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Identification of people with Lynch syndrome from those presenting with colorectal cancer in England: baseline analysis of the diagnostic pathway

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It is believed that >95% of people with Lynch syndrome (LS) remain undiagnosed. Within the National Health Service (NHS) in England, formal guidelines issued in 2017 state that all colorectal cancers (CRC) should be tested for DNA Mismatch Repair deficiency (dMMR). We used a comprehensive population-level national dataset to analyse implementation of the agreed diagnostic pathway at a baseline point 2 years post-publication of official guidelines. Using real-world data collected and curated by the National Cancer Registration and Analysis Service (NCRAS), we retrospectively followed up all people diagnosed with CRC in England in 2019. Nationwide laboratory diagnostic data incorporated somatic (tumour) testing for dMMR (via immunohistochemistry or microsatellite instability), somatic testing for *MLH1* promoter methylation and *BRAF* status, and constitutional (germline) testing of MMR genes. Only 44% of CRCs were screened for dMMR; these figures varied over four-fold with respect to geography. Of those CRCs identified as dMMR, only 51% underwent subsequent diagnostic testing. Overall, only 1.3% of patients with colorectal cancer had a germline MMR genetic test performed; up to 37% of these tests occurred outside of NICE guidelines. The low rates of molecular diagnostic testing in CRC support the premise that Lynch syndrome is underdiagnosed, with significant attrition at all stages of the testing pathway. Applying our methodology to subsequent years' data will allow ongoing monitoring and analysis of the impact of recent investment. If the diagnostic guidelines were fully implemented, we estimate that up to 700 additional people with LS could be identified each year.

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INTRODUCTION

At least 3% of cancers are attributable to constitutional (germline) pathogenic variants in a cancer susceptibility gene (CSG) [1]. Families harbouring these constitutional pathogenic variants were classically ascertained by clinical geneticists, based on familial clustering of related tumour types in several relatives, multiple primary tumours in some individuals, and tumour development at a younger age than typical for that cancer type. However, more widespread availability of molecular diagnostics has revealed other individuals who carry a similar genetic predisposition, but with a more subtle familial phenotype, or absence of a family history of similar cancers [2, 3]. Ascertainment has therefore been biased towards the classical familial pattern rather than the individual's own phenotype.

The Mismatch Repair (MMR) family of proteins is responsible for rectifying DNA replication errors that arise during the S-phase of the cell cycle. Germline pathogenic variants affecting any of the four MMR genes *MLH1*, *MSH2*, *MSH6* or *PMS2* underlie Lynch syndrome (LS), conferring a strong predisposition towards various cancers—predominantly colorectal and endometrial carcinoma, but also others including urothelial, ovarian, and upper gastrointestinal cancers, and sebaceous dermatological tumours [4]. Estimates of the true population prevalence of LS [5–7] indicate substantial underdiagnosis, hence NHS England's imperative to identify more cases. Outcomes for people diagnosed with LS could be improved by offering regular colonoscopy, aspirin and prophylactic gynaecological surgery, leading to reduced cancer incidence and earlier diagnosis. This could result in significant financial savings across the NHS [8], in addition to the primary objective of saving lives.

National Institute for Health and Care Excellence (NICE) guidelines (DG27) [9] issued in February 2017 state that all colorectal cancers (CRC) should be tested for MMR deficiency (dMMR) at the point of diagnosis, using either immunohistochemistry (IHC) or microsatellite instability (MSI) testing. Any tumours with evidence of dMMR should undergo further molecular tests, culminating in germline MMR gene testing for individuals at highest likelihood of having LS. In 2018, the charity Bowel Cancer UK initiated a Freedom

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of Information request [10] and campaign [11]—'Time To Test'—finding that MMR testing guidelines were being implemented by only 17% of hospitals in England, with cited barriers to testing including funding, staff capacity, awareness and local policy.

Whilst the diagnostic guidelines are clear, it is important to evaluate whether these are being consistently applied across the different NHS Cancer Alliances (regional healthcare partnerships that drive integration of local cancer services), and to highlight any inequities. This requires large scale, population-level collection and curation of molecular testing data, and robust linkage to cancer diagnoses. The National Disease Registration Service (NDRS) has developed a programme of work collating germline and somatic genetic testing data from NHS laboratories. By linking these data at patient- and tumour-level to national cancer registration records [12], we are, for the first time, able to describe the English national landscape of LS molecular diagnostic testing. The baseline data presented here refer to all colorectal cancers diagnosed in England in the year 2019, the first year for which national molecular data collections made this possible.

METHODS

Cancer registration

The National Cancer Registration and Analysis Service (NCRAS), part of NHS England, constructs the population-based cancer registry for England [12]. Somatic genomic testing data was derived from two sources: bespoke data extracts supplied by individual genomic laboratories, and pathology reports acquired through the nationally mandated Cancer Outcomes and Services Dataset (COSD). Laboratory germline data on MMR genes was submitted and processed via pseudonymisation and bioinformatics pipelines previously described [13], and linked at patient-level. Somatic data was linked at tumour-level. Where MMR testing was referenced in the initial pathology report, but there was no supplementary report containing the MMR test results, this was fed back to the relevant NHS Trust by the NCRAS Data Improvement Team, to maximise national data completeness.

Data analysis

From the 2019 end of year cancer registration table, 37,662 colorectal tumours (10th revision of the International Classification of Diseases (ICD-10) C18, C19 or C20) diagnosed in 2019 were identified. All tumours were linked to the genomic testing data up to the end of 2020 (latest available data at the time of writing).

