CLINICAL, IMMUNOLOGICAL, AND GENETIC CHARACTERIZATION OF COLOMBIAN PATIENTS WITH CUTANEOUS RECALCITRANT WARTS

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Gustavo Cerati

Dedicated to my siblings Felipe and Sofia for being my support and guide throughout my life; and to my father who has given all for us.

“Todo lo bueno tarda”
Alcolirykoz

TABLE OF CONTENTS

ABSTRACT

Inborn errors of the immunity (IEI) are monogenic diseases that result in a predisposition to a whole spectrum of infectious diseases. In humans, cutaneous warts are caused by infection with Human Papillomavirus (HPV). Warts are relatively common with an incidence of ~10% in the general population and regress spontaneously. However, warts persisting over two years in some patients despite aggressive treatments with conventional therapies are called cutaneous recalcitrant warts (RW). RW can be present in patients with IEI but also in otherwise healthy individuals. The host defense against HPV relies on functional cellular immunity, including T cells, natural killer (NK) cells, and skin-intrinsic immunity. Therefore, in patients with RW, concern for an immune defect may be considered.

Cutaneous RW has been documented in combination with a broad spectrum of infectious diseases in patients IEI such as severe combined immunodeficiency, common variable immunodeficiency, inborn errors of immunity with isolated or syndromic characteristics and NK deficiencies, and with acquired immunodeficiencies such as patients infected with Human Immunodeficiency Virus (HIV) and organ transplant recipient.

Also, patients with epidermodysplasia verruciformis (EV) which is characterized by macules like tinea versicolor and Tree Man Syndrome that present manifestations as cutaneous horns presents specific genetic predispositions to β- and α-HPV infection respectively, but only very few are known about genetic susceptibility to RW, and to our knowledge, no studies are searching for monogenic defects associated with RW. Therefore, we hypothesized that susceptibility to cutaneous RW due to HPV in otherwise

young healthy individuals might be due to underlying genetic defects in the intrinsic and innate immunity.

Aim: To describe the clinical, immunologic characteristics and genetic defects responsible for susceptibility to cutaneous recalcitrant warts (RW) in Colombian patients.

Methodology: We conducted a descriptive study. In our cohort, we included otherwise healthy Colombian patients affected with cutaneous RW, negative for HIV infection, and without history of cancer, primary immunodeficiency or acquired immunodeficiency. First, we reviewed medical records, including the history of HPV infection and pharmacological treatments. We obtained pedigrees and collected blood samples and performed immunophenotyping. Finally, Whole Exome Sequencing (WES) was performed in the genomic DNA of several patients and some of their relatives and performed extensive in silico analysis to search for potential candidate genes.

Results: We recruited 11 patients with ages between 9 to 34 years old (five males and six females), belonging to 11 families that fulfilled the inclusion criteria. We make the pedigrees for all families. Five patients have a family history of cervix HPV infections. Sequencing by WES was performed for 7 patients and 11 relatives, and after a deep analysis we obtained 7 candidate variants in *PYGO2*, *CASP9*, *CCNA2*, *CCNB3*, *GLTSCR2*, *PABPC1* and *CAD* that we postulate can explain the clinical phenotype of RW in these patients. We found no candidate variants in genes that have been previously associated with IEIs in which RW has been described. Likewise, immunophenotyping of peripheral blood was performed in one patient, which was normal in percentages and numbers.

Conclusions:

The presence of multiple affected individuals in different generations of the pedigrees, with no sex differences between affected individuals, suggests that susceptibility to cutaneous RW in our patients has a genetic component with an autosomal dominant inheritance pattern.

The lack of candidate variants in genes previously associated with inborn errors of the immunity (IEI) in patients with which RW, suggest that our patients probably have a new genetic defect not previously described in the literature.

The presence of variants in genes associated by connectome with genes previously associated with IEIs with presence of RW, in addition to the familial history of RW in the kindreds included in our study, supports the hypothesis that susceptibility to RW is the consequence of an IEI not previously described.

The clinical characteristics of our cohort of otherwise healthy patients with RW supported by the normal numbers of leukocyte subpopulations in peripheral blood in one patient, in addition to familial history of RW in the kindreds of our cohort suggest that the underlying causes of RW in our patients are caused by deficiencies of components of the immune system that are specific against HPV infection.

ABSTRACT (Spanish)

Los errores innatos de la inmunidad innata e intrínseca son enfermedades monogénicas que resultan en predisposición a un amplio espectro de enfermedades infecciosas. En humanos, las verrugas cutáneas son causadas por infecciones por el virus del papiloma humano (VPH). Las verrugas son relativamente comunes, con una incidencia de aproximadamente el 10% en población general y tienden a resolverse espontáneamente.

Sin embargo, verrugas persistentes en algunos pacientes incluso después de tratamientos agresivos con terapias convencionales que incluyen crioterapia, acido salicílico tópico o bleomicina, son llamadas verrugas recalcitrantes (VR). VR pueden estar presentes en individuos aparentemente sanos. La respuesta inmune contra VPH se apoya en una inmunidad celular funcional incluyendo células T, asesinas naturales (NK) e inmunidad intrínseca. Por lo tanto, en pacientes en los cuales las verrugas se convierten en recalcitrantes, se puede considerar un defecto inmunológico.

Las VR ha sido descritas en combinación con un amplio espectro de enfermedades infecciosas en pacientes con errores innatos de la inmunidad (EII) como pacientes con inmunodeficiencia severa combinada, inmunodeficiencia común variable, errores innatos de la inmunidad con características sindrómicas y deficiencias de células NK, y en pacientes con inmunodeficiencias adquiridas como pacientes infectados con virus de inmunodeficiencia humana (VIH) y pacientes con órganos trasplantados.

También, pacientes con Epidermodisplasia Verruciforme la cual se caracteriza por maculas similares a tinea versicolor y síndrome de “hombre árbol” que presentan manifestaciones de cuernos cutáneos los cuales presentan predisposiciones a infecciones por β y α-VPH respectivamente, pero se sabe poco sobre la susceptibilidad

genética a VR y en nuestras revisiones no hemos encontrado estudios de defectos monogénicos asociados con VR.

Por estas razones, nosotros hipotetizamos que la susceptibilidad a VR debido a infecciones por HPV en individuos aparentemente sanos puede deberse a un defecto genético subyacente a la inmunidad intrínseca o innata.

Objetivo: Describir las características clínicas e inmunológicas y los defectos genéticos responsables de la susceptibilidad a verrugas recalcitrantes cutáneas (VR) en pacientes colombianos.

Metodología: Nosotros realizamos un estudio descriptivo. En nuestra cohorte incluimos pacientes colombianos con diagnóstico de VR aparentemente sanos, negativos para infección por VIH y sin historial individual de cáncer. Primero, revisamos las historias clínicas de los pacientes incluyendo el historial de infecciones por VPH y tratamientos farmacológicos. También, obtuvimos los pedigríes de las familias, muestras de sangre y realizamos inmunofenotipificación. Finalmente, realizamos secuenciación completa del exoma (WES) en el ADN genómico de algunos pacientes y algunos de sus familiares y realizamos un análisis in sillico para buscar genes candidatos potenciales.

Resultados: Nosotros captamos 11 pacientes con edades entre los 9 y 34 años (cinco hombres y seis mujeres), pertenecientes a 11 familias que cumplieron todos los criterios de inclusión. Cinco pacientes presentan historia familiar de infecciones por VPH cervical.

Obtuvimos variantes candidatas de los datos de WES de 7 pacientes y 11 familiares, y después de un análisis exhaustivo se obtuvieron variantes en *PYGO2*, *CASP9*, *CCNA2*, *CCNB3*, *GLTSCR2*, *PABPC1* y *CAD* las cuales nosotros postulamos que pueden

explicar el fenotipo de VR en estos pacientes. Adicionalmente, en uno de los pacientes las subpoblaciones de sangre periférica revelaron porcentajes y conteos normales.

Conclusiones:

- La presencia de múltiples miembros afectados en diferentes generaciones en los pedigríes, sin predilección de sexo entre los miembros afectados, sugiere que la susceptibilidad especifica a VR en nuestros pacientes presenta un componente genético con un patrón de herencia autosómico dominante.
- La falta de variantes en genes previamente descritos en los que la susceptibilidad a VR está acompañada de otros fenotipos clínicos sugiere que estos pacientes presentan un defecto inmunológico no previamente descrito.
- La presencia de variantes asociadas por conectoma con genes previamente asociados con errores innatos de la inmunidad en los cuales se describe la presencia de VR acompañadas de un fenotipo clinic mas amplio, en adicion a la historia familiar de VR en las familias incluidas en este estudio apoya la hipotesis de que las VR son consecuencia de un error innato de la inmunidad no previamente descrito.
- Las caracteristicas clinicas de nuestra cohorte de pacientes aparentemente sanos con verrugas recalcitrantes, los conteos normales de subpoblaciones de leucocitos en sangre periferica en un paciente, en adicion a la historia familiar de VR en nuestra cohorte sugieren que las causas subyacentes de VR en nuestros pacientes son causadas por deficiencias del Sistema immune que son especificas en la respuesta immune contra PVH.

INTRODUCTION

Papillomaviruses (PV) are small non-enveloped DNA viruses that infect epithelium in different animal species including humans and are distributed in more than 50 different genera (1). The circular double-stranded DNA genome of PV is about 8 kb in length, contains three oncogenes (E5 not found in β-HPV genomes, E6 and E7), two regulatory protein-coding genes (E1 and E2), two structural protein-coding genes (L1 and L2), and a non-coding regulatory region called long control region (2). Taxonomically, PV "types" exhibits high degree conservation in E1, E2, L2, and L1 between species but, this conservation is higher in L1 ORF and therefore the taxonomic criteria for PV classification are based on the L1 sequence (2-4).

Human papillomaviruses (HPV)

Human Papillomavirus (HPV) species infect specifically human stratified epithelial cells, are common pathogens with a seroprevalence of 95% in the general population in USA (5-8). HPVs have been classified into five genera (α, β, γ, μ, and υ) and over 400 HPV genotypes (2, 7, 9). HPVs of the β, γ, μ, and υ genus have an exclusive cutaneous tropism. In contrast, α-HPVs have both cutaneous and mucosal tropism, and are associated with cutaneous warts, benign mucosal lesions, and carcinomas of the anogenital and oropharyngeal tracts. β-HPVs generally cause asymptomatic infections but are responsible for pytiriasis versicolor-like lesions and flat warts, and non- melanoma skin cancer (NMSC) in rare patients with epidermodysplasia verruciformis (EV). β, γ, μ, and υ-HPV genus infection is associated with benign cutaneous common and plantar warts. Due to the varying oncogenic capacity of the different HPV types such as those

found in the α- or β- genera, another classification has been proposed that divides HPVs in two main groups: high-risk types (HPV-16, HPV-18, etc.) and low-risk types (HPV-6 and HPV-11) (4, 10-13).

The infection caused by HPV requires the entry across the epithelium into the basal layer through microtraumas or micro-abrasions, leading the viral particles able to infect basal keratinocytes (14). The HPV particle binds to keratinocytes through heparan sulfate proteoglycans located on the epithelial cell surface and subsequently, cyclophilin B mediates the conformational changes necessary for the internalization of the viral capsid (15). After the entry of the virus, the E1-E2 viral proteins maintain low copy number replication while E6-E7 proteins inhibit the proapoptotic cell machinery (16).

Immune response to HPV infection.

Studies on the immunity against HPV infection are limited due to the diversity of species. The spectrum of species or "types" has allowed HPV to develop a wide range of adaptation mechanisms to intrinsic or extrinsic host factors, which in turn allows it to control resistance and chronicity of infections (17, 18). In addition, host genetic diversity plays a fundamental role in the control of HPV infection, and, in turn, of the clinical manifestations caused by HPV (19). Factors governing the immune response against HPV have so far been described as follows:

The keratinocyte is the cell for which HPV exhibits specific tropism (6). In this cell, intrinsic determinants of the immune response against HPV have been described, such as the presence of the restriction factors EVER1 (codified by *TMC6),* EVER2 *(TMC8)* and CIB1 (*CIB1)*, which in complex restrict β–HPV infection. The important role of these restriction

factors has been observed in three IEIs in patients with isolated EV (20). In addition, it has been described the presence of non-specific HPV viral restriction factors and non-coding RNAs that limit transcription and replication of HPV (21, 22).

The role of T $CD4^+$ cells subset in the HPV immune response, is shown in HIV/AIDS patients where the frequency of cutaneous HPV infections is significantly increased (23). Additionally, in patients with IEIs in whom the T-cell development and/or function is affected, HPV skin infections are increased (6, 24). This in turn is supported by wart regression studies in which an epidermal infiltrate of mainly CD4+ T cells is observed (25). The association between $CD4^+$ T cell deficiency and the presence of HPV infections can be observed in patients with a *CD28* and *CARMIL2* deficiencies affecting the TCR signaling through CD28 co-stimulation (6).

Serum antibody titers from patients with a natural HPV infection are usually low, suggesting that HPV efficiently evades the host antibody response (26). However, in the context of HPV vaccination, the neutralizing antibodies demonstrated a high level of protection against HPV infections, due to their ability to activate B lymphocytes and dendritic cells (26, 27). The immunological defects found in IEIs in which HPV infections are present will be reviewed later in this document.

Clinical manifestations of HPV infection

Clinical manifestations of HPV infections can be divided into two groups: 1) viral infections of the mucosae and 2) cutaneous viral infections. The first group includes patients with mucosal or genital lesions due predominantly of α-HPV infection and are related to the following diseases in patients with **recurrent respiratory papillomatosis** (28, 29), **head**

and neck squamous cell carcinoma (HNSCC) (30, 31), **penile cancer** (32, 33), **anal cancer** (34, 35), **and cervix cancer** (36, 37).

Cutaneous viral infections are those that are found in patients with skin lesions due to α–, β-, γ-, μ-, and υ-HPV infection and are divided into two other subgroups (6). The first subgroup describes cutaneous diseases with specific β-HPV infections. In this group, we can observe patients with epidermodysplasia verruciformis (EV) and patients with syndromic EV. We will describe these manifestations below. In the second group of cutaneous HPV manifestations are caused by α–, γ-, μ-, and υ-HPV types defined as self-limited lesions called warts or verruca (38). The diagnosis of warts is made by the classic physical examination or dermoscopy, and its classification is done based the warts' morphological features. In this group, warts are grouped in the following types:

- **Verruca vulgaris (common warts)** are flat or raised papules or nodules with irregular hyperkeratotic surfaces commonly found on the dorsum of the hand. They are usually caused by HPV types -2, -4, -7, -57 and histologically characterized by prominent papillomatosis, hyperkeratosis in a columnar arrangement (38-42).
- **Myrmecia warts** are round keratinized lesions found commonly on pressure points of the foot usually caused by HPV-1. These warts present epidermal growth endophytic, the cells are clarified and irregularly enlarged with eosinophilic cytoplasmic inclusion, and in the upper layers present basophilic nuclear inclusions and strongly basophilic parakeratotic cells (38-42).
- **Butcher warts** are exclusively present on both sites of the hand in butchers and meat and are rarely periungual. Butcher's warts are caused by HPV-7 and have prominent acanthosis and small vacuolated cells (38-42).

- **Plane warts** are multiple flat papules with a smooth but hyperkeratotic surface caused by HPV-3, -10 and -41. These warts present a less pronounced papillomatosis than common warts (38-42).
- **Plantar warts are** firm papules located on the plantar surface of the foot, characterized by “black dots” caused by thrombosed capillaries in the cornified layer of the skin, are usually caused by HPV-1, -2, -4, -27, and -57. Histologically these warts present a well-circumscribed area of epithelial thickening with parakeratosis, irregular downward proliferation of epidermal ridges, and papillomatosis (38-42).
- **Intermediate warts** are dermatological lesions generally found in immunosuppressed patients and present an intermediate morphology between common and plane warts. They are caused by HPV-26, -27, -28, -29 and present heavy hyper and parakeratosis, pronounced papillomatosis, and variable acanthosis (38-42)
- **Cutaneous horns** are rare keratinized skin protrusion with variable size and shape (43). These lesions are usually caused by HPV-2 infection and histologically these lesions are highly keratotic, conical, and circumscribed with or yellowish in color that varies in size and can hide benign or malign lesions (44-46).

Recalcitrant common warts (RW), the tree-man syndrome (TMS), and epidermodysplasia verruciformis (EV).

Although most cutaneous HPV infections are subclinical and persistent in the absence of immuno- suppression, they can cause benign common warts as well as extensive,

unusually severe conditions that can be divided into three distinct clinical categories: 1) the tree-man syndrome (TMS), 2) Epidermodysplasia Verruciformis (EV) and 3) cutaneous RW (47).

1. The “tree man” syndrome (TMS) is present in otherwise healthy subjects in which the cutaneous horns are abundant and persistent. In one family, TMS has been associated with autosomal recessive (AR) deficiency in the CD28-mediated response against α-HPV infections (44). These patients present decreased humoral response, decreased $CD4^+$ and $CD8^+$ central memory T cells populations, and loss of the Mucosal Associated Invariant T (MAIT) cell population and $CD4^+$ T reg lymphocytes in these patients (44). In these patients CD28 expression is absent and the cells do not respond to CD28 specific stimuli (47).
2. Isolated EV is caused by deficiency of specific viral restriction factors against β-HPV infections (*TMC6, TMC8*, and *CIB1* genes) (13). EV is characterized by the development of flat tinea versicolor-like macules and papules usually localized in the trunk, neck, arms, and face (48). The histology is like other HPV infection features such as koilocytosis and keratosis (like cervical intraepithelial neoplasia) with a predisposition to the development of cutaneous squamous cell carcinoma in UV exposed sites (49, 50). Syndromic EV is observed in patients with IEI commonly associated with T-cell deficiencies, such as those with deficiencies in *RHOH, GATA2, MST1, CORO1A, DCLE1C, DOCK8, IKBKG, IL12RG, IL7, IL7R, ITK, LCK, RASGRP1, SMARCAL1, STK4 and TPP2*. These patients can develop EV accompanied by a broad spectrum of infectious diseases (51).

3. The cutaneous warts usually resolve spontaneously within 1-2 years, but some of these lesions, defined as RW, persist for more than two years despite multiple treatments (47, 52). In rare cases, the warts can also transform into exophytic cutaneous lesions and giant horns, resulting in TMS (44).

In some cases, there is a familial susceptibility to the development of warts, as in EV, a heritable disorder. The presence of RW has been reported in three immunological groups of patients: **1) patients with acquired immunodeficiencies,** 2) **patients with known inborn errors of immunity and 3) patients without a predisposition factor** (53, 54).

1- Patients with acquired immunodeficiencies: There is a high prevalence of HPV infections in patients with acquired immunodeficiencies such as HIV-infected individuals, were these patients have an augmented susceptibility to HPV positive cervix and anal cancer (55). In the context of cutaneous HPV infections, HIV-positive patients have been described with atypical EV and increased susceptibility to plane warts. In addition, warts have been described in transplant recipients and atopic dermatitis (56-59).

