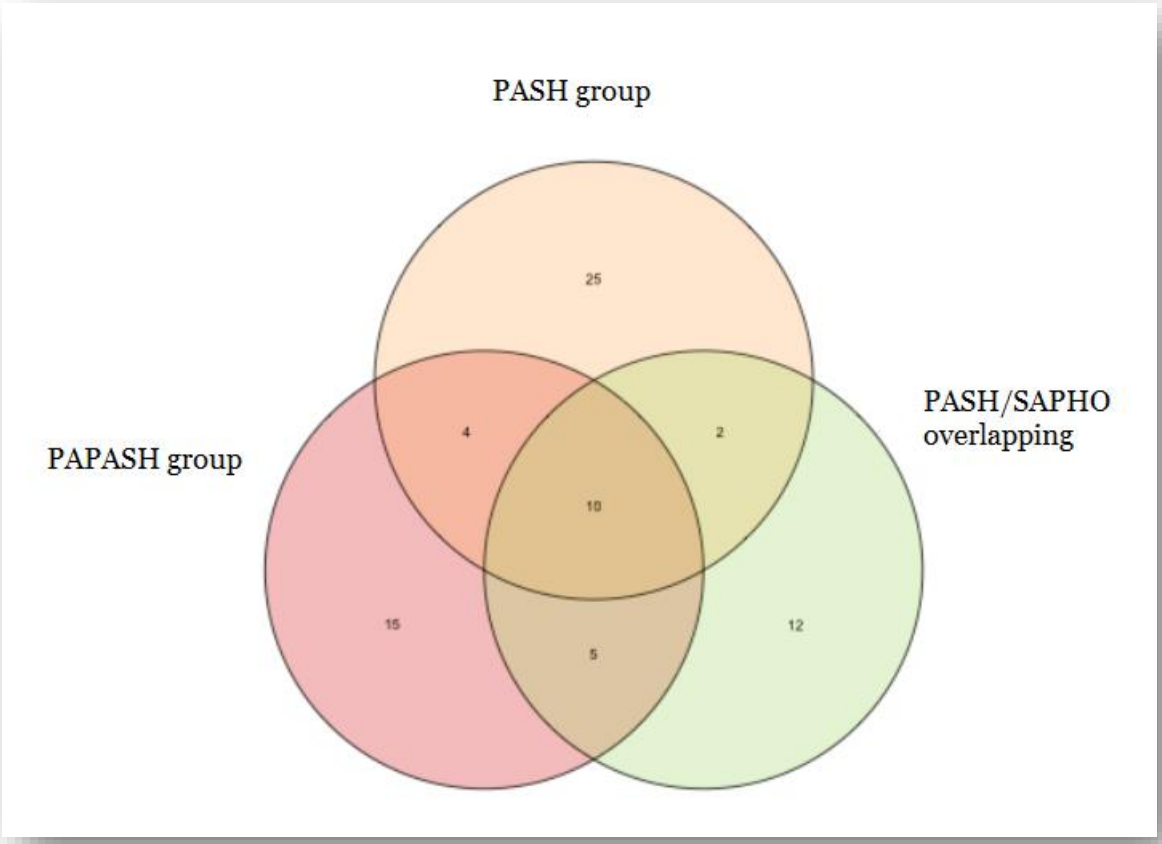

Figure 15: Venn diagram of common filtered exclusive enriched pathways for PASH, PAPASH and PASH/SAPHO overlapping. Adapted from Brandão LAC, et al. Variant Enrichment Analysis to Explore Pathways Functionality in Complex Autoinflammatory Skin Disorders through Whole Exome Sequencing Analysis. Int J Mol Sci. 2022; 23(4):2278. doi: 10.3390/ijms23042278.

4.6.2 EEP from PAPASH

As mentioned above, fifteen exclusive enriched pathways have been found for the PAPASH group (**Table 12**).