From the cancer registry data, information on patients' demographics and tumour information was retrieved. Patients were assigned a Cancer Alliance based upon their postcode of residence at diagnosis, using the 2019 geographical boundaries. Age groups were banded from 10–29 years, then by 10-year intervals between 30–49 years, 5-year intervals between 50–89 years, then 90 years+.

Self-reported gender and ethnicity information is recorded in the cancer registration data from clinical records; ethnicity was categorised according to the 16-category classification as used in the 2021 Census of England and Wales. This was then collapsed to seven ethnic groups: White, Asian, Chinese, Black, Mixed, Other, and Unknown. Each patient's socioeconomic deprivation quintile was assigned using the patient's residential postcode at the time of diagnosis and based upon the quintile distribution of the lower-layer super output area (LSOA) ranking of the Indices of Multiple Deprivation (IMD) 2019, with 1 being the most deprived and 5 being the least deprived. Tumour stage is recorded according to the Union for International Cancer Control (UICC) Classification of Malignant Tumours (TNM). Colorectal cancer grading is recorded as 1 to 4, with 1 representing well differentiated cancer cells through to 4 when cancer cells are poorly differentiated or undifferentiated.

Descriptive statistics, chi-squared and *t*-tests, and logistic regression analyses were carried out using R software [14].

Ethical and legal considerations

The data included in this study were collected and analysed under the National Disease Registries Directions 2021 [15], made in accordance with sections 254(1) and 254(6) of the 2012 Health and Social Care Act.

Before embarking upon the collection of genetic data, we sought courtesy permission from the Caldicott Guardian at each NHS Trust housing the relevant laboratories.

Patient and public involvement

Author JB has been involved with the patient group Lynch Syndrome UK (LSUK) from when it was established as a charity in 2014, initially as the Clinical Director. JB, GMB, FEM, IMF and KJM have all presented work in progress to LSUK at their annual conference, and are in regular contact, receiving patient feedback.

RESULTS

Somatic testing

In 2019, 37,662 CRCs (from 37,090 people) were diagnosed in England. Under half of these (44%; 16,463) were tested for dMMR. IHC was the preferred test method in 89% of cases; the remainder were tested by MSI (8%) or by both methods (3%). The dMMR detection rates were slightly higher for MSI (19% detection rate) than for IHC (16% detection rate) ($\chi^2 = 12.0$; df = 1; $p < 0.01$).

To triage individuals for germline testing as per NICE guidelines, dMMR tumours can be further subdivided according to MLH1 status. Individuals whose tumours are proficient for MLH1, but abnormal for one or more of the other MMR proteins (MSH2, MSH6 or PMS2), should be offered direct referral for germline testing; tumours with MLH1 abnormality require further somatic tests.

Overall, 16% ($n = 2576$) of CRCs were dMMR. Of these, 15% ($n = 386$ tumours from 372 patients) were deficient in MSH2, MSH6 or PMS2 (but MLH1-proficient), so were eligible for germline testing; 121 of these patients (33%) received a germline test. The remaining 85% ($n = 2190$) CRCs were MLH1-deficient or MSI-High, indicating requirement for further somatic tests. Downstream testing was, however, performed on only 54% ($n = 1178$) of these, comprising 1041 tumours tested for *BRAF* mutational status and a further 137 tested for *MLH1* promoter hypermethylation in the absence of *BRAF* testing.

Of those MLH1-deficient CRCs tested for *BRAF*, 34% ($n = 356$) had a normal (i.e. wild-type) result, of which 63% ($n = 224$) were reflex tested for *MLH1* promoter hypermethylation, as per NICE guidance. An additional 52 *MLH1* promoter tests were performed following abnormal or failed *BRAF* results. Thus a total of 413 tumours were tested for *MLH1* promoter hypermethylation as part of the Lynch screening pathway, of which 138 tumours (33%), from 137 patients, were unmethylated, and therefore eligible for germline testing. Full testing pathways and results are shown in Fig. 1.

Variation in MMR testing

Table 1 shows numbers and percentages of people having MMR testing according to patient and tumour characteristics. Females had a slightly lower testing rate than males (42.8% vs. 44.5%). The lowest testing rates were found among persons of White (43.4%) or unknown (39.8%) ethnicity, whereas the highest testing rate was observed among Black persons (56.5%). Testing rates were highest among persons from the least deprived areas (45.8%) and lowest among those from the most deprived areas (40.8%). Higher testing rates were observed for tumours with stage II and III (52.1% and 52.6%, respectively) than for stage I (40%) and IV (41%) and tumours with unknown stage (27.9%). Similarly, higher testing rates were found among grade 2 (52.8%) and 3 (53.7%) tumours than grade 1, 4 and unknown grade tumours (32.8%, 27.5% and 15.8%, respectively). The most striking difference in MMR testing rates was according to Cancer Alliance, where tumour MMR testing rates varied from 17 to 71% (Fig. 2). When compared to the Cancer Alliance with the highest testing rate (West Yorkshire and Harrogate), and apart from the surrounding Cancer Alliances (Humber, Coast and Vale, and South Yorkshire and Bassetlaw), tumours diagnosed in all other Cancer Alliances were significantly less likely to be tested; more markedly so when adjusting for