Due to the impaired T-cells response in transplanted patients, HPV tends to cause infection, in this case, the patients present a crescent risk of developing warts for a longer time post-transplantation (60, 61).

2- **Patients with inborn errors of immunity:** Inborn Errors of Immunity (IEIs) involve a heterogeneous group of more than 450 diseases that affect different components of the immune system. The clinical manifestations of IEIs include recurrent infections, autoimmune disorders, malignant lesions, and allergic diseases very similar to those caused by acquired immunodeficiencies (62). A broad spectrum of susceptibility to viral

infections has been reported in IEI patients where any of the most common infectious agents is HPV. In this context, two groups of patients have been described (54). The first group include patients with specific susceptibility to HPV infections such as patients with EV or TMS; and the second group patients who have a predisposition to develop RW accompanied by other infectious diseases. In the last group are included patients with the following IEIs:

- **Severe combined immunodeficiency (SCID)** is characterized by littler or absent immune response due to defects in T, B, and NK cells. RW have been observed in SCID patients with *IL12RG* and *JAK3* deficiency that causes an X-linked (XL) and autosomal recessive (AR) Common Gamma Chain deficiency. Also, in patients with AR deficiency in *CORO1A* (Coronin-1A), AR deficiency in *DCLRE1C* (Artemis deficiency), and AR *LIG4* deficiency (DNA ligase IV deficiency) (48, 63, 64).
- **Common Variable Immunodeficiency (CVID)** is characterized by failure of B-cell differentiation and impaired secretion of immunoglobulins that results in patients with antibody deficiency and hypogammaglobulinemia. RW have been observed in CVID patients with deficiency in *CARD11, and IKBKB* responsible for AR CVIDs (65, 66).
- **Combined Immunodeficiency (CID)** is a heterogeneous group of disorders characterized by T-cells dysfunction (67). RW has been reported in patients with CID with defects in *DOCK8, RHOH, STK4, ATM, SMARCAL1, SPINK5, IKBKG, RASGRP1, RLTPR, GATA2* and *CXCR4* (54, 68-77).

- RW has been reported in a patient with AR CD16 (*FCGR3A)* deficiency, a defect in the Fc Gamma Receptor IIIa, affecting NK cell's function (78, 79).

3- **Patients without a predisposition factor.** Cutaneous warts are usually asymptomatic, however they can cause pain, as in the case of plantar warts where abrasion caused by shoes and the constant movement can result in painful lesions, or they can cause a deformity as in the case of periungual warts where partial separation or lifting of the nails caused by the growth of the lesions can occur (80, 81). These warts in immunocompetent people can resolve spontaneously within months of years owing to natural immunity, but exist a possibility that spontaneous regression and persistence for many years in this point is considered recalcitrant warts, many studies report RW in immunocompetent patients has made managing these warts a challenge for clinicians (80, 82).

Based on the resistance of these lesions, different treatment guidelines have been published where it is usual to use strategies such as cryotherapy or salicylic acid as the first instance in the treatment of warts and in the case of more difficult to manage warts, more specialized options such as topical immunotherapies where all options show individual responses to treatment of more than 80%, considering this, it is possible that the presence of recalcitrant warts that are resistant to different treatment modalities may be a rare clinical event (24, 83). In Colombia, one of the option for RW treatment is the topical diphencyprone (DPCP) immunotherapy, which has been described as an effective treatment of RW (84).

Cutaneous warts in children

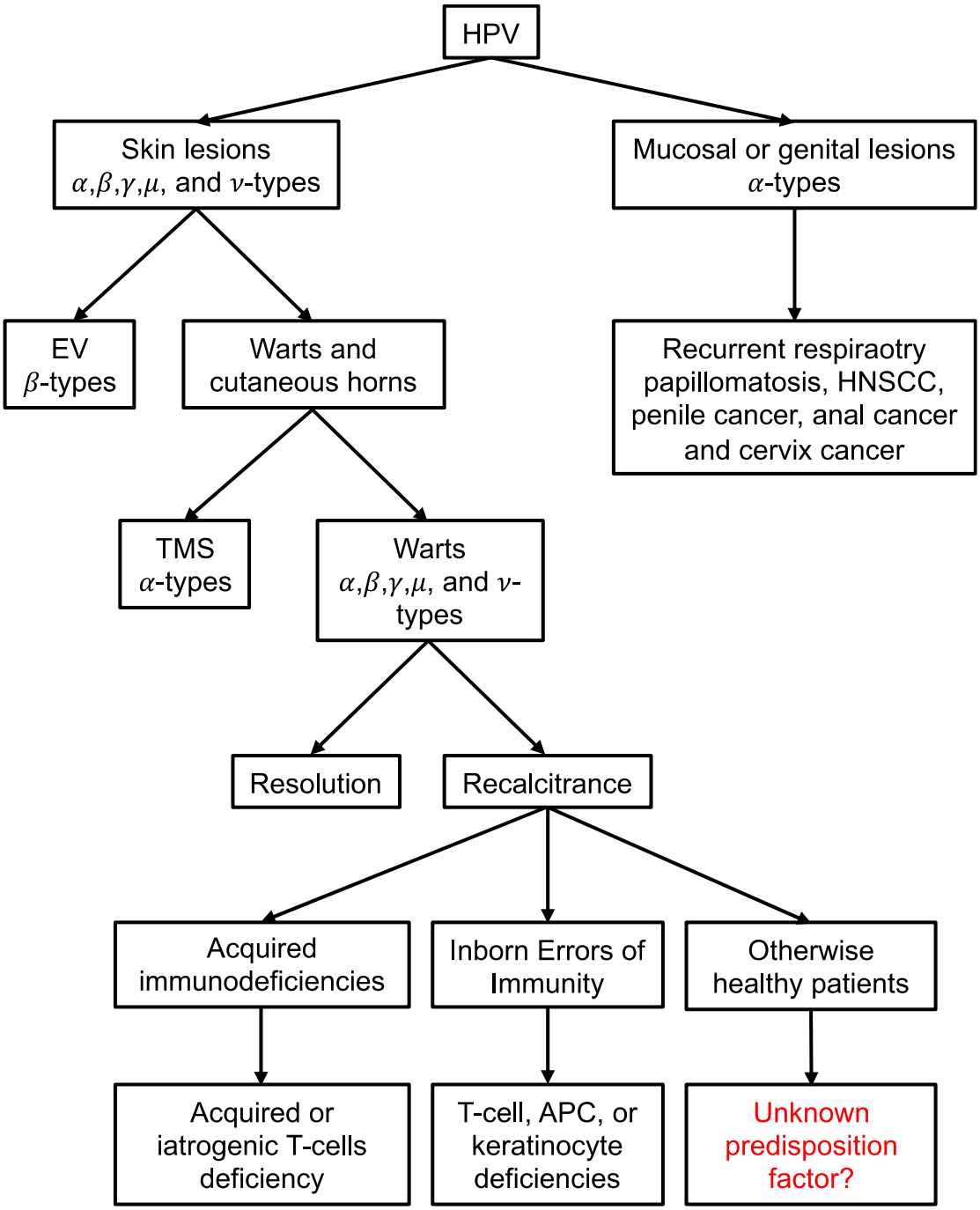
Cutaneous β and γ-HPV can be detected in 70% of children before 4 years of age and β-HPV types can be isolated in 90% of the general population. In addition, β-HPV types detected in parents are transmitted by mother-baby interaction due to skin contact (85). The prevalence of warts gradually increases in children of school age, peaking at 10–15 years of age (86). Common warts represent 70 % of lesions and flat warts 5% of lesions (87, 88). All types of warts can resolve spontaneously, usually, 30% of warts resolve before 0.5 years, 36% in the next 1.5 years, 9% in the following year and the remaining 25-30% tend to become persistent over time (39).

Studies of the natural history of CWs have shown that children previously affected with warts have an increased susceptibility to present warts in the future, than children that don't previously present these infections (52). In addition, CWs are not present in children before eight years of age, and involution of CW, is most usual in boys than girls and occur without treatment in two-thirds of the patients in a period before two years (52).

Epidemiologically, the common warts prevalence has been published in rates of (2-30%) in school-age children with variations due to risk factors, sociodemographic factors, and availability of medical facilities (89). And, due to the high number of risk factors associated with the presence of warts and the lack of studies in this regard, an estimated prevalence of recalcitrance in warts is not known. But the most common definition of RW is formulated by Massing et al in 1963 “Usually warts disappear within two years but the 35% of these warts can become persistent over time and resistant to treatment” (52, 90).

Cutaneous recalcitrant warts such as a monogenic inborn error of immunity

The persistence of this infection and the resistance to treatments may be explained by the deficiency of a component of the intrinsic cellular immunity of keratinocytes or a decrease in the specific response against papillomavirus in T cells., This is based on the specific susceptibility to present recalcitrant warts without other concomitant infectious diseases, like what is observed in patients with isolated EV with deficiency of *TMC6*, *TMC8* or *CIB1* or patients with Tree Man Syndrome with deficiency of *CD28* in which the anti-HPV specific immune response can be affected (6, 44). However, the literature currently does not describe any factor that explains the susceptibility to HPV infections in otherwise healthy young patients with only cutaneous RW. The early presentation, frequent relapses or persistence of infection, poor response to available treatments suggests a possible, not previously reported hereditary or genetic susceptibility to HPV infections. You can see the topics explained in the **graphical abstract** below.



Graphical abstract. In this summary, the different types of manifestations caused by HPV infection, the types associated with these infections, as well as the predisposing factors that lead to recalcitrance of RW can be observed. **HPV** Human Papillomaviruses, **HNSCC** Head and Neck Squamous cell carcinoma, **APC** Antigen Presenting Cells.

HYPOTHESIS

Otherwise healthy patients with diagnosis of recalcitrant cutaneous warts in whom the early age of infection, the frequent relapses, and resistance to conventional treatments suggest an increased genetic susceptibility to HPV infection.

Aims

General aim

- To describe the clinical, immunologic, and genetic characteristics of otherwise healthy young Colombian patients with cutaneous recalcitrant warts (RW).

Specific aims

- To constitute a well characterized cohort of otherwise healthy Colombian patients with cutaneous recalcitrant warts (RW) in terms of the inborn errors hypothesis of human immunity to papilloma virus (HPV) infections
- To describe the clinical and immunological characteristics of the patients.
- To discover by WES new disease-causing genetic defects in otherwise healthy Colombian patients with cutaneous recalcitrant warts (RW).

MATERIAL AND METHODS

Ethics statement

This study was conducted in accordance with the Helsinki Declaration, with written informed consent obtained from the patients, their parents (for minors), and other family members. Approval for this study was obtained from the Regional Ethics Committee of the Universidad de Antioquia, Medellín, Colombia.

Studied population

The study was carried out in nine patients from Antioquia and Valle del Cauca with the previous diagnosis of cutaneous RW when they were under topical diphencyprone (DPCP) immunotherapy at Dermatology Service (School of Medicine, Universidad de Antioquia), located at Hospital Universitario San Vicente Fundación. Two additional patients were referred by doctors Yolanda Caicedo (MD, Pediatrician) and Julio Cesar Orrego (MD, Immunologist).

All patients who present a diagnosis of cutaneous RW since childhood and who had a family history of warts were included. In our study, cutaneous RW was defined as lesions with failure response to at least one conventional treatment and that have been treated with diphencyprone (DPCP), or with at least 2 years of ongoing infection (52). Patients with conditions that may be related with immunosuppression such as HIV, pregnancy, diabetes, lupus, diagnosis of primary immunodeficiencies, or being treated with Immunosuppressants were excluded. A questionnaire (**supplementary materials 1 and 2)** was conducted, and patients' parents were also interviewed over the telephone when

possible. We included questions to obtain information related with: (i) Demographic characteristics: age, sex, occupation, (ii) history of the disease: age of onset of the disease, age of diagnosis by the doctor and family history or presentation of the disease, (iii) pathological background: allergic hypersensitivity reactions and autoimmune diseases, (iv) pharmacological background, (v) history of infections, (vi) cancer and other types of conditions that may be related to genetic defects or another pathology. To describe clinical characteristics of warts, photographic registers were realized.

Extraction of peripheral blood DNA

We purified gDNA from whole blood samples collected from patients in 4mL tubes anticoagulated with EDTA. Cells were lysed with Cell Lysis Solution (QIAGEN, Maryland) and incubated at 37° C overnight. The proteins were precipitated by Protein Precipitation Solution (QIAGEN, Maryland) and separated with centrifugation at 3200 rpm/15 min /4° C. gDNA was purified by extraction of isopropanol, precipitated in ethanol, and resuspended in 30 µL of DNA Puregene Hydration Solution (Gentra systems, Washington).

Whole-Exome Sequencing (WES), Bioinformatic Analysis, and Sanger Sequencing

Exome capture was performed with the Agilent Sureselect V4/V5, (Agilent Technologies) and paired-end sequencing was performed on a Hiseq4000 platform (Illumina) generating 100-base reads. The reads were aligned to the reference human genome GRCh37/Hg19 using the Burrows-Wheeler Alignment tool (BWA v0.7.12-r1039) [28]. Bioinformatic analyses were performed using an in-house software pipeline developed in collaboration with Rockefeller University (New York, USA).

Approach to the selection of candidate genes

To discover new genes related to RW, WES was combined, if possible, with analysis in families with inbreeding criteria (autosomal recessive or X- linked inheritance). Also, we searched for rare heterozygous variation with the hypothesis of an autosomal dominant inheritance. This approach generates massive clusters of data that were filtered as described.

Exome analysis: prioritizing the best candidate genes (Initial Filtering)

The databases that were used for the call of gene variants was Genome Aggregation Database (gnomAD) (http://gnomad.broadinstitute.org/), Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/about), NHLBI Exome Sequencing Project (ESP) (http://evs.gs.washington.edu/ EVS /) and 1000 Genome's data (1000 G) (https://www.genome.gov/27528684/1000-genomes-project/). Polymorphic variants were excluded, (up to a low frequency, using the conventional 1% cutoff score generally accepted by the definition of a polymorphic variant), according to the GnomAD genomes allelic frequency.

We prioritized genes with Gene damage index (GDI), this bioinformatics tool realizes metric profiling of the mutational damage accumulated in the general population in each gene. This profiling is related to selective evolutionary pressure, protein complexity, coding sequence length, and the number of paralogs. The variants with a GDI higher than or equal to 12.71 were excluded since no disease-caused mutation is expected (91).

In addition to this, we perform the *in-silico* prediction of the mutational impact of variants using the Combined Annotation Dependent Depletion (CADD) tool with the purpose to

scoring the deleteriousness of single nucleotide variants and using the Mutation significance cutoff (MSC) we define a significance threshold for each CADD score. In this same idea, all variants with a CADD minor than MSC are excluded (92, 93).

The blacklist (BL) of non-pathogenic NGS variants are rare variants that exhibit a minimal allelic frequency minor than 1% but in internal cohorts of patients are more common (for example PID and neurological diseases patients) and represents a group of false-negative variants in all the NGS data's, for this reason, we exclude the variants that are in the group of the blacklist in the Rockefeller Institute Cohort of PID patients (94). This process was performed using a tool developed in house using the Pandas dataframes library written in the Python language. This code was validated by our bioinformatics collaborators at the University of Antioquia (supplementary figure 3).

We can associate the variants in hypothetical cellular pathways with previously described genes that causes IEIs in which phenotype are involved RW (*TNFSF12, RLTPR, CXCR4, RHOH, STK4, GATA2, TMC6, TMC8, CIB1, FCGR3A, DOCK8, SPINK5, IKBKG, ATM, ADA, ICOS, CD19, CD20, CD81, BAFFR, TNFRSF13B, ITGB2, IL7, CD28,* and *CD40L*) using the Connectome Tool. In this case, we exclude variants that have a p-value greater than 0,05 (95).

We use the PopViz webserver (96) as a tool that allows us to visualize the genetic data of the populations according to Gnomad versus the prediction of the impact of variants in silico, because the reference human genome GRCh37/Hg19 has been used in the sequencing of patients, it was used for this analysis without discriminating between homozygous or heterozygous variants.

Exome Analysis: Familial Analysis Strategy (Trios Pointed Analysis)

In families with multiple cases of RW, we sequence not only the index case but also affected and unaffected family members. In this case, we excluded the variants shared between the index case and unaffected individuals and considered the variants shared between the index case and affected individuals in the family.

Exome Analysis: Candidate variant selection

To prioritize the variants, we search the possible association of each gene with HPV we perform a bibliographic search in the PubMed database, additionally, we perform different in silico predictions using these tools:

- Consensus Negative Selection (CoNeS): The negative selection affects human disease-causing genes, for this reason, in last years have been improved different tools with the finality to predict the possible process of negative selection in a gene. CoNeS performs a high performance of the prediction of Negative selection and the gene tends to Dominant or Recessive traits (97).
- Probability of Loss of function (pLof): This tool expresses the probability that having a gene of expected loss-of-function variants, this determination is likely under selection against LoF variants (98).
- Residual Variation Intolerance Score (RVIS): The score is designed to rank genes in terms of whether they have more or less common functional genetic variation relative to the genome-wide expectation given the amount of apparently neutral variation in the gene has (99).

- PopViz: this tool allows visualization of the minimum allele frequency versus the CADD score, which allows an assessment of the functional impact of the variant in the set of all variants reported with MAF less than 1% (96).
- CADD, SIFT, Polyphen scores were used to predict the deleteriousness of single-nucleotide variants in the human genome (92, 100).

We analyzed exonic, splicing sites, 5` UTR, and 3` UTR variants based on the genetic hypothesis autosomal dominant, autosomal recessive, or X-linked. Each variant was analyzed according to the following criteria: function of the gene and anatomical site of expression. The Genecards database was used to find the function of each gene and its expression in different cells of the body. The list of genes with rare variables was analyzed based on the prediction of the functional effect using SIFT, CADD, and Polyphen 2 scores. The quality in the number of reads and reports previously in the literature were validated using the software Alamut Visual v.2.14. All variants that accomplish these criteria were selected as possible candidate variants, to discriminate among the possible candidate variants, we used previously reported findings in the literature and in silico prediction criteria to choose one candidate variant per patient.