PathwayName	DB_ID	OR	p.adjust	ImportantVar	Gene Number	Protein ratio	Rna ratio
Endosomal/Vacuolar pathway	R-HSA-1236977	1,66	0	76	4	10,2	16,33
Alpha-defensins	R-HSA-1462054	2,24	0,0309	11	3	0	0
Synthesis of PC	R-HSA-1483191	1,57	0,0397	8	7	35,71	33,33
mTORC1-mediated signalling	R-HSA-166208	1,7	0,0273	5	4	80	84
Mineralocorticoid biosynthesis	R-HSA-193993	2,04	0,0257	4	3	0	0
Glucocorticoid biosynthesis	R-HSA-194002	2,08	0,003	4	3	6,25	6,25
Fatty acids	R-HSA-211935	1,82	0	11	3	10	15
Glycogen storage disease type 0 (liver GYS2)	R-HSA-3858516	3,94	0,0022	3	2	0	0
Smooth Muscle Contraction	R-HSA-445355	1,55	0,0001	5	4	51,22	48,78
Defective CYP11B2 causes CMO-1 deficiency	R-HSA-5579009	5,03	0	1	1	0	0
GLI proteins bind promoters of Hh responsive genes to promote transcription	R-HSA-5635851	2,49	0,0119	5	3	66,67	0
Formation of xylulose-5-phosphate	R-HSA-5661270	2,52	0,0377	1	1	60	20
Surfactant metabolism	R-HSA-5683826	1,57	0,0014	8	6	20,69	13,79
Noncanonical activation of NOTCH3	R-HSA-9017802	2,26	0,0034	1	1	75	75
Neutrophil degranulation	R-HSA-6798695	1,24	0	215	84	0*	0*

Table 12: List of 15 common exclusive enriched pathways in PAPASH group.

OR, odd ratios. Adapted from Brandão LAC, et al. Variant Enrichment Analysis to Explore Pathways Functionality in Complex Autoinflammatory Skin Disorders through Whole Exome Sequencing Analysis. Int J Mol Sci. 2022; 23(4):2278. doi: 10.3390/ijms23042278

Ranking the eEP according to important variants (ImportantVar), the following impaired pathways have been observed: i) “endosomal/vacuolar pathway” [Reactome ID (R-HSA)-1236977)], with the highest number of ImportantVar (n = 76), and ii) “alpha-defensins” (R-HSA-1462054) and iii) “fatty acids” (R-HSA-211935) pathways, both having 11 ImportantVar. Moreover, “defective CYP11B2 causes CMO-1 deficiency” (R-HSA-5579009) and “Glycogen storage disease type 0 (liver GYS2)” (R-HSA-3858516) achieved the greatest odds ratios (OR), 5.03 and 3.94, respectively.

Finally, based on the expression probability ratio (EPR), three eEP have been retrieved: i) “mTORC1-mediated signaling” (R-HSA-166208), ii) “noncanonical activation of NOTCH3” (R-HSA-9017802), and iii) “Neutrophil degranulation” (R-HSA-6798695). This latter, together with “alpha-defensins” (R-HSA-1462054) and “noncanonical activation of NOTCH3” is linked to the neutrophil inflammatory response.

These enriched pathways exclusively found in PAPASH patients showed that two of them are mainly related to autophagy: i) “endosomal/vacuolar pathway” (R-HSA-1236977) and ii) “mTORC1-mediated signaling pathway” (R-HSA-166208).

Autophagy is a crucial self-degradative process responsible for maintaining cellular homeostasis. It plays a housekeeping role in removing intracellular aggregates, damaged organelles as well as eliminating intracellular pathogens; it also promotes cellular senescence and antigen presentation, protects against genome instability, and prevents necrosis, giving it a key role in preventing diseases including, among others, infections, and autoimmune diseases [Glick et al., 2010]. An unregulated or absent autophagy is therefore related to a wide range of conditions, including skin diseases; it has been demonstrated, in mouse models, that autophagy inhibition in keratinocytes impairs skin repair. Autophagy deficiency in keratinocytes not only compromised keratinocyte function including proliferation and migration, but also suppressed fibroblast activation [Qiang et al., 2021]. Futhermore, autophagy deficiency in endothelial cells results in an unregulated leukocyte transendothelial migration (TEM), thus increasing neutrophil infiltration and tissue damage [Guo et al., 2019]. Leukocyte transendothelial migration is one of the most important steps in launching an adequate inflammatory immune response; upon tissue damage or infection, leukocytes exit blood vessels by adhering to and probing vascular endothelial cells (VECs), breaching endothelial cell-cell junctions, and transmigrating across the endothelium [Getter et al., 2019]. An uncontrolled leukocyte TEM, partially favoured by the loss of adhesion receptors that are not recycled by deficient autophagy events exacerbates several immune-mediated inflammatory diseases, including, among others, skin conditions such as HS.