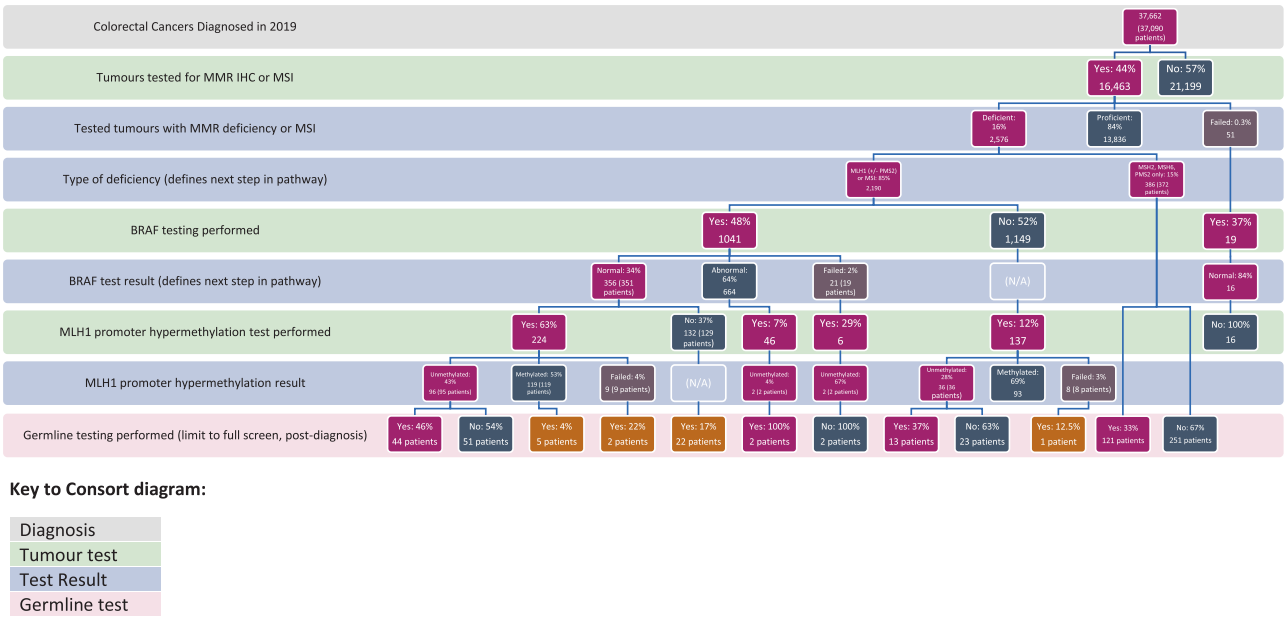

Fig. 1 Consort diagram showing Lynch syndrome testing pathway from cancer diagnosis to germline testing in 37,662 colorectal cancers (from 37,090 patients) diagnosed in England in 2019. For all levels of the Consort diagram, borderline results have been categorised as eligible to proceed to the next stage of the testing pathway, e.g. 'deficient' box in 'tested tumours with MMR deficiency or MSI' row includes both abnormal and borderline results; 'proficient' box includes normal results only; 'failed' box includes everything else (failed/not tested/unknown). Dark pink boxes represent the NICE DG27 'official' pathway to germline testing, defined as MMR deficiency with (in the case of MLH1 deficiency or MSI-High status), an unmethylated *MLH1* promoter. An unbroken line of pink boxes from top to bottom indicates the 'textbook' NICE-recommended pathway. Other pink boxes show paths to germline testing performed on samples that were incompletely tested, but were MLH1 deficient and unmethylated. Orange boxes indicate germline tests done under broader inclusion criteria, i.e. MLH1 deficiency with *BRAF* wild type but *MLH1* promoter methylated, failed testing, or untested. Dark grey boxes indicate either a lack of testing, or a test result that would signify a legitimate end to the testing pathway. Light brown boxes indicate failed tests.

demographic differences between Cancer Alliances. Full outcomes from the uni- and multivariable logistic regression analyses are shown in Supplementary Table 1.

Access to somatic follow up testing

Significant variation between Cancer Alliances was also observed when considering follow up of dMMR tumours (either germline testing for MSH2/MSH6/PMS2 deficient tumours, or further somatic testing for MLH1 deficient tumours). Performance of Cancer Alliances on follow up metrics did not necessarily correspond to their performance in arranging initial MMR testing (Supplementary Fig. 1).

Constitutional (germline) testing

Overall, 507 individuals with CRC were eligible for germline testing based on NICE guidelines—i.e. their tumours were either abnormal for MSH2/MSH6/PMS2 ($n = 372$) or abnormal for MLH1/MSI-High with no evidence of *MLH1* promoter methylation ($n = 135$). Of these 507 people, just 36% ($n = 180$) received a germline full screen test following their diagnosis. If eligibility for germline testing is instead based upon the NHS National Genomic Test Directory (indication R210) [16], this includes all patients whose MLH1-deficient/MSI-High tumours are *BRAF* wild-type (i.e. skipping *MLH1* promoter methylation testing). Adopting these broader eligibility criteria—i.e. at least one of *BRAF* wild type or failed, or *MLH1* promoter unmethylated or failed—786 people with CRC could have been offered a germline test. Of these 786 patients, 36 (5%) had either already received a germline test before diagnosis, or received a targeted germline test after diagnosis—i.e. they were members of families already known to genetics services. Thus 750 patients were, as a result of tumour molecular testing, newly identified as being eligible for germline testing, of whom only 210 (28%) actually received a germline test.