The multiple analysis strategies employed on patients WES data, as well as the workflow employed can be seen in **Figure 1.**

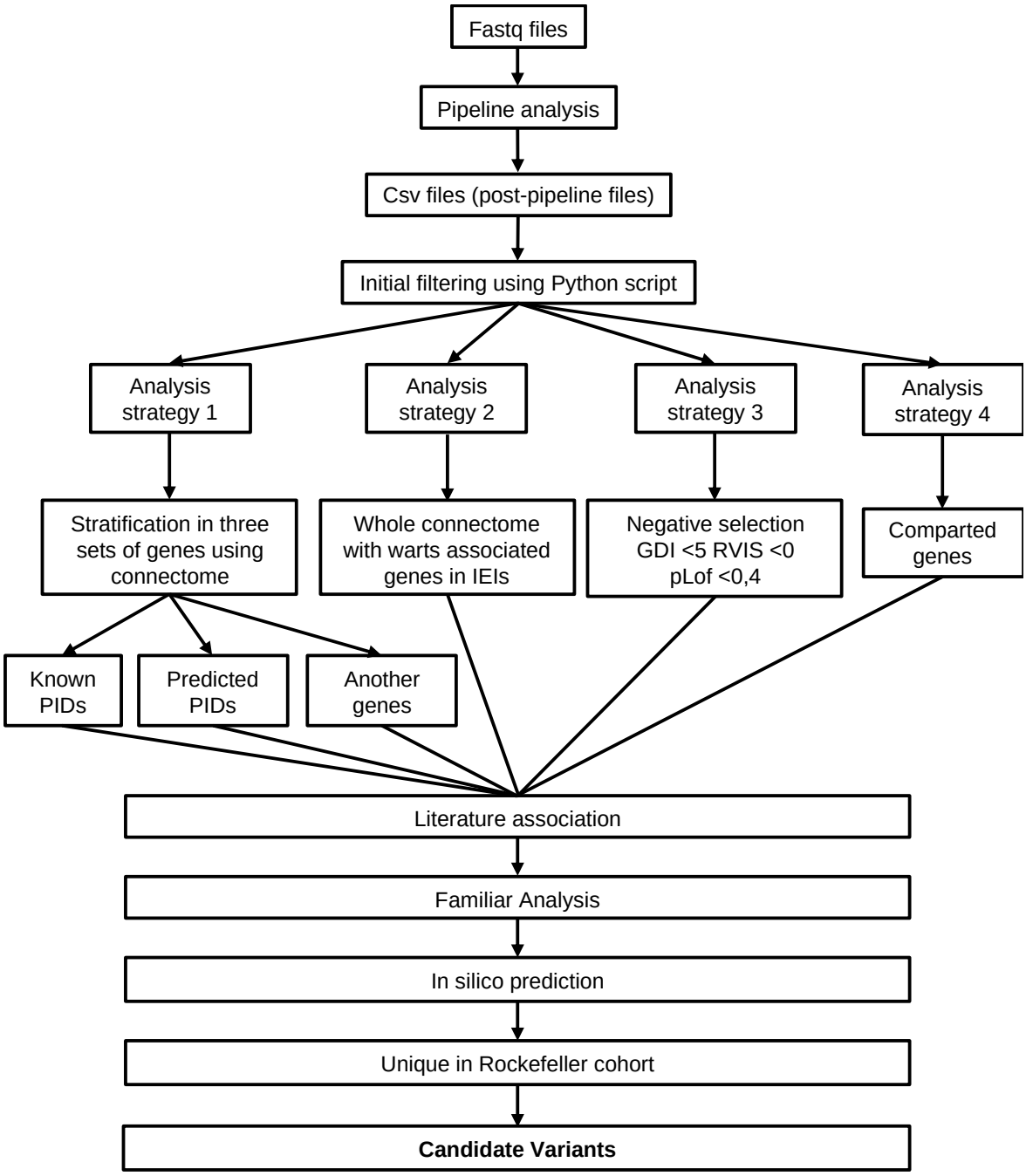


Figure 1. Analysis strategy of WES. Schematic of analysis of WES data from patients in our cohort analysis by Pipeline was performed using our in-house cohort of more than 10000 individuals. The Analysis in Python script was developed in this study. All determinations were performed using the human gene connectome using a $p<0.05$. The sets of genes with known PIDs, predicted PIDs and “another" genes were defined using the human gene connectome (91, 95, 97-99).

Flow cytometry

The leukocytes subpopulations were conducted with peripheral blood collected in EDTA tubes. The number of leucocytes was counted with a hemocytometer with 210µL of 3% glacial acetic acid and 10µL blood solution (22:1). Then, 100µL of blood was added in 3 cytometer tubes marked as (i) only cells, (ii) T and NK lymphocytes and (iii) B lymphocytes and monocytes. 5µL of these antibodies were used (T and NK lymphocytes tube anti-human CD45 FITC clone J33 (BD, USA), CD3 PECy7 clone 17A2 (BD), CD4 Pacific blue clone RM4-5 (BD), CD8 PE-Cy5 clone RPA-T8 (BD), CD16 PE clone 3G8 (BD), CD56 PE clone B159 (BD) and B lymphocytes and monocytes tube anti-human CD45 FITC clone J33 (BD), CD14 Pacific blue clone M5E2 (BD), CD21 PE clone Bly4 (BD), and CD19 APC clone HIB19 (BD).

The tubes were incubated for 20 minutes at room temperature protected from light. Then 1 mL of FACS lysis solution (BD, USA) was used for erythrocytes lysis during 10 minutes/ 20°C protected from light. Subsequently 2 mL of Dulbecco Phosphate Buffered Saline (PBS) (SIGMA-ALDRICH, St. Louis. MO) supplemented with 2% fetal bovine serum (FBS) (Invitrogen, USA) was used for washing per two centrifugations at 1700 rpm / 7 min. Finally, the pellet was resuspended in 300 µL of PBS and the tubes were analyzed in a Fortessa cytometer (BD, USA), using Flowjo (TreeStar Inc, Ashland).

Cell purification and culture

Blood samples Heparin-treated were taken from the proband. Blood (15 mL) was subjected to centrifugation on a Ficoll-Hypaque gradient separation for the isolation of peripheral blood mononuclear cells (PBMC). Then, PBMCs were treated with

cyclosporine and Epstein-Barr virus (EBV) for B-cell immortalization. EBV- transformed B-cell lines (EBV-B) were cultured in RPMI-1640 medium (Invitrogen, USA) supplemented with 10% heat-inactivated FBS (Invitrogen, USA) and cryopreserved for later use.

RESULTS

In this work, we studied the clinical, genetic, and immunologic characteristics of eleven patients with diagnosis of cutaneous RW from Colombia (South America). The study began with the evaluation of 40 individuals diagnosed with RW, 11 of whom met all the inclusion criteria. We evaluated the clinical information, elaborated pedigrees, and obtained gDNA samples from patients and relatives **(Table 1) (Figure 2**).

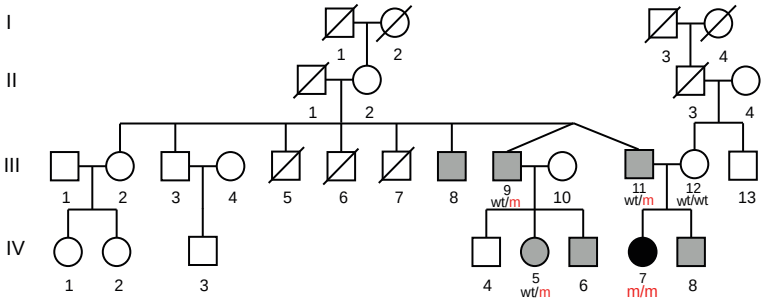
Table 1. **Clinical characteristics of patients.**

	City and department	Sex	Age (Years)	Age of Onset (Years)	Diphencyprone weekly sessions	Type of warts	Anatomical sites
P1	Medellín (Antioquia)	F	18	8	36	Plantar and periungual warts	Hands and feet
P2	Medellín (Antioquia)	F	15	2	39	Common warts	Face, knee, elbow and palm
P3	Medellín (Antioquia)	M	14	7	8	Periungual and common warts	Hands, face and feets
P4	Medellín (Antioquia)	M	16	10	20	Common and periungual warts	Face and hands
P5	Medellín (Antioquia)	F	18	7	42	Common and periungual warts	Hands, palm, elbow and knee
P6	Medellín (Antioquia)	M	15	7	16	Common warts	Hands, face and feets
P7	Medellín (Antioquia)	F	15	9	10	Periungual	Hand
P8	Medellín (Antioquia)	F	15	9	25	Periungual	Hand
P9	Medellín (Antioquia)	F	20	13	67	Periungual, common and plantar warts	Plant and hands
P10	Cali (Valle del Cauca)	M	14	14	N/A	Common and periungual warts	Face and hand
P11	Medellín (Antioquia)	M	34	14	N/A	Common warts	Face and hand

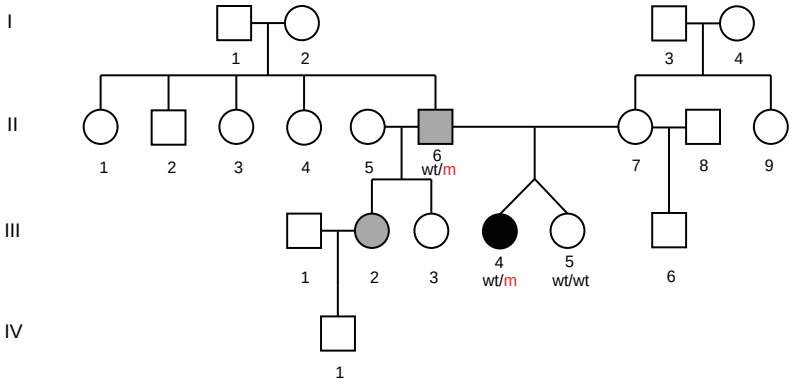
The patients are referenced as they are observed in the individual pedigrees. F= Female, M= Male, N/A= not apply.

Table 1. Clinical characteristics of patients. This table summarizes the cohort of patients and their epidemiological and clinical data such as city of residence, sex, current age, age of onset of RW, DCPC sessions, types of warts, anatomical sites and whether they have been vaccinated against HPV.

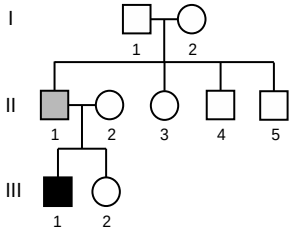
A. Kindred 1 ***PYGO2*, p.Gly223Glu**



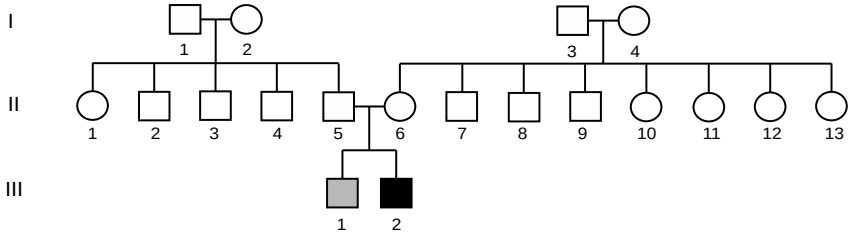
B. Kindred 2 ***CASP9*, p.Met39Ile**



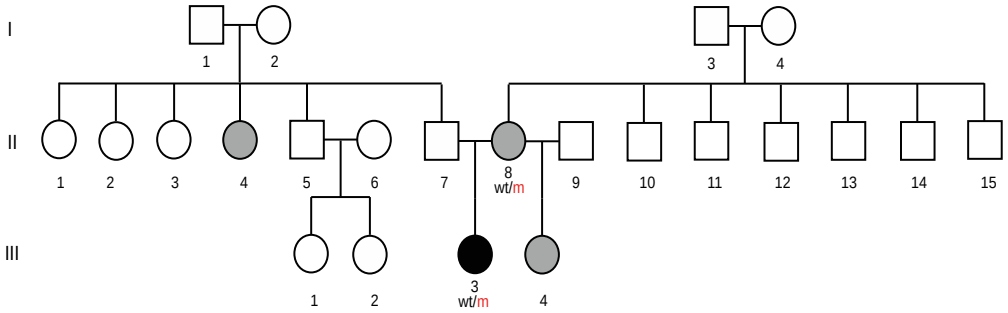
C. Kindred 3



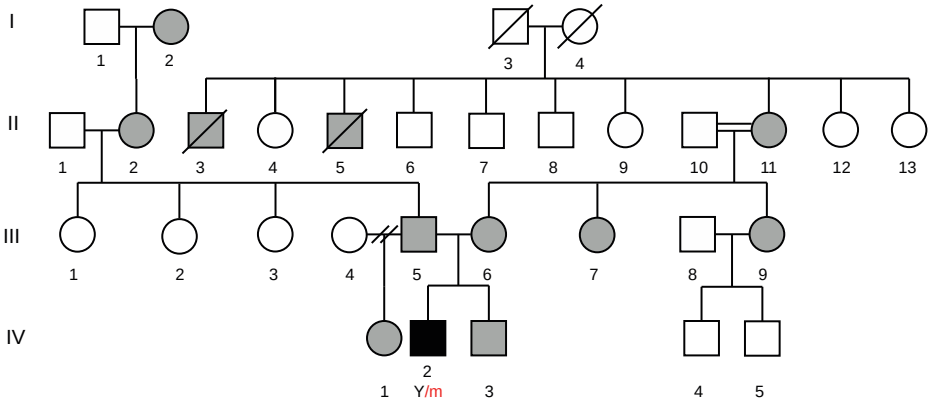
D. Kindred 4



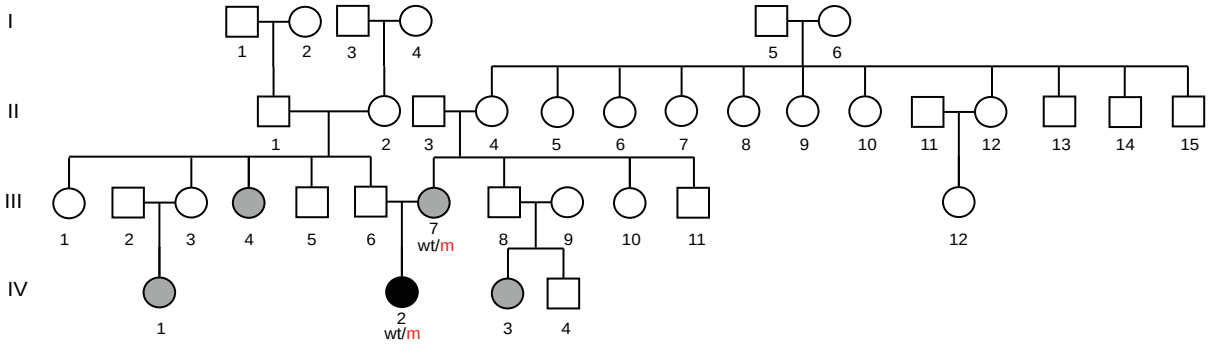
E. Kindred 5 ***CCNA2*, p.Cys193Arg**



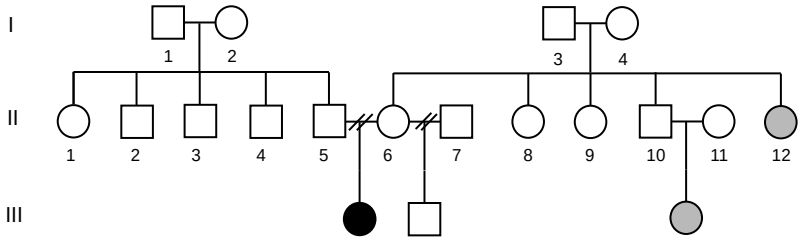
F. Kindred 6 ***CCNB3***, p.Lys494fs27*



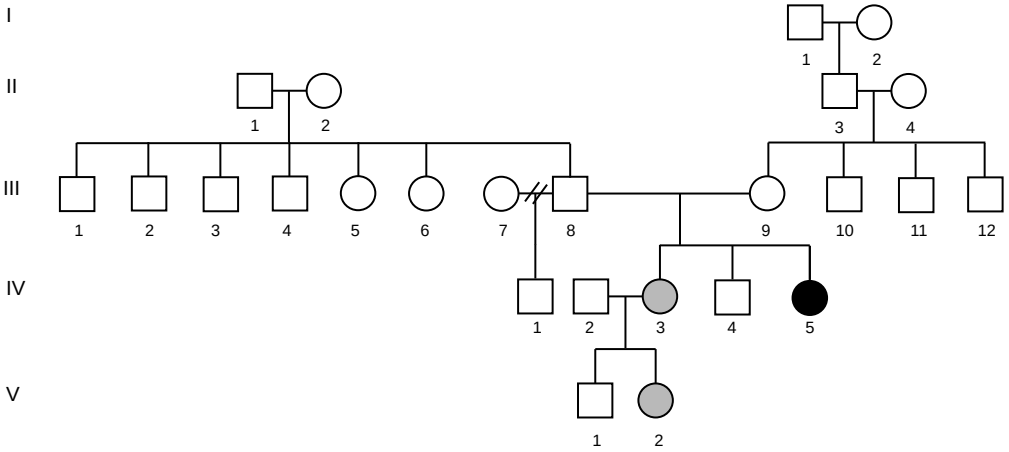
G. Kindred 7 ***GLTSCR2***, p.Arg387Gln



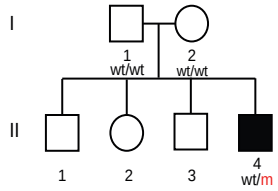
H. Kindred 8



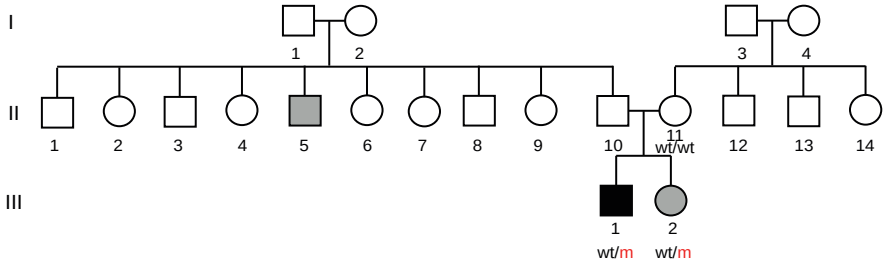
I. Kindred 9



J. Kindred 10 ***PABPC1***, p.Arg203*



K. Kindred 11 ***CAD***, c.6576-4_6576-5del



Non Afected **RW Affected** **RW like phenotype** **WES**

Figure 2. Pedigrees of eleven kindreds affected by RW. In black patients with a confirmed diagnosis of recalcitrant warts are observed. In gray, the members of the family that at some point had a phenotype like an index case are shown. Double diagonal stripes mean separation between couples.

Clinical characteristics of patients

Our study group is composed of 11 patients, 5 males and 6 females with ages ranging from 14 to 34 years and ages of onset of RW ranging from 2 to 14 years. The patients were born in the urban area of Medellín (Antioquia, Colombia) except for P10 who was born in Cali (Valle del Cauca, Colombia**) Table 1**. All of them were the result of no complicated pregnancies.

The presence of atopy was reported in two patients (P1 and P6). Both are under medical treatment and none of them present dermatitis in the anatomical sites of the lesions. No autoimmune diseases, severe episodes of infectious diseases or requiring hospitalization other than warts were observed in childhood of all patients, **Table 2**.

Table 2. Risk factors, familiar antecedents.