4.6.2 EEP from PASH

In the PASH patient group, 25 eEP have been found; the “Dectin-2 Family” (R-HSA-5621480) was the eEP with the highest number of ImportantVar (200 variants distributed in 10 genes) (**Table 13**), following by i) “Termination of O-glycan biosynthesis” (R-HSA-977068), ii) “Defective GALNT12 causes colorectal cancer 1 (CRCS1)” (R-HSA-5083636), iii) “Defective GALNT3 causes familial hyperphosphatemic tumoral calcinosis” (HFTC) (R-HSA-5083625), and iv) “Defective C1GALT1C1 causes Tn polyagglutination syndrome (TNPS)” (R-HSA-5083632).

PathwayName	DB_ID	OR	p.adjust	ImportantVar	Gene Number	Protein.ratio	Rna.ratio
Release of apoptotic factors from the mitochondria	R-HSA-111457	5,18	0	None	None	80	80
Cross-presentation of soluble exogenous antigens (endosomes)	R-HSA-1236978	1,74	0,0006	7	6	58,82	61,76
Ca2+ activated K+ channels	R-HSA-1296052	3,84	0	5	1	11,11	11,11
betaKlotho-mediated ligand binding	R-HSA-1307965	4,46	0,0019	1	1	33,33	0
Synthesis of PI	R-HSA-1483226	2,96	0,0465	1	1	80	60
FGFR4 mutant receptor activation	R-HSA-1839128	5,39	0,0007	1	1	0	0
Downstream TCR signaling	R-HSA-202424	1,73	0	61	10	41,38	43,1
Phosphorylation of CD3 and TCR zeta chains	R-HSA-202427	2,04	0	57	6	12,99	9,09
Translocation of ZAP-70 to Immunological synapse	R-HSA-202430	2,11	0	57	6	12,33	8,22
Generation of second messenger molecules	R-HSA-202433	1,98	0	57	6	21,59	12,5
Metabolism of ingested SeMet, Sec, MeSec into H2Se	R-HSA-2408508	3,75	0,0214	None	None	44,44	11,11
PD-1 signaling	R-HSA-389948	2,14	0	57	6	14,1	10,26
Dopamine receptors	R-HSA-390651	3,23	0,0152	None	None	0	0
Defective GALNT3 causes familial hyperphosphatemic tumoral calcinosis (HFTC)	R-HSA-5083625	1,52	0	199	9	9,76	4,88
Defective C1GALT1C1 causes Tn polyagglutination syndrome (TNPS)	R-HSA-5083632	1,52	0	199	9	0*	0*
Defective GALNT12 causes colorectal cancer 1 (CRCS1)	R-HSA-5083636	1,52	0	199	9	9,76	4,88
Defective MAT1A causes MATD	R-HSA-5579024	5,8	0,0149	None	None	0	0
Degradation of GLI2 by the proteasome	R-HSA-5610783	1,6	0,0295	6	5	61,25	63,75
Dectin-2 family	R-HSA-5621480	1,53	0	200	10	16,98	5,66
Metallothioneins bind metals	R-HSA-5661231	3,86	0,0001	4	3	18,18	36,36
Gluconeogenesis	R-HSA-70263	1,89	0,0101	7	6	44,74	36,84
Urea cycle	R-HSA-70635	2,94	0,0003	2	2	36,36	27,27
Protein methylation	R-HSA-8876725	1,95	0,0175	2	2	47,37	68,42
Termination of O-glycan biosynthesis	R-HSA-977068	1,52	0	199	9	10,42	6,25
Cell surface interactions at the vascular wall	R-HSA-202733	0,71	0	22	16	NONE	NONE

Figure 13: List of 25 common exclusive enriched pathways in PASH group.
OR, odd ratios. Adapted from Brandão LAC, et al. Variant Enrichment Analysis to Explore Pathways Functionality in Complex Autoinflammatory Skin Disorders through Whole Exome Sequencing Analysis. Int J Mol Sci. 2022; 23(4):2278. doi: 10.3390/ijms23042278

All these four eEP are involved in the biosynthesis of O-glycan oligosaccharides, sharing genes with the "Dectin-2 Family" pathway.
This latter plays an important role in both immunity and homeostasis. Dectin-2 family of C-type lectins are a family of proteins which recognize a wide range of ligands and includes Dectin-2, blood dendritic cell antigen 2 (BDCA-2), c-type lectin receptor (DCIR), dendritic cell immunoactivating receptor (DCAR), c-type lectin domain family 4 member D (CLEC4D/Clecsf8) and macrophage inducible Ca2+-dependent lectin receptor (Mincle). Dectin-2, BDCA-2, DCAR and Mincle are all associate with the γ subunit of the immunoglobulin Fc receptor (FcRγ) to give rise to cellular activation, including phagocytosis and cytokine production, while DCIR is the only member of the family that acts as an inhibitory receptor, showing to be crucial in preventing autoimmune disease by controlling dendritic cell proliferation [Graham et al., 2009].