Of all 37,090 patients diagnosed with CRC in 2019, 487 (1.3%) received germline MMR testing (Table 2). Those tested could be split into four groups, depending on (1) the timing of the germline test with respect to the 2019 CRC diagnosis (pre- or post-diagnosis), and (2) the scope of the germline test (full screening of all MMR genes, versus targeted testing for a specific pathogenic variant in a member of a known LS family) (Table 2). This distinction is important, as it reflects how patients were ascertained, and thus what proportion were identified through the NICE-recommended tumour testing pathway, as opposed to being already known to clinical genetics services.

A minority of germline tests (56/487; 11%) were targeted tests; these are indicated when a specific pathogenic variant has previously been identified in a relative. Of these, germline testing preceded the 2019 CRC diagnosis (i.e. predictive/pre-symptomatic testing) in 30 (54%); the remaining 26 (46%) underwent confirmatory germline testing following their CRC diagnosis.

Forty-one people (8% of all tested) had full screen testing prior to their 2019 CRC diagnosis; this could either follow an earlier cancer diagnosis, or be a clinical genetics referral for 'indirect testing' where family history or personal polyp status was sufficiently strong to warrant variant-agnostic germline testing.

Three hundred and ninety out of 487 germline tests (80%) were full screen, post-diagnosis tests; this group represents newly-identified LS families, as opposed to those already known to genetics services. However, not all 390 tests were performed as per NICE or National Genomic Test Directory guidelines (Table 3). Even taking the more liberal eligibility criteria for germline testing, as outlined above [16], only 210 out of 390 (54%) followed recommended diagnostic pathways. The remainder comprised 45 people whose tumour records showed no evidence of dMMR testing, 74 with MMR proficient tumours, 53 with MLH1 deficiency/MSI-High status but no evidence of downstream somatic testing,

Table 1. MMR testing according to patient and tumour characteristics.

	MMR tested?				
	No (*n* | %)		Yes (*n* | %)		χ^2 *p* value
Total	21,199	56.3%	16,463	43.7%	
Age					<0.001
10–29	40	38.1%	65	61.9%	
30–39	228	31.1%	504	68.9%	
40–49	450	30.9%	1006	69.1%	
50–54	675	40.4%	995	59.6%	
55–59	1289	47.2%	1444	52.8%	
60–64	1965	49.7%	1986	50.3%	
65–69	2239	50.4%	2203	49.6%	
70–74	3472	54.2%	2935	45.8%	
75–79	3196	57.4%	2374	42.6%	
80–84	3501	64.8%	1904	35.2%	
85–89	2670	76.0%	842	24.0%	
90+	1474	87.8%	205	12.2%	
Gender					0.001
Female	9581	57.2%	7161	42.8%	
Male	11,618	55.5%	9302	44.5%	
Ethnicity					<0.001
Asian	363	43.8%	466	56.2%	
Black	261	43.5%	339	56.5%	
Chinese	46	48.9%	48	51.1%	
Mixed	79	52.3%	72	47.7%	
Other	250	52.1%	230	47.9%	
Unknown	1689	60.2%	1117	39.8%	
White	18,511	56.6%	14,191	43.4%	
Socioeconomic deprivation quintile					<0.001
1—Most deprived	3571	59.2%	2463	40.8%	
2	3753	55.6%	3000	44.4%	
3	4584	57.3%	3410	42.7%	
4	4720	55.9%	3720	44.1%	
5—Least deprived	4571	54.2%	3870	45.8%	
Cancer alliance					<0.001
Cheshire and Merseyside	1497	82.6%	315	17.4%	
East Midlands	1691	49.5%	1724	50.5%	
East of England—North	1399	63.8%	793	36.2%	
East of England—South	1255	54.1%	1065	45.9%	
Greater Manchester	1424	80.8%	338	19.2%	
Humber, Coast and Vale	332	31.2%	731	68.8%	
Kent and Medway	874	70.2%	371	29.8%	
Lancashire and South Cumbria	883	67.0%	435	33.0%	
North Central and East London	580	41.2%	829	58.8%	
North East and Cumbria	1561	66.7%	780	33.3%	
North West and South West London	1015	59.0%	706	41.0%	
Peninsula	877	60.4%	576	39.6%	
Somerset, Wiltshire, Avon and Gloucestershire	1353	63.1%	792	36.9%	
South East London	304	34.9%	566	65.1%	
South Yorkshire and Bassetlaw	324	31.7%	698	68.3%	
Surrey and Sussex	1423	61.2%	904	38.8%	
Thames Valley	688	44.2%	870	55.8%	
Wessex	877	46.3%	1016	53.7%	
West Midlands	2366	56.8%	1798	43.2%	
West Yorkshire and Harrogate	476	29.2%	1156	70.8%	

Table 1. continued

	MMR tested?				
	No (*n* | %)		**Yes (*n* | %)**		**χ^2 *p* value**
Tumour stage					<0.001
I	3614	60.0%	2413	40.0%	
II	3740	47.9%	4076	52.1%	
III	4555	47.4%	5045	52.6%	
IV	4314	59.0%	3000	41.0%	
Unknown	4976	72.1%	1929	27.9%	
Tumour grade					<0.001
1	868	67.2%	424	32.8%	
2	10,842	47.2%	12,117	52.8%	
3	2200	46.3%	2550	53.7%	
4	29	72.5%	11	27.5%	
Unknown	7260	84.2%	1361	15.8%	

Data shown as absolute numbers and proportions.