	Atopies	Smoker	Infectious diseases (Childhood)	Familiar history of cancer	Consanguinity	Immunization obligatory plan in Colombia	HPV vaccine (Dose)
P1	Dermatitis	No	Tonsilitis (no repitition)	Breast cancer Cervix cancer	No	Yes	2
P2	No	Father	Bacterial urinary infection	Skin and kidney cancer	No	Yes	1
P3	No	No	No	No	No	Yes	N/A
P4	No	No	No	Cervix Cancer	No	Yes	N/A
P5	No	No	No	No	No	Yes	1
P6	Asthma	No	*Helicobacter pylori*	Cervix Cancer	Probably	Yes	N/A
P7	No	No	Pneumonia	Cervix Cancer	No	Yes	N/A
P8	No	No	No	Skin Stomach Breast Cervix cancer	No	Yes	N/A
P9	No	No	No	Cervix Cancer	No	Yes	2
P10	No	No	No	No	No	Yes	1
P11	No	No	No	No	No	Yes	N/A

Table 2. Risk factors, familiar antecedents. This table shows the family history of cancer of the RW patients obtained through follow-up phone calls.

All patients received all vaccines according to the guidelines of the national immunization program of Colombia with not reported complications. Five of the patients were vaccinated against HPV after the first lesions (P1, P2, P5, P9 and P10) with no reported improvement in warts. All patients report previous regimes of treatments for the management of warts after no response to treatments, patients are referred to for weekly topical DPCP immunotherapy (except for P10 and P11) **Table 2**.

None of the patients describes consanguineous marriages, except for P6 where a possible consanguinity was reported. Familial history of cancer was observed in 7 families (P1, P2, P4, P6, P7, P8 and P9), and all patients (except P10) reported family members affected with warts. The patients' risk factors and family history can be found in **Table 2**. Detailed information on the clinical characteristics of the patients can be consulted in the **supplementary material 4.**

Patients with recalcitrant warts may present lesions for a period longer than two years in the presence of last-line treatments.

We observed that the presence of RW and treatment with DPCP can become a clinical challenge that may require many years to resolve patient’s lesions. In our cohort, P10 was the shortest time affected by the lesions (2 years and 3 months) and P11 was the longest affected (more than 20 years) **(Figure 3 and 4. A).**

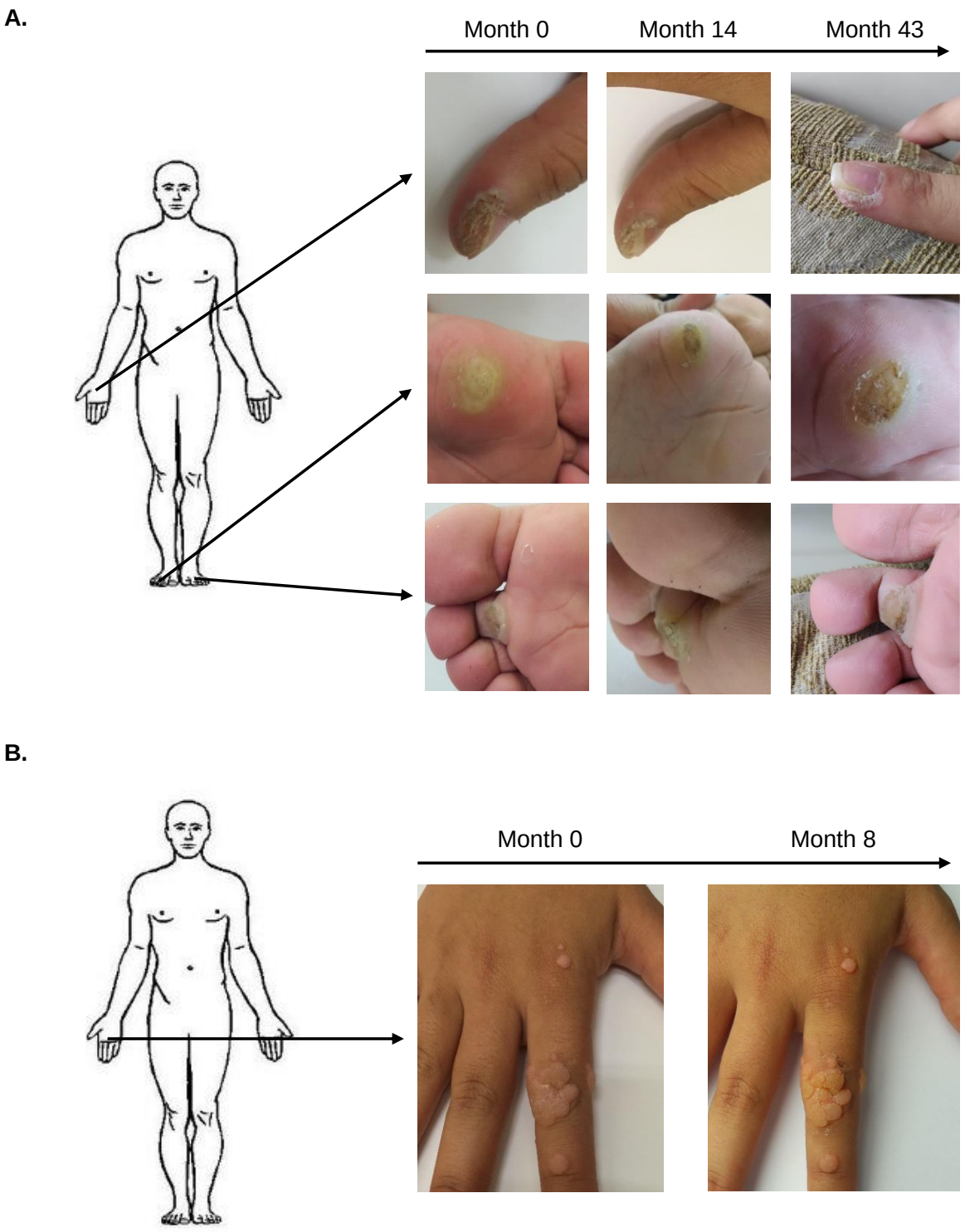
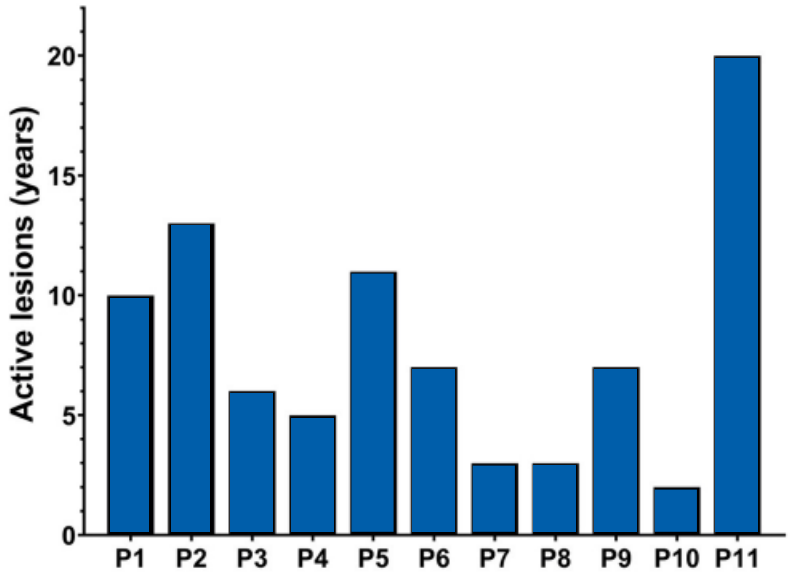


Figure 3. Persistance of lesions in A. P1 B. P5. Representative photos of patients in the RW cohort, photos were taken while patients were being followed up after they were diagnosed with RW.

A.



B.

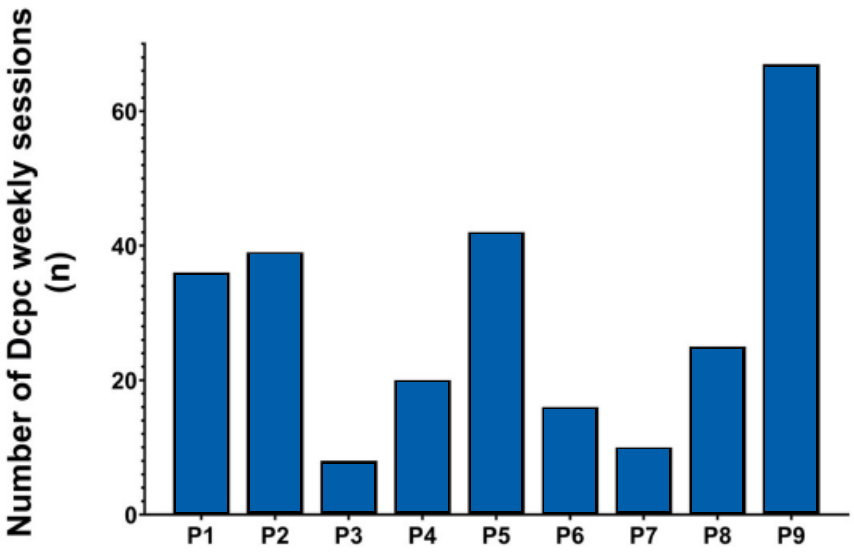


Figure 4. Recalcitrance of lesions A. Years of active lesions per patient **B.** Number of weekly doses in the diphencyprone (Dcpc) program of the dermatology unit of the University of Antioquia. Patients P1-P9 participated in this program.

Diphencyprone is the latest line of treatment available in Colombia for the management of recalcitrant warts, which is applied weekly to patients. In our cohort, we have found that patients with recalcitrant warts, even if they are under this treatment, may continue to present lesions for years (**Figure 4. B**). Currently only P1 and P11 continue with warts after 5 years of follow up.

Periungual warts may represent a clinical challenge to wart management.

We found patients with lesions in different anatomical sites. Periungual warts were the most common type of warts, followed by warts on hands, on sites other than palm and periungual region (the anatomical site and type of warts presented by each patient are reviewed in **Table 1**).

Patients with recalcitrant warts appear to have a specific susceptibility factor for HPV infection

Our patients do not present severe recurrent infections in childhood, nor neurological manifestations suggesting a syndromic disease of genetic origin. In addition, no presence of warts was observed in the family members with cancer.

To corroborate this hypothesis, we performed a phenotyping of blood leukocyte subpopulations in a random patient, which yielded normal results **(Supplementary figure 5)**.

Patients with cutaneous recalcitrant warts have a family history of warts.

We observed the presence of cutaneous warts in relatives of the index case in each kindred. Those individuals presented persistent lesions for long periods. In each kindred,

affected and unaffected individuals can be found, suggesting that skin-to-skin contact may be not sufficient for the development of recalcitrant warts, which in turn rules out a factor associated with constant inoculation among family members. Due to the presence of different affected individuals, regardless of gender in different generations, our main hypothesis is that recalcitrant warts could have segregation in an autosomal dominant (AD) inheritance pattern **(Figure 2).**

Genetic characterization

We performed WES from gDNA of patients **P1, P2, P5, P6, P7, P10 and P11**. The raw data, from which tables were obtained with initial filtering by 5% allele frequency based on GnomAD (**Table 3 first row**). Using the specific criteria mentioned in our methodology of allelic frequency less than 1%, GDI, MSC-CADD, BL, the results in terms of number of variants in terms of the strategy of analysis 2 can be seen in (**Table 3**). Based on the publications related to the genes, the prediction of the functional impact of each of the variants, as well as the negative selection process for each gene, a variant was selected for each patient, which will be explained below **(Table 4).**

Paramenter	P1	P2	P5	P6	P7	P10	P11
Initial Data	13646	16660	15947	23112	18152	17969	18355
Initial Filtering	798	801	845	734	957	664	636
Associated with warts in IEI by Connectome	245	233	263	223	271	158	132
Familiar Analysis	210	92	N/A	N/A	N/A	96	33
Private variants when compared to the Rockefeller University cohort.	2	1	3	2	2	4	2

Table 3. Results of WES analysis of patients P1, P2, P5, P7, P10, and P11. This table schematizes the number of variants resulting from each of the exome analysis steps according to the analysis strategy 2. **N/A** Not available.

Using a Human Gene Connectome (HGC) we found variants associated with genes previously reported to be affected IEI in which RW have been described (**Table 3**). The family analysis approach was performed in four families (**P1, P2, P10 and P11**) due to the availability of family members for sampling**.** By corroborating in public database (gnomAD version 2.1.1), and in our in-house database of >15,000 patients with diverse severe infectious diseases, we have found that our patients have unique variants of interest that are related by literature to HPV infection, further suggesting that the variant carried by the patients is disease-causing. The determinations of the negative selection, in addition to the report of the candidate variants, can be found in **(Table 4).** After analyzing the variants found in each index case and kindred using *in silico* impact predictors, we obtain following candidate variants:

Table 4. **Candidate variants in patients.**

PT	Loc	Chr	pos	ID	refNt	altNt	function	gene	gnomAD genomes AF	Change	Zygo	SIFT	Polyphen	CADD	MSC CADD	pLoF	RVIS	GDI
P1	**IV.7**	**1**	154931778	.	C	T	missense	***PYGO2***	-	p.Gly233Glu	Het	D	PD	11.49	4.31	0.28	-0.34	1.80
P2	**III.4**	**1**	15850579	.	C	A	missense	***CASP9***	-	p.Met39Ile	Het	PD	D	25.7	2.31	0.97	0.82	8.01
P3	**III.3**																	
P4	**III.2**																	
P5	**III.3**	**4**	122741914	rs531485009	A	G	missense	***CCNA2***	-	p.Cys193Arg	Het	T	B	16.51	2.31	0.13	0.37	1.46
P6	**IV.2**	**X**	50052648	.	GA	G	indel-frameshift	***CCNB3***	-	p.Lys494fs	Het	-	-	22.3	2.31	0.62	-0.40	1.72
P7	**IV.2**		48258711	rs769427571	G	A	missense	***GLTSCR2***	-	p.Arg387Gln	Het	T	D	17.58	2.31	0.49	0.40	5.36
P8	**III.1**																	
P9	**III.5**																	
P10	**II.4**	**8**	101721899	rs142985461	C	A	stop-gained	***PABPC1***	0.003962	p.Arg203*	Het	-	-	39.0	15.15	0.27	-0.38	12.49
P11	**III.1**	**2**	27466258	rs759140005	CTT	C	splicing	***CAD***	0.000940687	-	Het	-	-	8.55	5.0	0.25	-3.84	3.14

Table 4. Candidate variants in patients. This table shows the candidate variants resulting from the analysis of patient exome data, in silico impact determinations of the variants and negative selection measurements on the genes. **Chr:** Chromosome, **Pos:** genomic position, **refNt:** reference nucleotide, **altNt:** alternative nucleotide**, D:** deleterious, **PD:** probably damaging, **T:** tolerable, **B:** benign.

Patient 1 (P1): We found on chromosome 1 a heterozygous missense variant (g.154931778C>G, c.698G>C, p.Gly233Glu) in *PYGO2* not previously reported in gnomAD that predicts for a glycine to glutamine change at position 233 of the protein **(Figure 6A).**

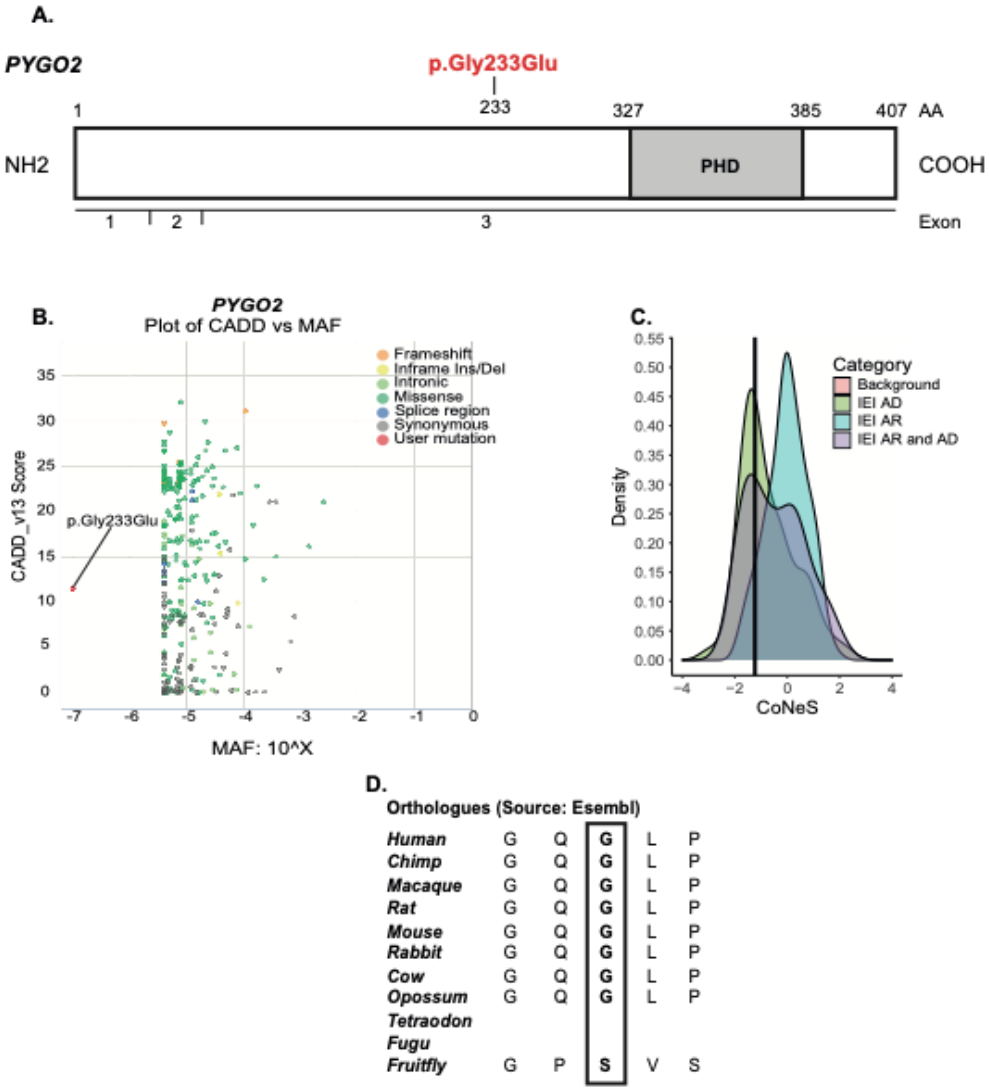


Figure 6. Patient P1 analysis of candidate variant (g.154931778C>G, c.698G>C, p.Gly233Glu) in *PYGO2* gene: A. protein-level representations of the patient's variant. **B.** Population genetics, minor allele frequency (MAF) and CADD scores shown for all *PYGO2* variants reported in the gnomAD database. The patient mutation showed a CADD score of 11.49. **C.** *PYGO2* mutations are predicted to underlie AD traits as determined by CoNeS. **D.** Amino acid conservation in different species, this amino acid presents a high conservation between species.