Returning to the discovered eEP in the PASH group, "Downstream TCR signaling" (R-HSA-202424) and its related pathways have been also observed.
T cell activation requires extracellular signals mainly mediated by T cell receptor (TCR) complexes. The TCR recognizes antigens on major histocompatibility complex (MHC) molecules with the synergy of CD4 or CD8 coreceptors; after antigen recognition, TCR promotes intracellular signaling cascades that regulate specific cellular functions. Consequently, several features of T cell-mediated immune responses are determined by these signal transduction pathways and only a stringent regulation of T cell activation is fundamental for T cell homeostasis and a proper immune response. A dysregulated TCR complex can lead to autoimmunity or global immunologic unresponsiveness [Hwang et al., 2020].

In the second step of the analysis, according to OR and thus considering all eEP carrying at least one ImportantVar, two eEP have been observed: "FGFR4 mutant receptor activation" (R-HSA-1839128) and "betaKlotho-mediated ligand binding" (R-HSA-1307965). Both these pathways are involved in fibroblast growth factor receptor 4 (FGFR4) signaling pathway, which

is of considerable importance in a plethora of processes during embryonic development and adult homeostasis by regulating cellular differentiation, proliferation, and apoptosis [Xie et al., 2020].

Finally, according to the EPR, the following five pathways have been detected: i) “Release of apoptotic factors from the mitochondria” (R-HSA-111457), ii) “Degradation of GLI2 by the proteasome” (R-HSA-5610783), iii) “Cross-presentation of soluble exogenous antigens (endosomes)” (R-HSA-1236978), iv) “Synthesis of PI” (R-HSA-1483226), and v) “Cell surface interactions at the vascular wall” (R-HSA-202733).
This latter pathway describes some of the key interactions involved in the process of platelet and leukocyte interaction with the endothelium, in response to injury [https://reactome.org/content/detail/R-HSA-202733].
Under physiological conditions, endothelium mediates vascular dilatation, prevents platelet adhesion and activation, blocks thrombin formation and reduces fibrin deposition; in addition, the adhesion and transmigration of inflammatory leukocytes are attenuated, thanks to the action of various mediators, and oxygen radicals are scavenged. When the endothelium is functionally altered by different conditions such as acute and/or chronic inflammation, completely opposite actions occur, such as vasoconstriction, externalization and upregulation of adhesion and pro-inflammatory molecules and - in platelet-leukocyte aggregates -, increased inflammatory interaction [Becker et al., 2000].
Furthermore, autophagy regulates leukocyte TEM by recycling the molecular adhesion receptors on the cell surfaces of EC and an impaired autophagy process can also experience increased neutrophil infiltration and tissue damage. Neutrophils have been reported to be involved in skin inflammation of neutrophilic dermatoses (ND), a group of conditions including, among others, PG and HS (present in both PASH and PAPASH syndrome) and caused by an altered neutrophil recruitment and activation [Marzano et al., 2019].

4.6.2 EEP from PASH/SAPHO overlapping

Twelve eEP have been identified in the PASH/SAPHO patient group (**Table 14**).

Based on highest numbers of ImportantVar, the following eEP have been found: i) "Signaling by Rho GTPases" (R-HSA-194315), ii) "Macroautophagy" (R-HSA-1632852) and iii) "Crosslinking of collagen fibrils" (R-HSA-2243919).

PathwayName	DB_ID	OR	p.adjust	ImportantVar	Gene Number	Protein.ratio	Rna.ratio
Macroautophagy	R-HSA-1632852	1,58	0	16	14	57,14	72,11
Signaling by Rho GTPases	R-HSA-194315	1,52	0	88	58	60,66	44,31
Crosslinking of collagen fibrils	R-HSA-2243919	1,79	0	11	6	44,44	27,78
The canonical retinoid cycle in rods (twilight vision)	R-HSA-2453902	2,05	0	3	2	16	12
SDK interactions	R-HSA-373756	2,19	0,0026	2	2	50	0
Mitochondrial tRNA aminoacylation	R-HSA-379726	1,61	0,006	7	4	59,26	59,26
XBP1(S) activates chaperone genes	R-HSA-381038	1,53	0,0002	7	5	60,78	74,51
cGMP effects	R-HSA-418457	1,58	0,0209	5	3	47,06	0
Josephin domain DUBs	R-HSA-5689877	2,02	0,0037	7	1	58,33	83,33
Invadopodia formation	R-HSA-8941237	2,29	0,0146	2	2	25	50
Antigen activates B Cell Receptor (BCR) leading to generation of second messengers	R-HSA-983695	1,53	0,0001	3	3	54,55	45,45
Collagen formation	R-HSA-1474290	1,26	0	42	30	None	None

Table 14: List of 12 common exclusive enriched pathways in PASH/SAPHO overlapping group.