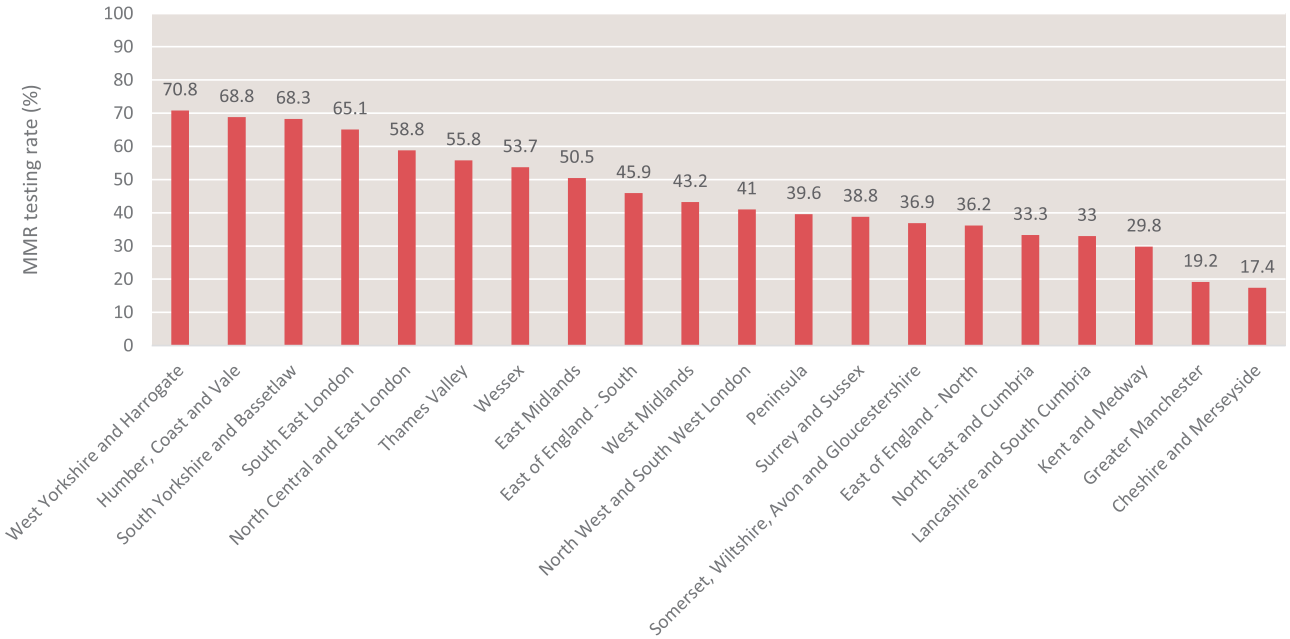

Fig. 2 Geographical variation in compliance with guidelines to test all CRCs for dMMR. Proportion of 2019-diagnosed colorectal cancers tested for dMMR, stratified by NHS England Cancer Alliance (using 2019 geographical boundaries and based upon patient postcode of residence at diagnosis).

Table 2. Number of germline MMR tests performed in 2019, split by test timing and scope.

		Timing of germline test, with respect to CRC diagnosis in 2019		
		Pre-diagnosis germline test	**Post-diagnosis germline test**	**Total**
Scope of germline test	Full screen test (Interrogates all MMR genes for an unknown variant)	41	390	431
	Targeted test (Looks for a specific MMR gene variant already known to segregate in family members)	30	26	56
	Total	71	416	487

Table 3. Full screen, post-diagnosis germline tests, split by route to testing (somatic test status), and outcome of the germline test.

Key: (a) Insufficient somatic testing performed; (b) germline testing not based on NICE guidelines; (c) NICE pathway followed correctly.

	Germline test result				
Somatic test status	**Total tested**	Normal	VUS (Class 3)	Pathogenic (Class 4/5)	**% pathogenic**
No MMR test done[a]	**45**	34	2	9	**20.0**
MMR tested — all genes proficient / MSS[b]	**74**	68	3	3	**4.1**
MMR tested — MSH2/MSH6/PMS2 deficient[c]	**121**	41	2	78	**64.5**
MLH1 deficient/MSI — no further somatic done[a]	**53**	31	4	18	**34.0**
MLH1 deficient/MSI & *BRAF* wt +/or *MLH1* unmeth[c]	**89**	63	1	25	**28.1**
MLH1 deficient/MSI & *BRAF* mut + *MLH1* meth (or 1 abnormal, the other untested)[b]	**8**	8	0	0	**0.0**
TOTAL	**390**	**245**	**12**	**133**	**34.1**