The population genetics graph shows a variant not previously reported in the GnomAD database with a moderate impact according to CADD **(Figure 6B)**. CoNeS analysis, a sequence-based metric for quantifying gene-level selection (101) predicts this gene presents a tendency towards autosomal dominant (AD) defects **(Figure 6C)**. The population genetics graphics shows that Bioinformatic analysis predicts this amino acid is highly conserved between species **(Figure 6D).** Based on different metrics, this gene is predicted with a residual variation intolerance score (RVIS) of −0.34; a gene damage index (GDI) of 1.80 (102); and a probability of loss of function intolerance (pLI) score of 0.28 (103). Taken together, *PYGO2* is under strong negative selection, consistent with that seen in genes underlying autosomal dominant IEI (101) **(Table 4)**, p.Gly233Glu predicts as deleterious and probably deleterious by SIFT and Polyphen **(Table 4)**.This variant was found in 3 affected individuals of kindred 1 and in his father, and was not present in his mother who is not affected **(Figure 2A)**.

Patient 2 (P2): We found on chromosome 1 a heterozygous missense variant (g.15850579C>A, c.117G>T, p.Met39Ile) in *CASP9* that predicts for a Methionine to Isoleucine change at position 39 of the protein **(Figure 7A)** with a population genetics of a previously undescribed variant and a high impact according to CADD **(Figure 7B)**. The CoNeS analysis presents a tendency towards autosomal recessive (AR) defects **(Figure 7C)** and this gene is predicted with a residual RVIS of −0.82 ; GDI of 8.01 (102); and a pLof of 0.97 (103) **(Table 4).** Bioinformatic analysis predicts this amino acid is highly conserved between species **(Figure 7D).** This variant was present in the father of the

patient who is affected, and it was not present in his healthy sister that shows a possible AD inheritance **(Figure 2B)**.

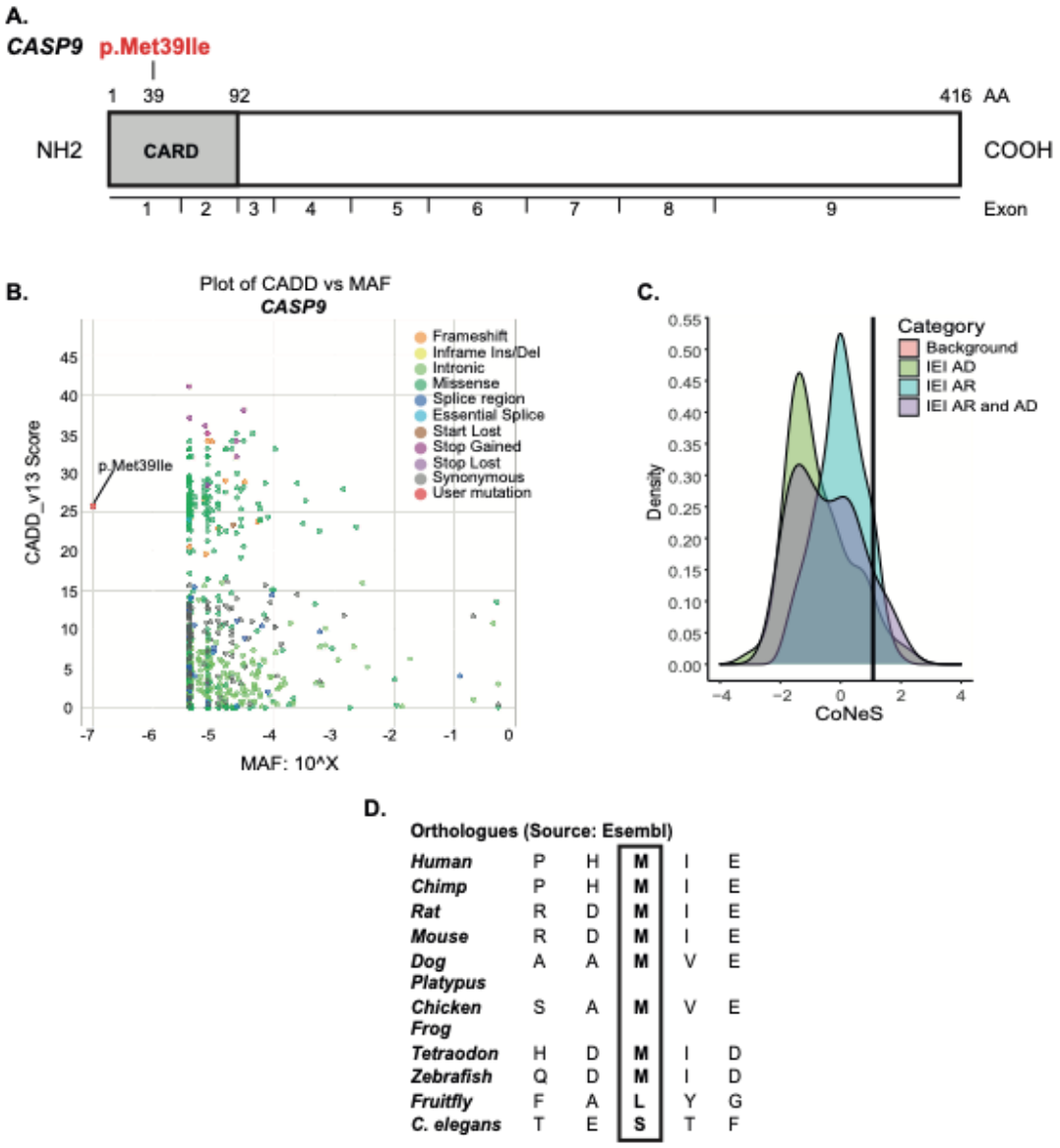


Figure 7. Patient P2 analysis of candidate variant (g.15850579C>A, c.117G>T, p.Met39Ile) **in *CASP9* gene: A.** protein-level representations of the patient's variant. **B.** Population genetics, minor allele frequency (MAF) and CADD scores are shown for all *CASP9* variants reported in the gnomAD database. The patient mutation showed a CADD score of 25.7. **C.** *CASP9* mutations are predicted to underlie AR traits as determined by CoNeS. **D.** Amino acid conservation in different species, this aminoacid presents a high conservation between species.

Patient 5 (P5): We found on chromosome 4 a heterozygous missense variant (g.122741914A>G, c.577T>C, p.Cys193Arg) in *CCNA2* that predicts for a Cysteine to Arginine change at position 193 of the protein **(Figure 8A)** with a population genetics of a previously undescribed variant and a moderate impact according to CADD **(Figure 8B)**. CoNeS analysis predicts this gene presents a tendency towards autosomal dominant (AD) defects **(Figure 8C)**, this gene is predicted with a RVIS) of 0.37; GDI of 1.46 (102); pLI of 0.13 (103) **(Table 4)**. In addition, bioinformatic analysis predicts this amino acid is moderate conserved between species **(Figure 8D)**. In kindred 5 the only individual sequenced was P5 **(Figure 2E)**.

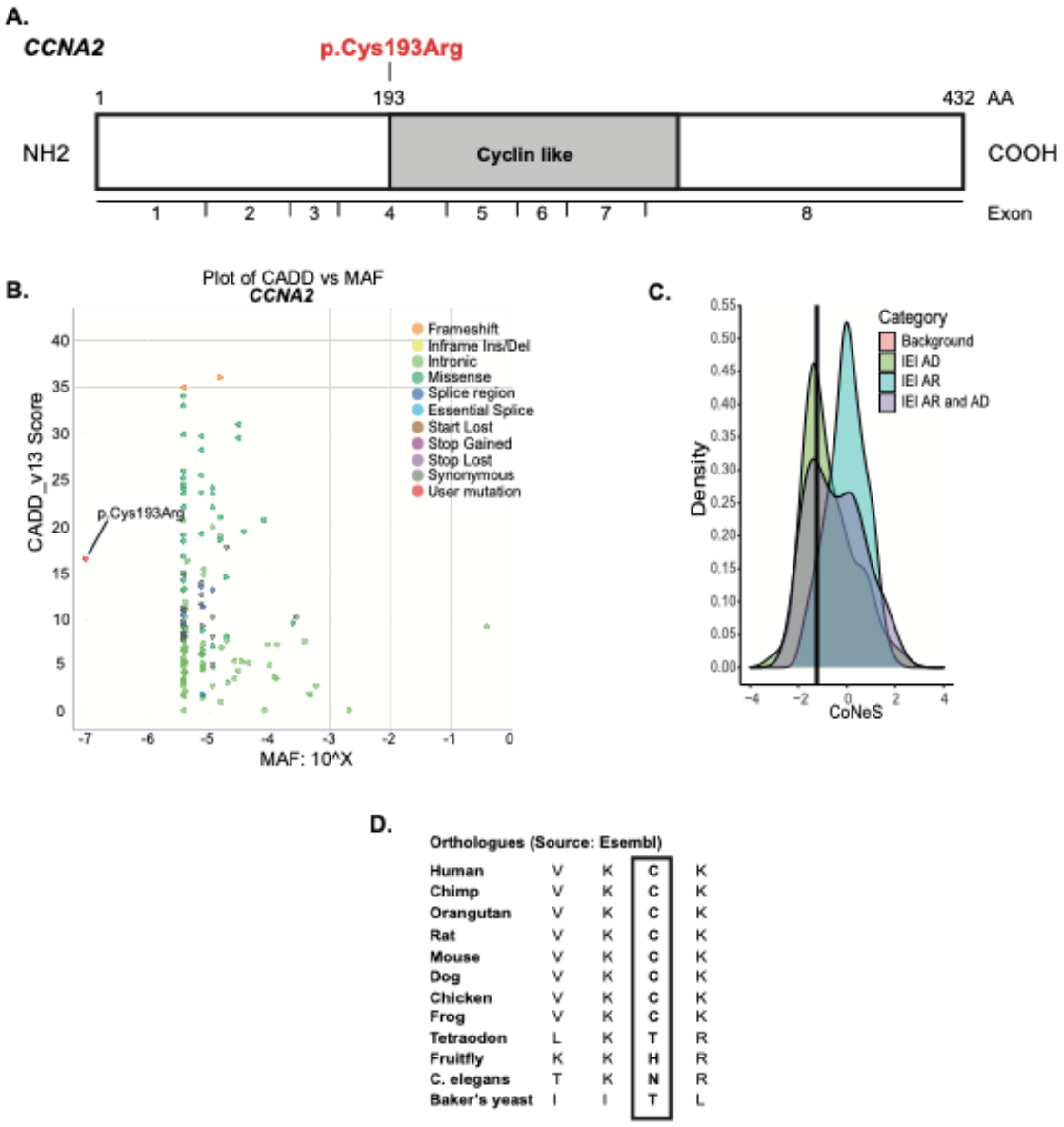


Figure 8. Patient P5 analysis of candidate variant (g.122741914A>G, c.577T>C, p.Cys193Arg) **in *CCNA2* gene: A.** protein-level representations of the patient's variant. **B.** Population genetics, minor allele frequency (MAF) and CADD scores are shown for all *CCNA2* variants reported in the gnomAD database. The patient mutation showed a CADD score of 16.51. **C. *CCNA2*** mutations underlie AD traits as determined by CoNeS. **D.** Amino acid conservation in different species, this aminoacid presents a moderate conservation between species.

Patient 6 (P6): We found on chromosome X a hemizygous deletion of an adenine that induces a frameshift (g.50052648_50052649del, c.1479_1480del, p.Lys494Alafs*27) that predicts for a Lysine to Alanine change at position 494 of the protein and a frameshift leading to a stop codon after 27 amino acids in *CCNB3* **(Figure 9A)** with a population genetics of a previously undescribed variant and a moderate impact according to CADD **(Figure 9B)**. The CoNeS analysis predicts this gene presents a tendency towards X-linked (XL) defects **(Figure 9C)**, also, this gene is predicted with a RVIS of -0.40; GDI of 1.72 (102); pLI score of 0.62 (103) **(Table 4).** In kindred 6, the only individual sequenced was P6 **(Figure 2F)**.

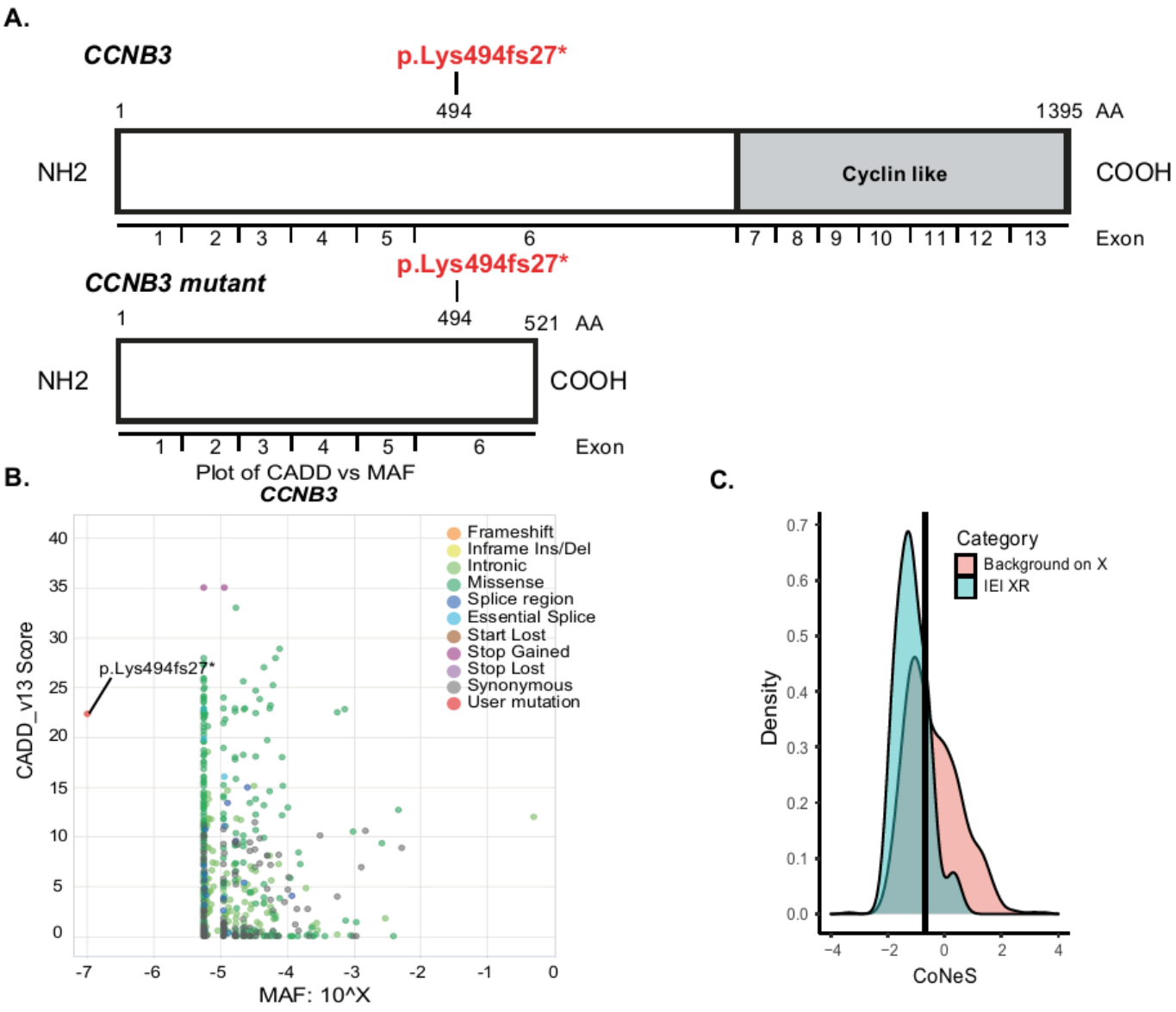


Figure 9. Patient P6 analysis of candidate variant (g.50052648_50052649del, c.1479_1480del, p.Lys494Alafs*27) **in *CCNB3* gene: A.** protein-level representations of the patient's variant. **B.** Population genetics, minor allele frequency (MAF) and CADD scores are shown for all *CCNB3* variants reported in the gnomAD database. The patient mutation showed a CADD score of 22.3. **C. *CCNB3*** mutations underlie XL traits as determined by CoNeS.

Patient 7 (P7): We found on chromosome 19 a Heterozygous missense variant (g.48258711G>A, c.1160G>A, p.Arg387Gln) in *GLTSCR2* that predicts for a Arginine to

Glycine change at position 387 of the protein **(Figure 10A)** with a population genetics of a MAF close to 10^{-4} and a moderate impact according to CADD **(Figure 10B)**. The CoNeS analysis, predicts this gene presents a tendency autosomal recessive (AR) defects **(Figure 10C)** and this gene is predicted with a RVIS of 0.40; a gene damage index (GDI) of 5.36 (102); pLI score of 0.49 (103) **(Table 4)**. This variant (in kindred 7) was also found in the patient's mother, who is also affected **(Figure 2G)**.

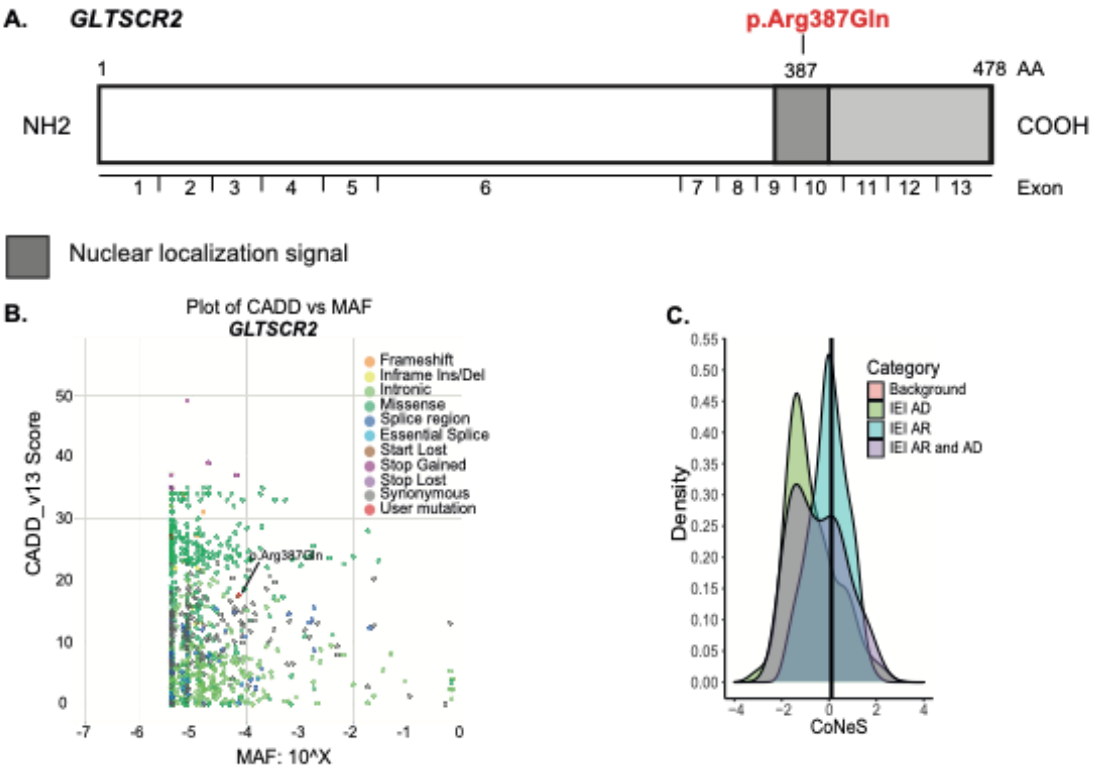


Figure 10. Patient P7 analysis of candidate variant (g.48258711G>A, c.1160G>A, p.Arg387Gln) **in *GLTSCR2* gene: A.** protein-level representations of the patient's variant. **B.** Population genetics, minor allele frequency (MAF) and CADD scores are shown for all *GLTSCR2* variants reported in the gnomAD database. The patient mutation showed a CADD score of 17.58. **C. *GLTSCR2*** mutations underlie AR traits as determined by CoNeS.