OR, odd ratios. Adapted from Brandão LAC, et al. Variant Enrichment Analysis to Explore Pathways Functionality in Complex Autoinflammatory Skin Disorders through Whole Exome Sequencing Analysis. Int J Mol Sci. 2022; 23(4):2278. doi: 10.3390/ijms23042278

As mentioned above, autophagy is regarded as a “self-digestion” process, which degrades a cell's own cytoplasmic content through lysosomes, to maintain a proper cellular homeostasis [Klionsky et al., 2007]. Four forms of autophagy have been identified: macroautophagy, microautophagy, chaperone-mediated autophagy (CMA), and crinophagy [Csizmadia et al., 2020]. The macroautophagy represents the most common type of autophagy and consists of the formation of a phagophore, the shallowing of cytoplasmic components - by the phagophore -, the elongation of the phagophore membrane, and fusion of its edges to form an autophagosome; the fusion of autophagosomes with lysosomes then leads to degradation of the cargo [Levine et al., 2008]. Macroautophagy at its optimal level ensures cell homeostasis, while its deregulation compromises cell survival [Maiuri et al., 2007]; it has in fact been demonstrated that a macroautophagy deregulation leads to the onset of several conditions such as neurodegenerative disorders, cancers, and inflammatory diseases [Levine et al., 2008]. Notably, the macroautophagy process is deregulated via distinct mechanisms in each disease; in inflammatory disorders, the machinery for autophagosome nucleation is mislocalized, and in turn inhibits macroautophagy [Levine et al., 2008].

Four additional eEP have been found in according to their OR: “Invadopodia formation pathway” (R-HSA-8941237), “SDK interactions (R-HSA-373756)”, “Canonical retinoid cycle in rods” (R-has-2453902), and “Josephin domain DUBs” (R-HSA-5689877), whereas five eEP have been revealed by EPR: “Josephin domain DUBs” (R-HSA-5689877), “XBP1(S) activates chaperone genes” (R-HSA-381038), “Macroautophagy” (R-HSA-1632852), “Mitochondrial tRNA aminoacylation” (R-HSA-379726), and “Collagen formation” (R-HSA-1474290).

The eEP “Invadopodia formation pathway” (R-HSA-8941237), “Crosslinking of collagen fibrils” (R-HSA-2243919) and “Collagen formation” (R-HSA-1474290) are related to extracellular matrix organization, suggesting its possible involvement in pathogenesis of HS and its syndromic forms (as discussed in the session **4.2**).

5. CONCLUSIONS

The overall aim of this PhD dissertation is to expand the knowledge on the genetic basis underlying the susceptibility, onset, severity and clinical course of HS and its syndromic forms. The delineation of the genetic background of these patients could pave the way to pathogenesis-targeted treatments and improved clinical follow-up.

The pathogenesis of HS is very complex and, to date, has not yet been fully elucidated; there is a close interaction between genetic, epigenetic factors, host-specific aspects, and environmental influences, such as bacterial microbiomes and cigarette smoking. Furthermore, an innate and adaptive immunity dysfunction leading to autoinflammation has been reported to play a crucial role with overexpression of proinflammatory cytokines such as IL-1β and IL-17, chemokines, and tumour necrosis factor (TNF)-α both in the lesional skin and in the serum of HS patients.

A family history of HS has been reported in one-third of cases, and some HS familial forms have been suggested to be disorders with an autosomal dominant inheritance pattern; genetic defects underlying familial HS have been reported only in few patients, with the majority of mutations in genes encoding for the gamma-secretase complex (GSC) being associated with some familial pedigrees and specific disease subsets.