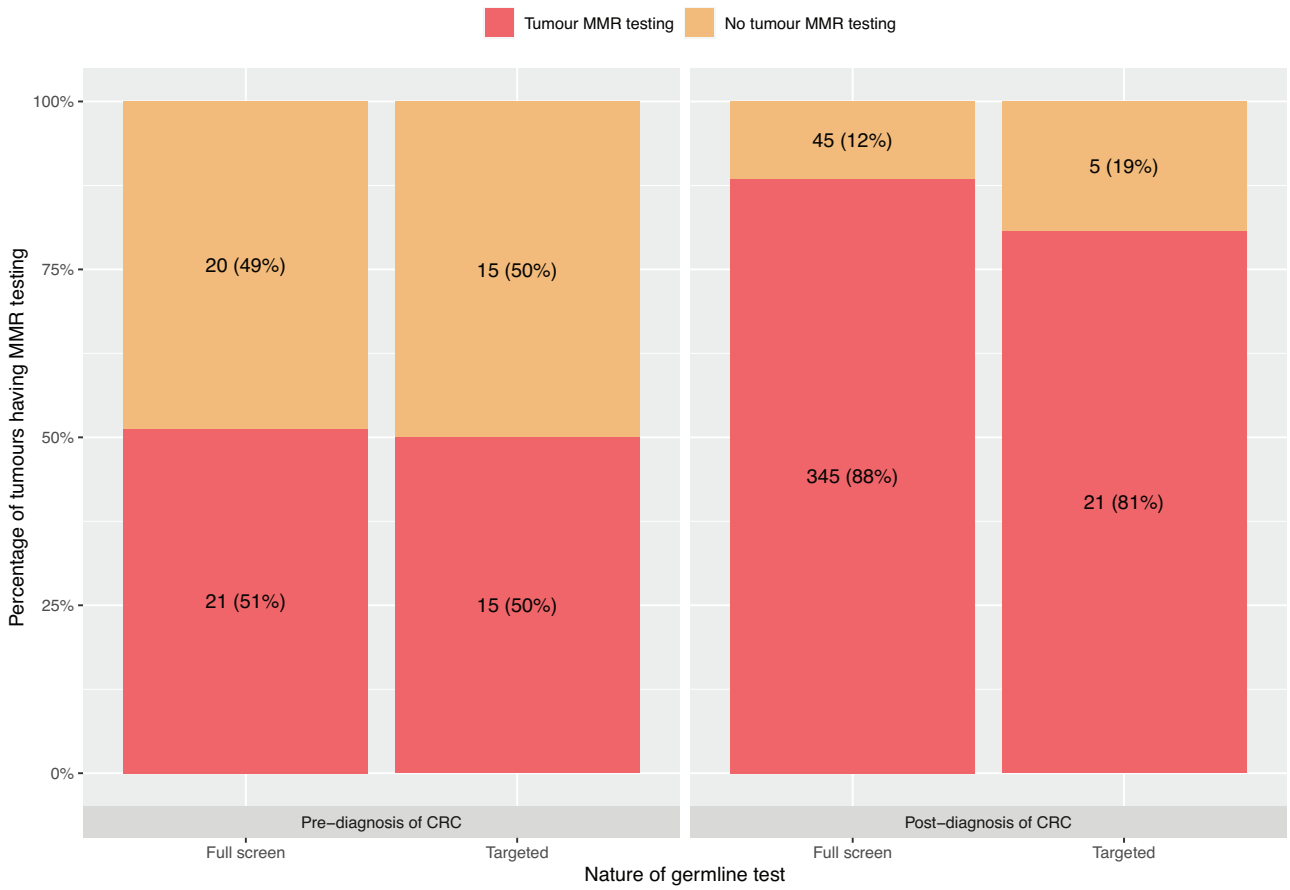

Fig. 3 Number and percent of tumours having dMMR testing, grouped by timing of patient's germline genetic test (pre- or post-2019 diagnosis of CRC) and scope of their germline test (full screen or targeted). Bars from L to R: full screen germline test performed pre-2019 cancer diagnosis; targeted germline test performed pre-2019 cancer diagnosis; full screen germline test performed post-2019 cancer diagnosis; targeted germline test performed post-2019 cancer diagnosis. Red bars signify that tumour dMMR testing has taken place; orange bars indicate no tumour dMMR test was performed.

and eight with MLH1 deficiency but mutant *BRAF*/*MLH1* promoter hypermethylation.

Thus of the total 390 full screen, post-diagnosis germline tests carried out, 210 patients (54%) were tested appropriately, 98 (25%) with no or insufficient somatic testing, and 82 (21%) following somatic results that did not indicate germline testing.

Overlap between somatic and germline testing

Individuals having a germline test post-diagnosis were significantly more likely ($\chi^2 = 58$; $p < 0.0001$) to have had MMR testing on their 2019-diagnosed tumour(s) (366/416; 88%) than those whose germline test had preceded their 2019 CRC diagnosis (36/71; 51%). The group most likely to have had MMR tumour testing were the full screen, post-diagnosis germline test group, at 88%) (Fig. 3).

Outcome of germline testing

A germline MMR pathogenic or likely pathogenic (P/LP) variant was reported in 206/487 (42%) people tested, comprising variants detected in 156/431 (36%) people undergoing full screen testing,

and 50/56 (89%) people undergoing targeted (familial) testing. Abnormal germline results were distributed between the four MMR genes and *EPCAM* as expected [4], with variants in *MLH1* and *MSH2* comprising 65% of cases, and *PMS2* just 15% (Table 4).

When full screen, post-diagnosis germline tests were stratified according to prior somatic testing status, variant detection rates ranged from 0–64%, (Table 3). A P/LP variant was detected in 103/210 (49%) of people whose tumour testing pathway followed NICE guidelines, in 27/98 (28%) of those where somatic testing was absent or incomplete, and in 3/82 (4%) of those where somatic testing results did not indicate germline testing (Table 3).

Of patients undergoing full screen, post-diagnosis germline testing, those with MMR-proficient (pMMR) tumours were significantly younger than those with dMMR tumours (mean 48.1 years vs. 56.8 years; Welch two sample *t*-test statistic = 4.29 (95% CI = 4.67–12.68, df = 111.78, $p = 0.001$).

Table 4. Mutated gene spectrum for all 2019-diagnosed colorectal cancer patients who had an abnormal germline Lynch test ($n = 206$; includes all germline test scopes and timings).