Patient 10 (P10): We found on chromosome 8 a Heterozygous Stop-gain variant in the codon 203 of *PABPC1* gene (g.48258711G>A, c.1160G>A, p.Arg203*) **(Figure 11A)** with

a population genetics of a MAF close to 10^{-1} and a high impact according to CADD **(Figure 11B)** and a CoNeS with tendency to autosomal dominant (AD) defects **(Figure 11C)**, the Arginine 203 is highly conserved between species **(Figure 11D)**, and this gene is predicted with a RVIS of -0.38; a GDI of 12.49 (102); and a pLI score of 0.27 (103) **(Table 4)**. This variant could not be found in either parent in kindred 10, so it is hypothesized to occur *de novo* in the patient. **(Figure 2J)**.

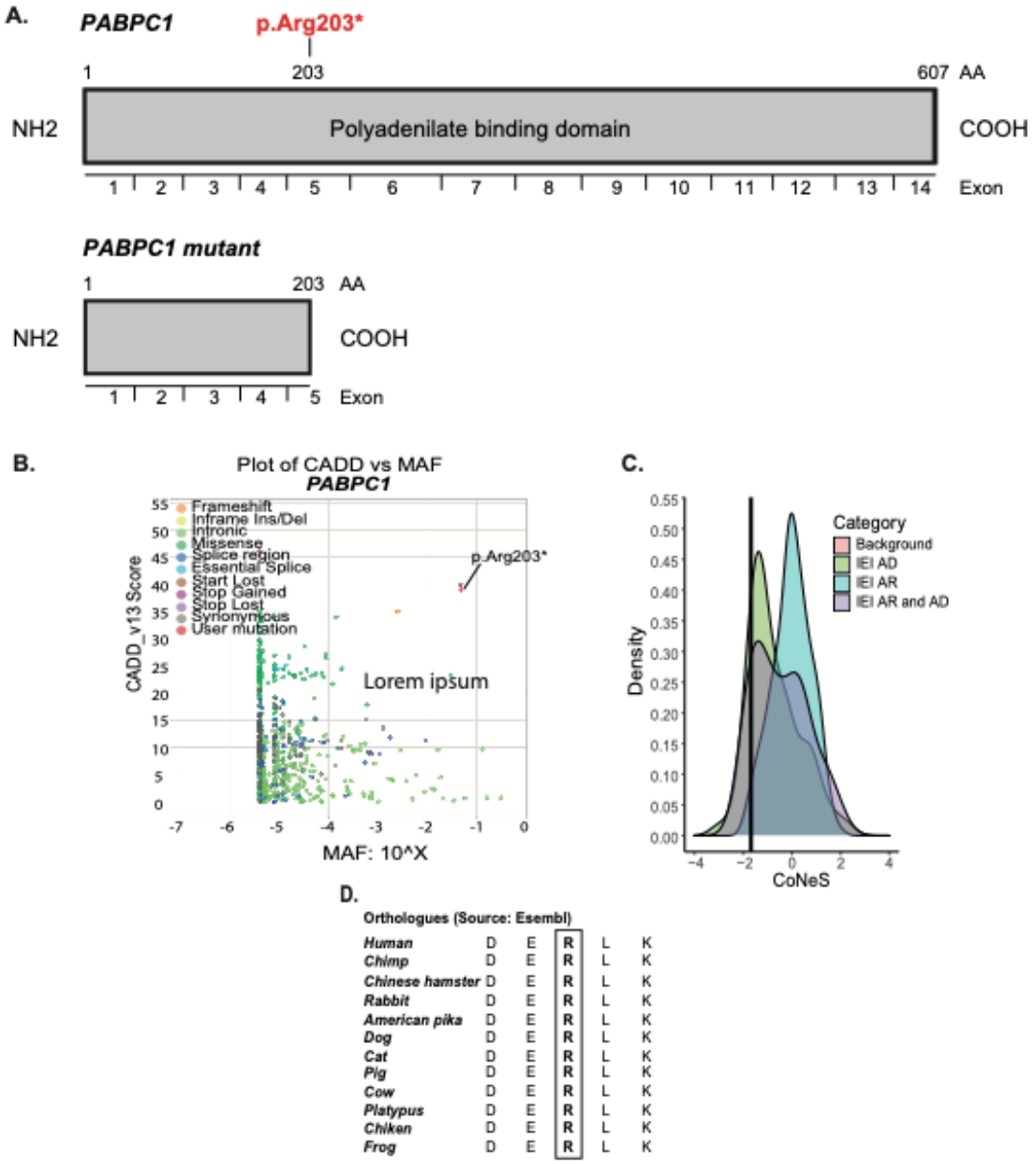


Figure 11. Patient P10 analysis of candidate variant (g.48258711G>A, c.1160G>A, p.Arg203*) **in *PABPC1* gene: A.** protein-level representations of the patient's variant. **B.** Population genetics, minor allele frequency (MAF) and CADD scores are shown for all *PABPC1* variants reported in the gnomAD database. The patient mutation showed a CADD score of 22.3. **C. *PABPC1*** mutations underlie AD traits as determined by CoNeS. **D.** Amino acid conservation in different species, this aminoacid presents a high conservation between species.

Patient 11 (P11): We found on chromosome 8 a heterozygous Splicing variant (g.27466258_27466260del, c.6576-26_6576-24del) in CAD that predicts for a splicing defect in the exon 44 of the protein **(Figure 12A)** with a population genetics of a MAF close to 10^{-1} and a moderate impact according to CADD **(Figure 12B)**. The CoNeS analysis predicts this gene presents a tendency to autosomal dominant (AD) defect **(Figure 12C)**, this gene is predicted with a RVIS of -3.84; GDI of 3.14 (102); pLI score of 0.25 (103) **(Table 4).** This variant also be found in the affected sister of patients and is absent in their mother in kindred 11 **(Figure 2K)**.

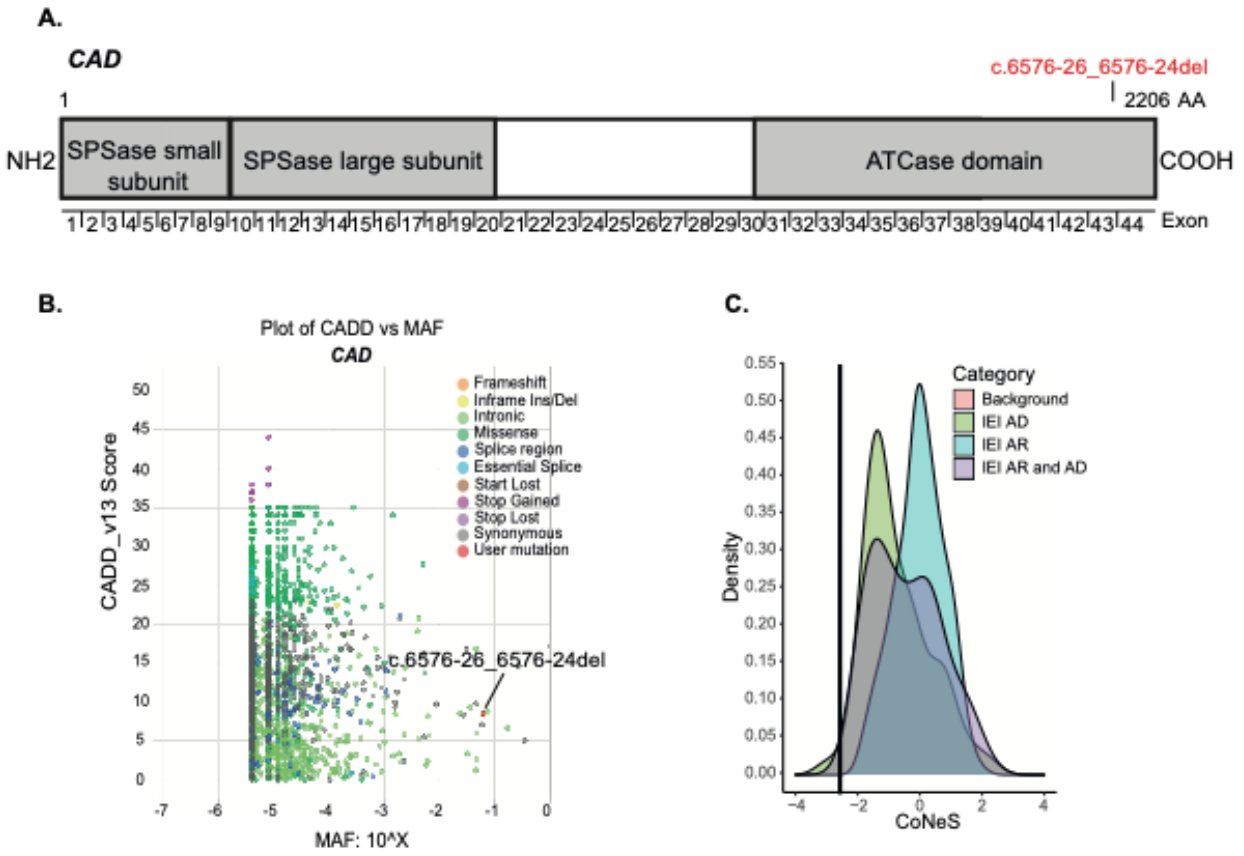


Figure 12. Patient P11 analysis of candidate variant (g.27466258_27466260del, c.6576-26_6576-24del) **in CAD gene: A.** protein-level representations of the patient's variant. **B.** Population genetics, minor allele frequency (MAF) and CADD scores are shown for all *CAD* variants reported in the gnomAD database. The patient mutation showed a CADD score of 8.55. **C.** *CAD* mutations underlie AD traits as determined by CoNeS.

In patients P3, P4, P8 and P9, WES was not performed, therefore they do not have candidate variants.

DISCUSSION

In this study, we report a cohort of eleven, otherwise young healthy patients with a unique diagnosis of cutaneous RW. These patients belong to eleven unrelated Colombian families from Antioquia and Valle del Cauca. We found patients with age years of age of onset of CRWs between 2 and 14 years of age, with no sex differences, which is consistent with studies of the prevalence and natural history of warts (39, 52, 88).

The HPV vaccine has demonstrated a 66% lesion resolution rate in adult patients with recalcitrant warts, however, we found that in our cohort none of the patients had resolution of lesions after HPV vaccination (104). This is interesting because it could be explained by differences attributable to the vaccine, the age of the patients' or the genetics our population group.

Diphencyprone (DCPC) is a contact immunotherapy treatment, which is used as a last-line treatment in the management of recalcitrant warts. Studies in the efficacy of this treatment exhibits cure rates of 60% and the cure rate are considered to drop to 0% after 5.3 months of weekly treatment, which is consistent with what we found in our study as patients who left the DCPC program and resolved the lesions reported sporadic resolution in periods longer than 6 months after the last application of the treatment (105).

We found these recurrent lesions occur mainly on fingers with periungual warts, hands, knees, lips, mouth, nose, elbow, and the sole of the feet, as reported before (106-108). Periungual warts are difficult to treat because they present a high recurrence rate (109), the HPV types most associated with these lesions are 1, 2, 4, 5, 7, 27, 57 (110).

We have reported the presence of atopies in two patients (P1 and P6) in which only one had atopic dermatitis (P1), this disease has been related to an increased risk of warts in children (59), but in the case of P1 no warts were found in the same anatomical site where the dermatitis was present.

We have reported the presence of family members of the patients affected by cervical cancer; however, we have not found the presence of cancer in the index cases. No warts were also found in individuals affected by cervical cancer of our cohort. This can also be consistent with the broad spectrum of possible susceptibility phenotypes to oncovirus infections suggesting that depending on the severity of the genetic defect a spectrum of variant complexity could be induced in oncogenic virus infections (24).

Patients with IEIs who present with warts usually have other concomitant infections, these infections occur repeatedly and usually require hospitalization of the patient to resolve the clinical lesion (54). This in turn is associated with abnormalities in the function or number of T-cells, which play a key role in the immune response against HPV (24, 54). Our cohort are otherwise young patients presenting in some cases unique, not recurrent, and sporadic infections (urinary tract infection and pneumonia of unknown etiology) in P2, P7; *H. pylori* infection one patient (P6). These clinical findings, associated with normal parameters in general blood leukocyte subpopulations and together with the specific tropism of HPV for keratinocytes suggest a type of patient with specific defects in keratinocyte intrinsic immunity (6, 47).

EVER1, EVER2 and CIB1 form a complex that restricts β-HPV infection in keratinocytes, and defects in that complex can explain the clinical phenotype in patients with EV and since the discovery of this protein complex, it was hypothesized that there may be others

proteins controlling the HPV infections (13). The persistence of HPV-related warts in our cohort of patients without obviously impaired immune and familial susceptibility to the development of warts, suggests the presence of genetic impaired immunity to cutaneous HPVs, supporting the idea of a possible IEI (6, 47).

To study the presence of a genetic component that could explain this susceptibility to recalcitrant warts in our patients, we performed WES in index cases (P1, P2, P5, P6, P7, P10 and P11) and relatives in kindreds 1, 2, 7, 10 and 11 in which we searched for the presence of variants related to the immune response to HPV and the persistence of its infection **(Figure 13).**

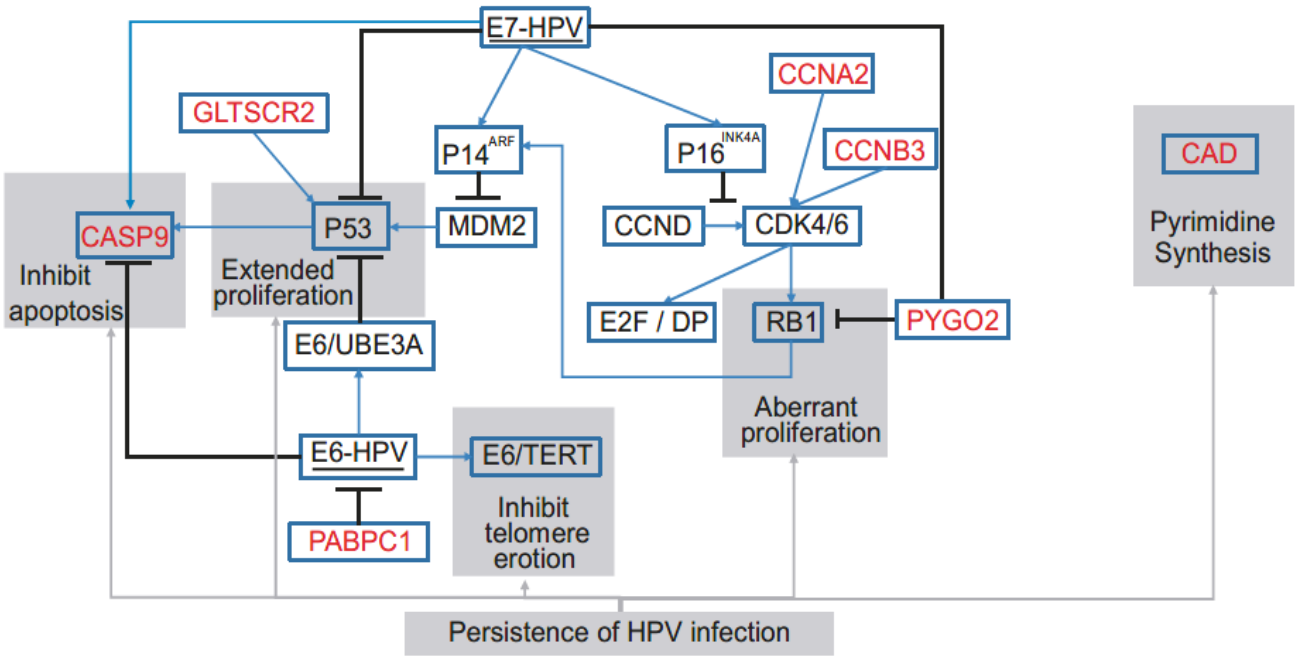


Figure 13. Candidate genes schematized in biological processes associated with persistence of HPV infections. Schematic analysis of candidate genes of patients with cutaneous RW and their possible interactions with E7- and E6-HPV proteins in the context of the recalcitrance of HPV infections. Adapted from (111-117).

The use of WES for the analysis, diagnosis, and study of new IEI has proven to be a robust, reliable, and cost-effective tool. Due to the high rate of false positives, it is necessary to implement analysis strategies based on other in silico predictions at the variant and gene-level that allow us to reduce the number of false positives and focus the search on variants that can explain the genotype-phenotype relationship (118).

The use of metadata connectivity tools to evaluate hypothetical cell signaling pathways, while extrapolation of different sets of loss-of-function variants in different population data

sets, as well as theoretical association with the HPV infectious cycle, proved to be an efficient strategy for searching and correlating variants in WES of patients with RW in which candidate variants were found that could explain the persistence of infection and recalcitrance in these patients (91, 95).

Analysis of the patients WES data showed variants in different genes (*PYGO2, CASP9 CCNA2, CCNB3, GLTSCR2, PABPC1*, and *CAD*) which are associated by connectome with the HPV infectious process ,all these genes are expressed in skin (Except for CCNB3 which has no expression data available in protein atlas) (119), and could explain the clinical phenotype of this disease. The discussion of each of the genes is presented below:

Due to its participation in the Wnt/β-catenin pathway as a critical factor in the transcriptional complex, pygopus (PYGO) has been associated with the development of neoplasms in humans (120). *PYGO2* $^{-/-}$ mice, presents impairment in the morphogenesis and regeneration of epithelial progenitor cells (121). In the context of HPV infection, it has been reported that HPV E7 protein induces PYGO2 expression by inhibiting retinoblastoma protein, which in turn is related to the decreased activity of p53 as the main regulator of uncontrolled growth in the cervical cancer (111). Cutaneous HPV infection that cause skin cancer present similar mechanisms of p53 attenuation highlighting the importance HPV and the control cell cycle in the tissues, and in the control mechanisms of the neoplastic process such as PYGO2 induction and how its possible genetic alterations may be related to the processes of persistence of HPV infection (122). The familial segregation (Kindred 1) of p.Gly233Glu in *PYGO2* and the multiple *in silico* predictions, as well as the absence of this variant in our in house cohort,

and allele frequency databases, suggest that this variant may be considered a good candidate explaining the clinical phenotype of P1.