Genetic diagnosis of syndromic forms of HS, in spite of novel variations recently identified, is just descriptive, since most cases are not sharing the same mutations, and the mutations themselves are not informative of the biological signaling pathways commonly disrupted in these patients. As an example, some recent works failed to reveal mutations in genes already described in the literature, in patients suffering from syndromic forms of HS, leading to hypothesize novel mechanisms at the basis of these conditions. To date, a main pathway associated to HS is the Notch pathway; in fact, a deficiency in Notch signaling has been observed in some cases (mainly familial cases of HS), associated with loss of function pathogenic variants in GCS genes []. Nevertheless, functional studies demonstrated that several *NCSTN* missense variants causing HS are functional and promote Notch signalling, counteracting the view that pathogenic variants in genes encoding γ-secretase components cause disease simply as a result of haploinsufficiency that leads to impaired Notch signalling pathway.

In this critical context, - omic approach constitutes an essential diagnostic and research tool for the detection of rare variants, enriched in syndromic HS, that can provide the identification of several common genes and cellular pathways potentially associated to the onset and progression

of these disorders, additionally prompting the development of tailored and personalised treatments to be administered successfully to selected patients carrying specific mutations.

1. In the first part of this project, we reported impaired shared biological signaling pathways in syndromic HS patients (PASH and PAPASH), mainly vitamin D metabolism and keratinization. Although these results – based on a novel WES approach - need a further functional validation, at least for vitamin D pathway, we found direct confirmation in its low levels seen in all our patients. It is well known that vitamin D possesses immunomodulating properties by increasing the expression of Toll-like receptor 2 and the production of antimicrobial peptides that are direct transcriptional targets of Vitamin D, thus suppressing the production of inflammatory cytokines and promoting the expression of anti-inflammatory ones. In another of our studies, we retrospectively evaluated 250 HS patients non-supplemented with vitamin D, measuring serum levels of 25-hydroxyvitamin D (25OHD) at diagnosis before starting any specific HS treatment. The main finding of our study was the inverse correlation between 25OHD levels and HS severity; moreover, an inverse correlation between 25OHD levels and systemic inflammation was also found by measuring C-reactive protein, thus confirming that HS severity and inflammation are associated with major hypovitaminosis D.

   Concerning keratinization pathway, our results are consistent with a previous study that proposed HS as a potential subtype of autoinflammatory keratinization disease.

2. Three clinical settings, in ten syndromic HS patients (PASH, PAPASH, PASH/SAPHO overlapping), have been identified based on presence/absence of gut and joint inflammation. We reported, through WES approach, in four PASH patients also affected by gut inflammation, three different variants in *NOD2* gene, two variants in *OTULIN*, and a variant in *GJB2*, respectively. Three PAPASH and three PASH/SAPHO overlapping patients, who had also joint inflammation, showed two different variants in *NCSTN*, one in *WDR1* and *PSTPIP1*, and two variants in *NLRC4* gene, one of whom was present in a patient (PAPASH) with gut and joint inflammation. This study represents a first preliminary step for a genotype-phenotype correlation and confirms the polygenic autoinflammatory nature of syndromic HS, in this context, closely related to joint and gut inflammation. We found variations - some of which are being validated – in autoinflammatory genes (e.g., *PSTPIP1, NOD2, NLRC4*) as well as in

keratinization genes (e.g., *NCSTN*, *GJB2*) confirming that a genetically disrupted keratinization pathway may contribute to the pathogenesis of cutaneous inflammation in syndromic HS.

Furthermore, WES should be recommended as a fundamental diagnostic and research tool for correctly classifying these conditions.

3. In the third part of the project, we applied VEA to unravel novel pathways altered in patients with complex autoinflammatory skin diseases, PAPASH, PASH and PASH/SAPHO overlapping syndrome. We identified pathways related to neutrophils and endothelial cells homeostasis, as disrupted in our patients, which lead us to assumed that unregulated neutrophil transendothelial migration could elicit increased neutrophil infiltration and tissue damage. This reflects the classification of HS and its syndromic forms in neutrophilic dermatoses, conditions hallmarked by an accumulation of neutrophils in the skin and, rarely, internal organs, sharing clinicopathological aspects with the autoinflammatory diseases.

Our VEA approach is applicable in situations of individual reports or in cases with low numbers of subjects; moreover, although skin transcriptomics and proteomics of our patients is not, to date, available, this novel VEA approach - based only on WES data - contributes to changing the vision of genetic analysis and its objectives (i.e., genetic variants associated with certain diseases), thus representing a modern tool for translating exome variants in a broader pathological context.

This PhD dissertation represents a solid contribution to the pathophysiological study of HS and its syndromic forms, although further studies are needed to shed more light on the pathomechanisms underlying these conditions to bring us closer to pathogenesis-targeted treatments.

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