Gene	Number of patients with pathogenic/likely pathogenic variant, split by gene ($N = 206$)	Proportional distribution by gene of all patients with pathogenic/likely pathogenic variant (%)
MLH1	65	31.6
MSH2	68	33.0
MSH6	41	19.9
PMS2	31	15.0
EPCAM	1	0.5

Timeline of complete molecular diagnostic pathway for LS

The median time between CRC diagnosis and functional MMR testing (IHC and/or MSI) was 24 days (mean 58 days), with a further 34 days elapsing before follow up somatic testing, i.e. the total median time to complete somatic testing was 58 days (mean 129 days). The main diagnostic pathway delay occurred between somatic and germline testing, the latter being performed at median 315 days (mean 368 days) following initial CRC diagnosis. For all tests, there was a long right-hand tail in the distribution, indicating delays exceeding 1000 days for some individuals (Fig. 4).

DISCUSSION

This is the first comprehensive analysis of a policy to identify people with Lynch syndrome (LS) across a national healthcare system serving 55 million people. Despite being a snapshot in time, prior to coordinated expansion of testing [17], it provides a baseline for assessment of future developments, and is a likely reflection of underdiagnosis of this treatable disorder in other developed countries [18, 19].

In depth analysis of comparable populations suggests a LS birth prevalence of 1 in 280–1 in 500 [5–7], implying a population prevalence of one to two hundred thousand in England. Pooled data across clinical and laboratory genetics services indicates under 10% are known. A health economic analysis [8] indicated the clinical utility of testing all CRCs for dMMR; on this evidence, NICE introduced the current pathway in 2017 [9]. The rationale for identifying LS carriers is

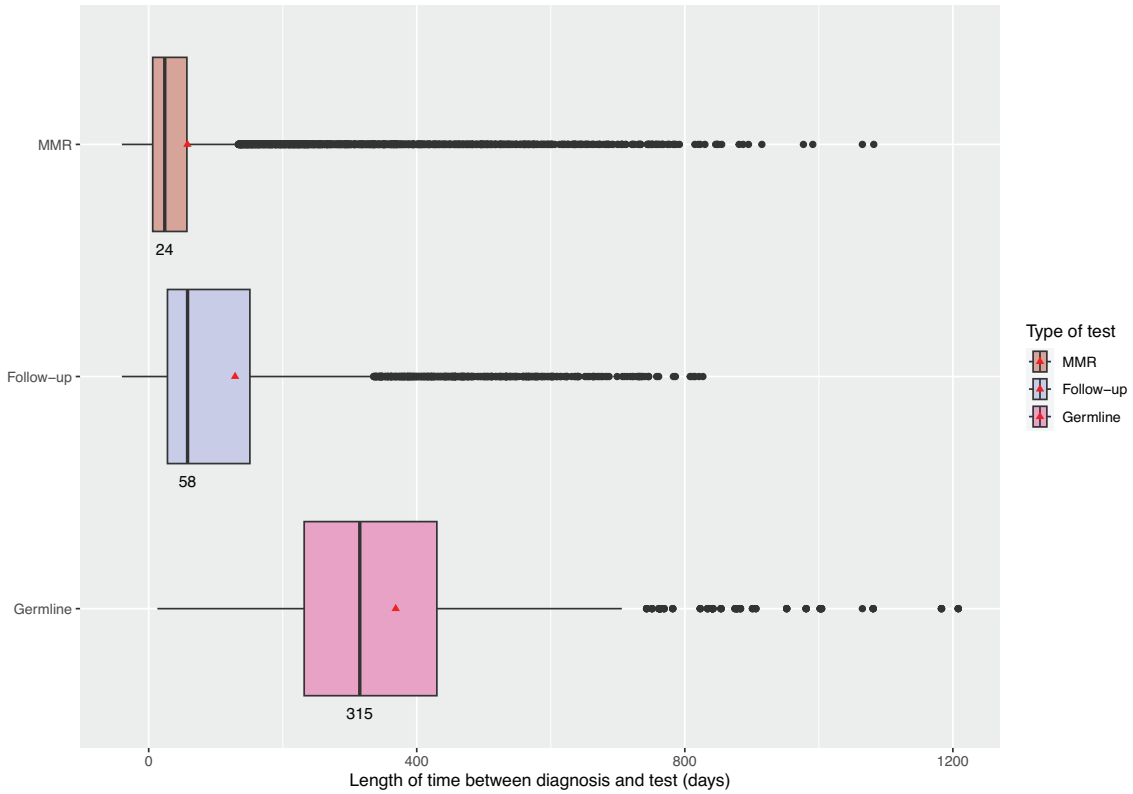

Fig. 4 Distribution and average time from initial diagnosis (at day 0) to functional testing (MMR IHC/MSI), subsequent follow-up (somatic *BRAF*/*MLH1* promoter methylation testing following an MMR test) and germline testing. Within each box, vertical black lines denote median values (enumerated below the box), and red triangles denote mean values; boxes extend from the 25th to the 75th percentile of each group's distribution of values and denote the interquartile range (IQR). Horizontal extending black lines denote adjacent values (i.e. the most extreme values within 1.5 x IQR of the 25th and 75th percentile of each group); black dots denote the observations outside the range of adjacent values (i.e. the outliers). Only full screen, post-diagnosis germline tests are included here (pre-diagnosis tests went back ~18 years).

further enhanced by the demonstration of a 50% reduction in their CRC incidence following daily aspirin [20] (now also a NICE guideline) [21], and the highly significant reduction in their non-CRC LS-associated cancer risk when prescribed dietary supplementation with resistant starch [22]. Identification of dMMR cancers as a target for immunotherapy [23–26] provides further justification for functional testing of all tumours, regardless of patient LS status.