HPV guarantee the persistence of the controlling the expression and activity of proapoptotic molecules that can lead to the interruption of the infectious cycle through programmed cell death (122). HPV alters the expression of caspase 3, 8, and 9, decreasing the proapoptotic capacity of these molecules, in turn, this sensitizes keratinocytes to genetic alterations caused by UV rays which is a mechanism that leads to cancer in EV (123). Also, *CASP9* is involved in the resistance to different treatments; It can be explained by observations where altered caspase 9 render HPV-infected cells resistant to chemotherapeutic stimuli, which may, in turn, suggest that modifications in *CASP9* may modify the outcome of a benign neoplastic phenomenon such as a wart and turn it into a recalcitrant lesion (112). *CASP9*$^{-/-}$ mice are prone to die perinatally due to failures in apoptosis during cerebellum formation (124). The presence of p.Met39Ile in *CASP9* as well as the presence of a family history of cancer and the in silico predictions suggesting suggest the involvement of *CASP9* in the susceptibility to recalcitrant warts in P2.

HPV regulate the cell cycle as a mechanisms to maintain the cell in the epigenetic and regulatory conditions necessary for constant cell division as well as the expression of viral proteins (122). Cyclins and cyclin-dependent kinases are important families of proteins that have been found in the control of the cell cycle, and also the replication, transcription, and translation (113). HPV also induces control over cyclins, arresting the cell cycle that promote oncotic activity and persistence of the expression of the viral genome (125). The presence of variants in *CCNA2* and *CCNB3* with high *in silico* mutational impact in cyclins

in two kindreds (Kindred 5 and 6) of our cohort suggests that this family of proteins and their regulatory activity in the cell cycle may be an interesting phenomenon in the study of the recalcitrance of warts.

GLTSCR2, also called *NOP53,* is a gene that codifies to a protein (GLTSCR2) composed by multiple nucleolar localization sequences (126). GLTSCR2 shows direct interactions with viral proteins, such as ICP22 and ICP0 of Herpes Simplex Virus and Ks-Bcl2 of Karposi's Sarcoma-associated Herpes Virus in in-vivo models (127, 128). These interactions enable GLTSCR2 to attenuate IFN-β which facilitates viral replication (114). In addition, GLTSCR2 participates in the p53 attenuation process in different neoplassic phenomena (129). In the context of HPV infections, GLTSCR2 expression is inversely proportional to the grade of the lesions in cervical lesions (130). the presence of the variant in *GLTSCR2* in P7, as well as its multiple relationships with the HPV infectious cycle make this variant of interest to us in the study of RW in our patients.

The cytoplasmic poly(A) binding protein 1 (*PABPC1*) is a coding gene to a protein called PABPC1 that promotes 60S ribosomal unit joining at the start codon (131, 132). In the context of viral infections PABPC1 is a common target in different viral cycles, and in the case of HPV infection, PABPC1 regulates of HPV RNA expression (116, 133). In addition, PABPC1 plays a crucial role in the HPV induced telomerase activation, for these reasons, the study of *PABPC1* p.Arg203* variant in P10 is an interesting variant in the study of the presence of RW (115).

The *CAD* is a coding gene that codifies a trifunctional protein called CAD (134). It has been observed that due to the crucial role of CAD in the synthesis of pyrimidines, this protein plays a crucial role in the process of hepatitis delta virus infection and Ebola virus

(135, 136). CAD$^{-/-}$ mice presents increased skin carcinogenesis in chemical induced stimuli (137). In the context of HPV infectious cycle, HPV-E6/E7 have the ability to amplify CAD sequences (138). The importance of CAD in the pyrimidine synthesis cycle, as well as the involvement in the HPV infectious cycle suggest that the variant may be of interest in the presence of HPV.

In our study we were able to find variants in genes that are related to the immune response against HPV, in addition, which by their relationship with the literature could generate possible etiologies for the presence of RW in patients, in our study we did not observe shared variants in the different patients with in-silico impact that could explain a possible relation phenotype-genotype.

In our study we did not perform determinations to calculate the ancestral components that characterize our cohort, but it has been shown that common warts are more common in Caucasian people (139). In addition to this we have found a possible autosomal dominant inheritance pattern in most families, as well as a family history of cancer in most families, for this reason we questioned whether it is possible that this genetic susceptibility to CRWs and the cancer history of the patients are being inherited by an ancestral component.

Only two patients present active infections at the moment (P1 and P11), the rest of the patients in the cohort resolved their lesions upon entering early adulthood (before the age of 20), this allows us to question whether it is possible that we are observing two possible patterns of presentation of RW, the first in which patients do not resolve the lesions and present a **"chronic recalcitrance"** and the second where there is a factor that appears in early adulthood that helps the resolution of the warts.

Considering this model, there may be hormonal factors that explain this phenomenon, such as variability in the production of sex hormones during puberty, which stabilize during early adulthood. In this context, it is possible that this mechanism is due to the activity of hormones such as estrogen, which has been shown to be an important immunomodulator (140). The possible interaction between HPV and estrogen has been most extensively studied in genital infections where a relationship between sex hormones and the development of HPV-positive cancer has been found, but the evidence is contradictory in some population groups (141). Taking this into account, the study of a possible interaction between hormones and immune response against HPV in skin may be of interest to understand this type of phenomena.

CONCLUSIONS

By doing the genetic and immunological characterization of this cohort, we help dissecting the genetic components of the innate and intrinsic immune response against HPV viral infections. This study describes the clinical phenotype of patients presenting with cutaneous RW in otherwise healthy young patients. Our results demonstrated that patients with RW in our cohort have an early age of symptom onset, with highly variable responses to treatments (like Diphencyprone), and that these kindreds present genetic variants that could be associated with the HPV infectious cycle and recalcitrance during the cutaneous RW. The presence of multiple affected individuals in different generations of the pedigrees, with no sex differences between affected individuals, suggests that susceptibility to cutaneous RW in our patients has a genetic component with an autosomal dominant inheritance pattern.

The persistence of HPV-related warts in our cohort of patients without obviously impaired immune and familial susceptibility to the development of warts, suggests the presence of genetic impaired immunity to cutaneous HPVs, supporting the idea of a possible inborn errors of immunity (IEIs). In addition, the clinical characteristics of our patients and their lesions, which do not assimilate to any of the genotypes previously reported in the literature, suggest that the susceptibility to RW of our patients is caused by an immunologic defect different from those previously described **(Figure 14).**

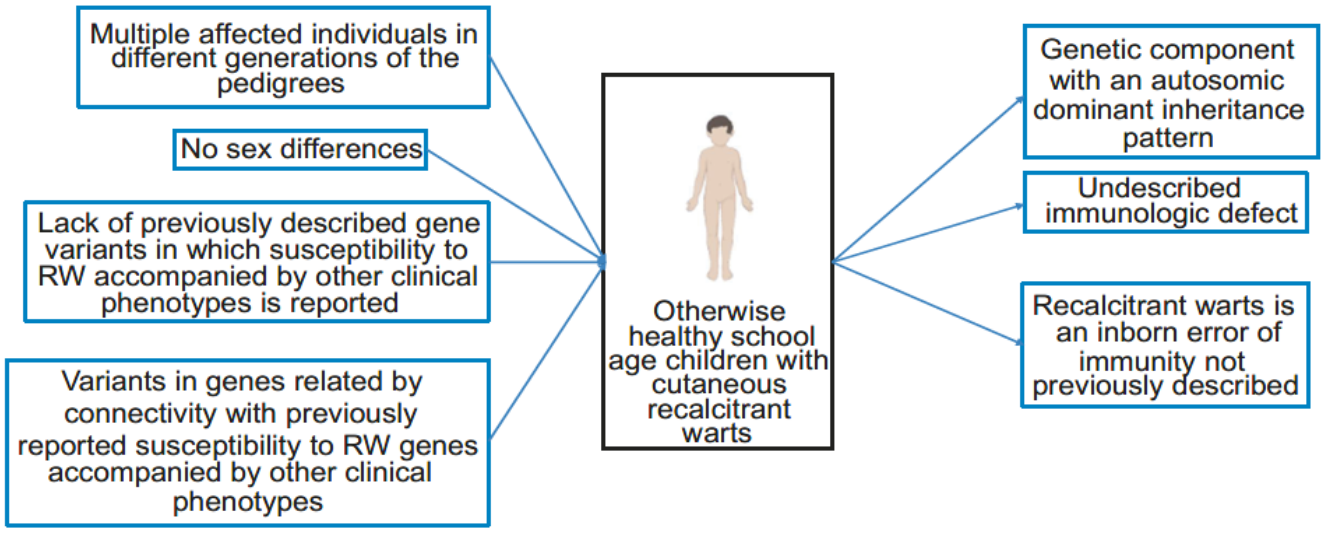


Figure 14. Schematic summary of conclusions. This summary shows the graphic representation of the conclusions obtained in this study based in the information of the patients, their clinical and genetic characterization.

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SUPPLEMENTARY MATERIALS.

CLINICAL IMMUNOLOGIC AND GENETIC CHARACTERIZATION OF COLOMBIAN PATIENTS WITH CUTANEOUS RECALCITRANT WARTS

Presented by:

Julian Rojas Silva, Microbiologist

Supplementary material 1

CUESTIONARIO PROYECTO EPIDERMODISPLASIA VERRUCIFORME

Nombre:

Edad:

Identificación:

Lugar de residencia:

Fecha de nacimiento:

Lugar de nacimiento:

Estado civil:

Nivel de educación

Ocupación:

Teléfono 1:

Teléfono 2:

Dirección:

EPS:

Acompañante:

Teléfono del acompañante

Por favor conteste con una X en los campos.

1. ¿Tiene manifestaciones clínicas de Epidermodisplasia Verruciforme (EV)? Sí ___ No ___

2. ¿Tiene manifestaciones clínicas de verrugas? Sí ___ No ___

3. ¿Edad de inicio de la enfermedad?

a. _____ Años

b. _____ Meses

4. ¿Edad del diagnóstico por el médico?

a. _____ Años

b. _____ Meses

5. Antecedentes Patológicos (Atopia):

a. Conjuntivitis Sí ___ No ___

b. Dermatitis Sí ___ No ___

c. Asma Sí ___ No ___

d. Rinitis Sí ___ No ___

e. Otra: ¿Cual(es)? _____

6. Antecedentes Patológicos (Enfermedades autoinmunes):

a. Artritis reumatoide Sí ___ No ___

b. Lupus Sí ___ No ___

c. Tiroiditis autoinmune Sí ___ No ___

d. Otra: ¿Cual(es)? _____

7. Antecedentes Farmacológicos:

a. ¿Toma medicamentos permanentemente (uso mayor a un mes)?

b. ¿Ha desarrollado reacciones alérgicas a medicamentos?

c. Sí ___ No ___ ¿A cuál medicamento?_____

8. ¿Ha sido usted fumador? Sí ___ No ___

a. ¿A qué edad comenzó a fumar? _____

b. ¿Cuántos cigarrillos fuma diariamente? _____

9. ¿Ha tenido otras enfermedades infecciosas de la piel?

a. Virales Sí ___ No ___ ¿Cuál(es)? _____

b. Fúngicas Sí ___ No ___ ¿Cuál(es)? _____

c. Parasitarias Sí ___ No ___ ¿Cuál(es)? _____

d. Bacterianas Sí ___ No ___ ¿Cuál(es)? _____

¿Alguna de estas enfermedades se presenta de manera repetida?

Si ___ No ___ Cuál? ____

¿Alguna de estas enfermedades ha requerido hospitalización? _____

10. ¿Tiene historia de enfermedades infecciosas en el sistema respiratorio?

a. Virales Sí ___ No ___ ¿Cuál(es)? _____

b. Fúngicas Sí ___ No ___ ¿Cuál(es)? _____

c. Parasitarias Sí ___ No ___ ¿Cuál(es)? _____

d. Bacterianas Sí ___ No ___ ¿Cuál(es)? _____

¿Alguna de estas enfermedades se presenta de manera repetida?

Si ___ No ___ Cuál? ____

¿Alguna de estas enfermedades ha requerido hospitalización? _____

11. ¿Tiene historia de enfermedades infecciosas en el tracto genitourinario?

a. Virales Sí ___ No ___ ¿Cuál(es)? _____

b. Fúngicas Sí ___ No ___ ¿Cuál(es)? _____

c. Parasitarias Sí ___ No ___ ¿Cuál(es)? _____

d. Bacterianas Sí ___ No ___ ¿Cuál(es)? _____

¿Alguna de estas enfermedades se presenta de manera repetida?

Si ___ No ___ Cuál? ____

¿Alguna de estas enfermedades ha requerido hospitalización? _____

12. ¿Tiene historia de enfermedades infecciosas en el tracto gastrointestinal?

a. Virales Sí ___ No ___ ¿Cuál(es)? _____

b. Fúngicas Sí ___ No ___ ¿Cuál(es)? _____

c. Parasitarias Sí ___ No ___ ¿Cuál(es)? _____

d. Bacterianas Sí ___ No ___ ¿Cuál(es)? _____

¿Alguna de estas enfermedades se presenta de manera repetida?

Si ___ No ___ Cuál? ____

¿Alguna de estas enfermedades ha requerido hospitalización? _____

13. ¿Tiene historia de enfermedades infecciosas en el sistema circulatorio?

a. Virales Sí ___ No ___ ¿Cuál(es)? _____

b. Fúngicas Sí ___ No ___ ¿Cuál(es)? _____

c. Parasitarias Sí ___ No ___ ¿Cuál(es)? _____

d. Bacterianas Sí ___ No ___ ¿Cuál(es)? _____

¿Alguna de estas enfermedades se presenta de manera repetida?

Si ___ No ___ Cuál? ____

¿Alguna de estas enfermedades ha requerido hospitalización? _____

14. ¿Tiene historia de enfermedades infecciosas en el sistema nervioso?

a. Virales Sí ___ No ___ ¿Cuál(es)? _____

b. Fúngicas Sí ___ No ___ ¿Cuál(es)? _____

c. Parasitarias Sí ___ No ___ ¿Cuál(es)? _____

d. Bacterianas Sí ___ No ___ ¿Cuál(es)? _____

¿Alguna de estas enfermedades se presenta de manera repetida?

Si ___ No ___ Cuál? ____

¿Alguna de estas enfermedades ha requerido hospitalización? _____

15. ¿Tiene historia de otro tipo de enfermedades infecciosas?

a. Virales Sí ___ No ___ ¿Cuál(es)? _____

b. Fúngicas Sí ___ No ___ ¿Cuál(es)? _____

c. Parasitarias Sí ___ No ___ ¿Cuál(es)? _____

d. Bacterianas Sí ___ No ___ ¿Cuál(es)? _____

¿Alguna de estas enfermedades se presenta de manera repetida?

Si ___ No ___ Cuál? ____

¿Alguna de estas enfermedades ha requerido hospitalización? _____

16. ¿Le han realizado colposcopias? Sí ___ No ___

17. ¿Le han diagnosticado cáncer de piel? Sí ___ No ___

a. ¿A qué edad? _____

b. Cáncer Basocelular _____ Cáncer escamocelular ___ Melanoma ____ No sabe ____

18. ¿Usted ha sufrido otros tipos de cáncer? Sí ___ No ___

¿Cuál(es)? _____

19. ¿En su familia alguien ha sufrido de cáncer? Sí ___ No ___

¿Qué tipo de cáncer? _____

Si su respuesta anterior fue sí, indique con una X qué parentesco tiene con el familiar:

Padre ___ Madre ___ Hermano(a) ___ Hijo(a) ___ Tío(a) ___

Primo(a) ___ Abuela(o) ___ Esposo(a) ___

Otros ____ ¿Cuál(es)?_____

20. ¿Ha tenido cáncer de cuello uterino? Si __ No__

21. ¿Tiene problemas de coagulación o sangra fácilmente? Sí ____ No ____ No sabe ___

a. Trombosis Sí ___ No ___

b. Embolismo Sí ___ No ___

c. Otros ¿Cuál(es)? _____

22. ¿Ha tenido algún aborto? Sí ___ No ___

En el caso en que su respuesta sea sí:

¿Cuántos? ____ ¿Por qué razones?_____

23. ¿Tiene historia de problemas cardiacos? Sí ___ No ___ ¿Cuál? _____

24. ¿Tiene o ha tenido problemas de audición? Sí ___ No ___ ¿Cuál? _____

25. ¿Le han hecho diagnóstico de Alzheimer o Parkinson u otras enfermedades neurodegenerativas?

Sí ___ No ___ ¿Cuál? _____

26. ¿Ha tenido dificultades para tener hijos? Sí ___ No ___ ¿Cuál? _____

27. ¿Le han hecho diagnóstico de Diabetes Mellitus o le han encontrado glicemias alteradas?

Sí ___ No ___

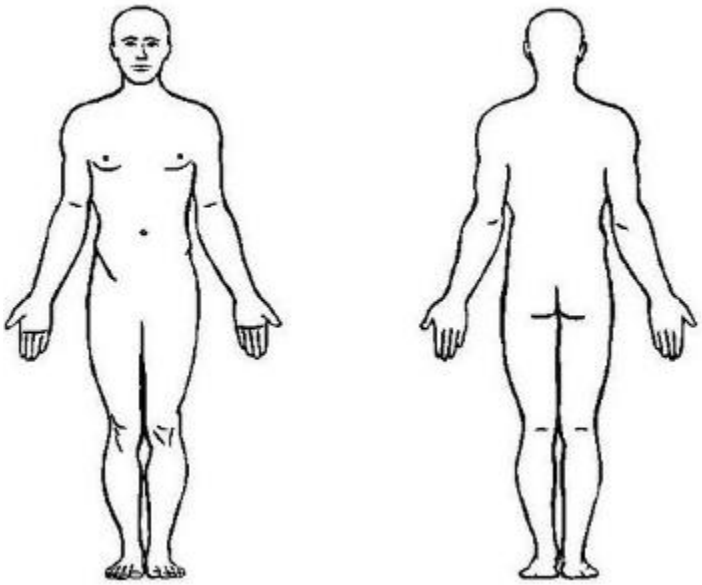
28. ¿Ha tenido alguna dificultad en el desarrollo psicomotor o en el aprendizaje?

Sí ___ No ___

29. ¿Ha recibido la vacuna contra virus del papiloma humano? Sí ___ No ___

¿Cuántas dosis? ____ ¿Hace cuánto? _____

30. Localización anatómica de las verrugas (Señale el lugar de su cuerpo donde presenta las lesiones)



31. ¿Ha presentado verrugas en otros lugares anteriormente? Sí ____ No ____

¿Donde? _____

Supplementary material 2

CUESTIONARIO NUMERO 2

(Re-entrevista)

PROYECTO VERRUGAS RECALCITRANTES

Fecha de aplicación de la encuesta _____

Quien recibe la llamada_____

Nombre:

Edad:

Identificación:

Lugar de residencia:

Fecha de nacimiento:

Lugar de nacimiento:

Correo electrónico:

Estado civil:

Nivel de educación:

Ocupación:

Teléfono 1:

Teléfono 2:

Dirección:

¿Presenta en este momento verrugas? Sí ___ No ___

Si no las presenta, ¿hace cuánto tiempo desaparecieron?_____

¿Desaparecieron de manera parcial o completa? _____

¿En qué parte(s) del cuerpo las presentó (presenta)?_____

¿Cuantas? _____

¿Ha tenido reaparición de las lesiones? ¿Con qué frecuencia? _____

¿Se encuentra en algún tratamiento médico para las verrugas? Sí ___ No ___

¿Cuál?_____

¿Se ha realizado algún tratamiento casero para las verrugas? Sí ___ No ___

¿Cuál?_____

¿Que otros tratamientos para verrugas ha recibido y en qué frecuencia? _____

¿Se encuentra en algún tratamiento (diferente) para la piel? Sí ___ No ___

¿Cuál?_____

¿Hace cuanto no se trata con difenciprona? _____

¿Por qué razón dejó el tratamiento? _____

¿Cuántas sesiones recibió de difenciprona antes de dejar el tratamiento?_____

¿Con qué frecuencia recibe-recibió el tratamiento? _____

Especifique si hubo alguna discontinuidad al recibir el tratamiento _____

¿Ha tenido alguna enfermedad durante o después del tratamiento? Sí ___ No ___

¿Cuál?_____

¿Consume algún suplemento vitamínico (homeopático o farmacéutico)? Sí ___ No ___

¿Cuál, desde cuándo y con qué frecuencia? _____

Fecha de la última citología _____

Resultado de la última citología _____

¿Se ha aplicado vacuna para el VPH? _____

¿Cuantas dosis de la vacuna ha recibido? _____

¿Actualmente, algún familiar presenta verrugas? Sí ___ No ___

Especifique qué familiar _____

¿En qué parte(s) del cuerpo las presentó (presenta)?

_____

¿Cuantas? _____

¿Ha tenido reaparición de las lesiones? ¿Con qué frecuencia?

_____

¿Se encuentra en algún tratamiento médico o casero para las verrugas? Sí ___ No ___

¿Cuál?_____

Supplementary material 3

```
import pandas as pd
# import openpyxl

#para crear tabla


#importar csv
datos=pd.read_csv('COLXXXX.V4.2.gnomGN_0.05.csv',header=0,low_memory=0)
print(datos)
#ans_tags.append(len(datos.index))

frec = 0.01
Dp = 5
#filtrar por frecuencias

filtro1=datos.loc[(datos['gnomADGenomes_AF'] <frec) |
(datos.gnomADGenomes_AF.isnull())]
print(filtro1)

# filtrado por GDI

filtro2=filtro1.loc[(filtro1['GDI'] <12.71) | (filtro1.GDI.isnull()) ]
print(filtro2)

# filtrado por MSC

filtro1=filtro2.loc[(filtro2['MSC_99%_Pred'] == 'HIGH') |
(filtro2['MSC_99%_Pred'] == None) | (filtro2['MSC_99%_Pred'] ==
"Unknown")]
print(filtro1)
#ans_tags.append(len(filtro1.index))

#Backlist
filtro2=filtro1.loc[filtro1['BL'] == 'no' ]
print(filtro2)
#ans_tags.append(len(filtro2.index))

#DP
filtro1=filtro2.loc[filtro2['DP'] > Dp ]
print(filtro1)
#ans_tags.append(len(filtro1.index))

filtro1.reset_index(drop=True, inplace=True)

filtro1.to_csv('COLXXXX.V4.2.gnomGN_0.01_Filtered.csv',header=True,index=F
alse)
```

Supplementary material 3. Code developed in house for the analysis of WES data. In the code you can see the data import, as well as the different modifiable parameters for the analysis of a patient's WES data.

Supplementary material 4.

Clinical Characteristics

Patient 1 (**P1**) is a female born in 2003 in the urban area of Medellín (Antioquia, Colombia, South America). She was born from the first uncomplicated pregnancy of apparently no consanguineous parents. She has 1 (one) younger affected brother. She shared the living space with domestic animals. She received all required vaccination by the Colombian Official immunization program (PAI) without complications. At the age of 8-years-old, she developed cutaneous warts lesions in the right hand, under the left thumb foot, and on the sole of the right foot. She reports having undergone multiple cauterization treatments with the prompt reappearance of the lesions, after several years of unsuccessful treatments she is referred to the dermatology unit of the Universidad de Antioquia where she started treatment with weekly doses of diphencyprone where she attended 36 weekly doses and abandoned the treatment due to lack of response (Table 1). The patient currently continues to have active lesions (Figure 2. A). The patient presents dermatitis in anatomical sites other than those affected by warts and rhinitis with constant medication with oral antihistamines. His medical history shows no autoimmune diseases, she does not smoke or live with smokers, and as for infectious diseases, he only reports mild, non-repetitive episodes of tonsillitis and laryngitis without requiring hospitalization. The patient has no apparent family history of consanguinity, but different family members have presented cancer without association with the presence of warts in these individuals (Table 2). The patient received 2 doses of tetravalent HPV vaccine without improvement of the lesions. The clinical characteristics of this patient are summarized in Table 1,2.

Patient 2 (**P2**) is a female born in 2006 in the urban area of Medellín (Antioquia, Colombia, South America). She was born from the first uncomplicated pregnancy of apparently no consanguineous parents. She has 1 dizygotic twin and an older half-sister. She doesn't share the living space with domestic animals. She received all required vaccination by the Colombian Official immunization program (PAI) without complications. At the age of 2-years-old, she developed cutaneous warts lesions in the region of the eyelids and on the palm of the left hand. She also reports having undergone multiple cauterization treatments with the prompt reappearance of the lesions, multiple cryotherapy sessions and after 5 years of unsuccessful treatments, she is referred to the dermatology unit of the Universidad de Antioquia where she started treatment with weekly doses of diphencyprone where he attended 52 weekly doses and abandoned the treatment due to economic factors that impeded him from continuing with therapy (Table 2). The patient currently has no active lesions. The patient does not report the presence of atopies or autoimmune diseases. She does not smoke but lives with his father who is a smoker, and as for infectious diseases, she only reports mild, non-repetitive episodes of bacterial urinary infections without requiring hospitalization. The patient has no apparent family history of consanguinity, but your father has liver cancer and non-melanoma skin cancer and the presence of warts on his hands (Table 2). The patient received 1 dose of tetravalent HPV vaccine without improvement of the lesions. The clinical characteristics of this patient are summarized in Tables 1-3.

Patient 3 (**P3**) is a male born in 2005 in the urban area of Medellín (Antioquia, Colombia, South America). He was born from the first uncomplicated pregnancy of apparently no

consanguineous parents. He has an unaffected younger sister. He doesn't share the living space with domestic animals. He received all required vaccination by the Colombian Official immunization program (PAI) without complications. At the age of 10-years-old, he developed cutaneous warts lesions in the region of the corner of the lips, both hands, and both soles. He also reports having undergone multiple cauterization treatments with the prompt reappearance of the lesions, multiple cryotherapy sessions and after 2 years of unsuccessful treatments, he is referred to the dermatology unit of the Universidad de Antioquia where she started treatment with weekly doses of diphencyprone where he attended 35 weekly doses and abandoned the treatment due to satisfactory response to treatment (Table 1). The patient currently has no active lesions.

The patient does not report or autoimmune diseases. She does not smoke or live with smokers, and as for infectious diseases, he has not reported episodes of infections in their childhood. The patient has no apparent family history of consanguinity and no family history of cancer (Table 2). The patient did not receive HPV vaccine doses. The clinical characteristics of this patient are summarized in Tables 1-2.

Patient 4 (**P4**) is a male born in 2007 in the urban area of Medellín (Antioquia, Colombia, South America). He was born from the first uncomplicated pregnancy of apparently no consanguineous parents. He has an older affected sibling. He shared the living space with domestic and farm animals. He received all required vaccination by the Colombian Official immunization program (PAI) without complications. At the age of 7-years-old, he developed periungual skin warts on both hands. He also reports having undergone multiple cauterization treatments with the prompt reappearance of the lesions, multiple cryotherapy sessions and after 3 years of unsuccessful treatments, he is referred to the

dermatology unit of the Universidad de Antioquia where she started treatment with weekly doses of diphencyprone where he attended 8 weekly doses and abandoned the treatment due to satisfactory response to treatment (Table 1). The patient currently has no active lesions. The patient reports rhinitis controlled with oral and inhaled antihistamines. His medical history shows no autoimmune diseases, he does not smoke or live with smokers, and as for infectious diseases, he has not reported episodes of infections in their childhood. The patient has no apparent family history of consanguinity, and his mother was diagnosed with cervical cancer and reports no warts (Table 2). The patient did not receive HPV vaccine doses. The clinical characteristics of this patient are summarized in Tables 1-2.

Patient 5 (**P5**) is a female born in 2003 in the urban area of Medellín (Antioquia, Colombia, South America). She was born from the first uncomplicated pregnancy of apparently no consanguineous parents. She has a younger affected half-sister. She shared the living space with domestic animals. She received all required vaccination by the Colombian Official immunization program (PAI) without complications. At the age of 7-years-old, she developed periungual cutaneous warts and cutaneous warts lesions in the right hand, right knee, and left elbow. She reports having undergone multiple cauterization treatments with the prompt reappearance of the lesions, after 7 years of unsuccessful treatments she is referred to the dermatology unit of the Universidad de Antioquia where she started treatment with weekly doses of diphencyprone where he attended 35 weekly doses and abandoned the treatment due to lack of response (Table 1). The patient currently has no active lesions (Figure 3. B). The patient does not report the presence of atopies. Her medical history shows no autoimmune diseases, she does not smoke or live

with smokers, and as for infectious diseases, she has not reported episodes of infections in their childhood. The patient has no apparent family history of consanguinity and no family history of cancer (Table 2). The patient has received 1 HPV vaccine dose without improvement of the lesions. The clinical characteristics of this patient are summarized in Tables 1-2.

Patient 6 (**P6**) is a male born in 2006 in the urban area of Medellín (Antioquia, Colombia, South America). He was born from the first uncomplicated pregnancy of apparently no consanguineous parents. He has an affected younger brother with a genetic diagnosis of tuberous sclerosis. He doesn't share the living space with domestic animals. He received all required vaccination by the Colombian Official immunization program (PAI) without complications. At the age of 7-years-old, he developed periungual skin warts on both hands, nose, soles, and right leg. He also reports having undergone multiple cauterization treatments with the prompt reappearance of the lesions, multiple cryotherapy sessions and after 4 years of unsuccessful treatments, he is referred to the dermatology unit of the Universidad de Antioquia where she started treatment with weekly doses of diphencyprone where he attended 16 weekly doses and abandoned the treatment due to satisfactory response to treatment (Table 1). The patient currently has no active lesions

The patient report asthma controlled with inhaled corticosteroids. His medical history shows no autoimmune diseases, he does not smoke or live with smokers, and as for infectious diseases, he reports helicobacter pylori intestinal infection without requiring hospitalization in their childhood. The patient has a history of consanguinity on the maternal side of the family. and his aunt was diagnosed with cervical cancer and reports

no warts (Table 2). The patient did not receive HPV vaccine doses. The clinical characteristics of this patient are summarized in Tables 1-2.

Patient 7 (**P7**) is a female born in 2006 in the urban area of Medellín (Antioquia, Colombia, South America). She was born from the first uncomplicated pregnancy of apparently no consanguineous parents. She has no siblings. She doesn't share the living space with domestic animals. She received all required vaccination by the Colombian Official immunization program (PAI) without complications. At the age of 9-years-old, she developed periungual cutaneous warts. She reports having undergone multiple cauterization treatments with the prompt reappearance of the lesions, after 2 years of unsuccessful treatments she is referred to the dermatology unit of the Universidad de Antioquia where she started treatment with weekly doses of diphencyprone where he attended 6 weekly doses and abandoned the treatment due to complete response (Table 1). The patient currently has no active lesions.

The patient does not report the presence of atopies. His medical history shows no autoimmune diseases, he does not smoke or live with smokers, and as for infectious diseases, she reports an episode of pneumonia of unknown etiology that required hospitalization for 4 days, with no recurrence. The patient has no apparent family history of consanguinity and presents a family history of cervical cancer in a maternal cousin with no association with warts (Table 2). The patient did not receive HPV vaccine doses. The clinical characteristics of this patient are summarized in Tables 1-2.

Patient 8 (**P8**) is a female born in 2006 in the urban area of Medellín (Antioquia, Colombia, South America). She was born from the first uncomplicated pregnancy of apparently no consanguineous parents. The patient has an unaffected half-sibling. She doesn't share

the living space with domestic animals. She received all required vaccination by the Colombian Official immunization program (PAI) without complications. At the age of 9-years-old, she developed periungual cutaneous warts. She reports having undergone multiple cauterization treatments with the prompt reappearance of the lesions, after 2 years of unsuccessful treatments she is referred to the dermatology unit of the Universidad de Antioquia where she started treatment with weekly doses of diphencyprone where he attended 18 weekly doses and abandoned the treatment due to complete response (Table 1). The patient currently has no active lesions.

The patient does not report the presence of atopies. Her medical history shows no autoimmune diseases, she does not smoke or live with smokers, and as for infectious diseases, she doesn't report any episodes of infectious diseases in childhood. The patient has no apparent family history of consanguinity and presents a family history of skin cancer in the maternal grandfather and stomach, breast, and cervical cancer in maternal aunts with no association with warts (Table 2). The patient did not receive HPV vaccine doses. The clinical characteristics of this patient are summarized in Tables 1-2.

Patient 9 (**P9**) is a female born in 2001 in the urban area of Medellín (Antioquia, Colombia, South America). She was born from the first uncomplicated pregnancy of apparently no consanguineous parents. She has an unaffected older brother. She doesn't share the living space with domestic animals. She received all required vaccination by the Colombian Official immunization program (PAI) without complications. At the age of 13-years-old, she developed periungual cutaneous warts. She reports having undergone multiple cauterization treatments with the prompt reappearance of the lesions, after 2 years of unsuccessful treatments she is referred to the dermatology unit of the

Universidad de Antioquia where she started treatment with weekly doses of diphencyprone where he attended 44 weekly doses and is currently continues under treatment with 2 weeks doses (Table 1). The patient currently has active lesions.

The patient does not report the presence of atopies. Her medical history shows no autoimmune diseases, she does not smoke or live with smokers, and as for infectious diseases, she doesn't report any episodes of infectious diseases in childhood. The patient has no apparent family history of consanguinity and presents a family history of cervical cancer in maternal grandmother with no association with warts (Table 2). The patient received 2 doses of tetravalent HPV vaccine without improvement of the lesions. The clinical characteristics of this patient are summarized in Tables 1-2.

Patient 10 (**P10**) is a male born in 2007 in the urban area of Cali (Valle del Cauca, Colombia, South America). He was born from the first uncomplicated pregnancy of apparently no consanguineous parents. He has four unaffected siblings. He doesn't share the living space with domestic animals. He received all required vaccination by the Colombian Official immunization program (PAI) without complications. At the age of 12-years-old, he developed cutaneous warts lesions in the region of the corner of the lips, face, and both hands. He also reports having undergone multiple cauterization treatments with the prompt reappearance of the lesions, multiple cryotherapy sessions and after 2 years of unsuccessful treatments he is referred to infectiology and he has been treated with HPV tetravalent vaccine without resolution of lesions (Table 1). The patient currently has no active lesions.

The patient does not report the presence of atopies. His medical history shows no autoimmune diseases, he does not smoke or live with smokers, and as for infectious

diseases, he has not reported episodes of infections in their childhood. The patient has no apparent family history of consanguinity and no family history of cancer (Table 2). The patient did not receive HPV vaccine doses in the Colombian PAI. The clinical characteristics of this patient are summarized in Tables 1-2.

Patient 11 (**P11**) is a male born in 1987 in the urban area of Medellín (Antioquia, Colombia, South America). He was born from the first uncomplicated pregnancy of apparently no consanguineous parents. He has an affected younger sister. He doesn't share the living space with domestic animals. He received all required vaccination by the Colombian Official immunization program (PAI) without complications. At the age of 14-years-old, he developed cutaneous warts lesions in the region of the corner of the lips, face, and both hands. He also reports having undergone multiple cauterization treatments with the prompt reappearance of the lesions, multiple cryotherapy sessions and after 18 years of unsuccessful treatments, he is referred to immunology without changes in treatment (Table 1). The patient currently has active lesions.

The patient does not report the presence of atopies. His medical history shows no autoimmune diseases, he does not smoke or live with smokers, and as for infectious diseases, he has not reported episodes of infections in their childhood. The patient has no apparent family history of consanguinity and no family history of cancer (Table 2). The patient did not receive HPV vaccine doses in the Colombian PAI. The clinical characteristics of this patient are summarized in Tables 1-2.

Supplementary figure 5.

Description	Relative value (%) of the patient	Average of the expected relative value for 10 to 16 years of life	Absolute value of the patient	Average of the expected absolute value for 10 to 16 years of life
Leukocytes	-	-	6985	6.000 (4.700-7.300) [a]
Total lymphocytes	31,5	46 (38-53) [a]	2200	2.400(1.400-4.200) [b]
CD3+ lymphocytes	71,3	68 (52-90) [b]	1569	1.600 (850-3.200) [b]
CD3+/CD4+ lymphocytes	45,9	36 (20-65) [b]	1010	900 (400-2.100) [b]
CD3+/CD8+ lymphocytes	20,8	24 (14-40) [b]	458	600 (300-1.300) [b]
CD3+/CD4+/CD8+ lymphocytes	1,55	0.13 (0.013-1) [b]	24	9.1 (3.2-26) [b]
CD16+/CD56+CD3- NK lymphocytes	8,0	14 (4-51) [b]	175	330 (92-1.200) [b]
CD19+ lymphocytes	13,9	13 (7-24) [b]	306	300 (120-740) [b]
CD19+/CD21+ lymphocytes	13,7	-	301	-
CD45+/CD14+ monocytes	10,4	4 [c]	726	-
CD4/CD8 relation	2,2	1.7 (0.9-3.4)		-

Supplementary figure 5. Leukocytes general subpopulation of P5. References values from [a] Hannet L. et al. 1992. Immunology Today, 13 (6): pp215-2. [b] Schatorjé EJH, *et al*. Pediatric reference values for the peripheral T cell compartment. Scand. J Immunol. 2012 vol. 75 (4) pp. 436-44, [c] Berrio M. *Et al*. El Hemograma: Análisis e interpretación con las tres generaciones. Ed. Universidad de Antioquia, pp 65. 2003.