The health economic benefit can be maximised by offering cascade testing to relatives to identify other at-risk carriers. Management guidelines for LS are gene-specific: colonoscopic surveillance should be offered at least every 2 years, starting from age 25 for carriers of pathogenic or likely pathogenic variants (PVs) in *MLH1* or *MSH2*, and from age 35 for those with PVs in *MSH6* or *PMS2* [27]. From 2023, colonoscopic surveillance of LS carriers will be incorporated into the NHS national bowel cancer screening programme.

Any guidelines, however good, are only beneficial if properly implemented. The Bowel Cancer UK investigation in 2018 indicated that only 17% of hospitals in England were following NICE recommendations for tumour MMR testing [10]; however this questionnaire-based investigation was limited in its design, potentially had a response-bias, and was set up to ask the question at hospital-level rather than patient-level. The current study is therefore the first national evaluation of MMR testing in England, covering the entire LS diagnostic pathway from initial tumour testing (IHC/MSI) through to germline testing, and is only possible due to the systematic collection, curation, and linkage of comprehensive NHS laboratory data within the National Disease Registration Service (NDRS).

Our data show that only 44% of 2019-diagnosed CRCs were tested for MMR status (IHC and/or MSI), and highlight large disparities in provision across England. There was more than a four-fold difference in MMR testing rates between the best- and worst-performing Cancer Alliance. Notably, the three best performing Cancer Alliances (West Yorkshire and Harrogate, South Yorkshire and Bassetlaw, and Humber, Coast and Vale) belong to the Yorkshire and Humber (YH) region, where, between April 2017 and March 2019, the Yorkshire Cancer Research Bowel Cancer Improvement Programme (YCR BCIP) funded pilot MMR screening for all CRC patients in the region who were not already covered by the previous inclusion criteria (<50 years of age) [28]. Although the YH pilot overlapped this NDRS evaluation only for the first 3 months of 2019, the region performed consistently well throughout the year, indicating the ongoing positive legacy of the YCR BCIP programme, and its implementation of suitable infrastructure, education, and co-ordination.

Of the 16% of tested tumours found to be dMMR, only 51% were followed up as per diagnostic guidance: 121/372 (33%) patients with MSH2/MSH6/PMS2 deficient tumours had germline testing, and 1178/2190 (54%) tumours with MLH1 deficiency or MSI-High status had further somatic testing. The latter facilitates distinction between sporadic (tumour-confined) dMMR versus potential constitutional dMMR underpinned by a germline pathogenic variant. As with initial MMR testing, the follow up of dMMR tumours was observed to vary significantly across Cancer Alliances.

There are some caveats here around data completeness, with potential gaps in *BRAF* data particularly affecting London and the Thames Valley region. Additionally, due to database challenges at genomics laboratories, we are missing a small number of germline MMR testing records from Great Ormond Street from December 2019 onwards, and from Bristol since the inception of their MMR testing service in summer 2019. Nevertheless, these gaps constitute a very small proportion of the overall national LS-related testing activity, and do not alter our overall conclusions. In 2019, 2 years after publication of the NICE guidance [9], MMR testing and appropriate follow up were generally poorly implemented, with major geographical inequities, substantial attrition from all levels of the testing pipeline, and very long time lags between initial functional MMR tumour testing and germline follow up. This long delay in germline testing limits the analysis that can be performed on more recently diagnosed tumours, as the data need time to mature with respect to the time period between diagnosis of cancer and genetic diagnosis of Lynch syndrome. It also evidences the need to develop and implement more efficient LS testing pathways, e.g. those co-ordinated via mainstream oncology services.

Where germline testing was performed, we observed a relatively high detection rate of pathogenic/likely pathogenic (P/LP) variants. Amongst full screen, post-diagnosis tests, the detection rate was 34.1%; this is somewhat higher than the 28% reported for all full screen MMR testing carried out in English labs since 2008 [13]. The difference probably reflects the biased nature of the 2019 CRC-diagnosed cohort, most of whose tumours had been pre-screened for dMMR. In contrast, most historical full screen germline testing would have been performed based on family history and/or young age of cancer development. Accordingly, by restricting the 2019 analysis to patients whose tumour-screening adhered properly to the NICE guidelines, the germline detection rate increased to 49%. Strikingly, a germline P/LP MMR variant was detected in 65% of patients whose tumours were abnormal for MSH2/MSH6/PMS2, indicating the clinical utility of this as a biomarker of LS.

Overall, 133 (65%) of the total 206 people with MMR germline P/LP variants were identified following a full screen, post-diagnosis germline test, i.e. represented new LS families not previously known to genetics services. This demonstrates the importance of the NICE-recommended tumour testing pathway in identifying new cases. Were the pathways to be implemented fully, both lives and health service resources could be saved [8, 29]. Based on extrapolations from all tumour and germline data, we estimate that, were NICE guidelines to be fully executed in all cases of CRC, up to 700 additional LS index cases (above this 2019 baseline) could be diagnosed per year; others could then be identified through familial testing.

Since the current reporting period of 2019 diagnoses, there has been more recognition of the importance of detecting LS, and a national transformation project is now underway [17]. This report provides a baseline for the anticipated improvement in LS detection. To facilitate comparison, and provide figures for subsequent reporting years beyond this baseline, we have made regional and national data available online at https://cancerstats.ndrs.nhs.uk/molecular/lynchsyndrome (requires an NHS network connection and login).

The national-scale collection, collation, curation and standardisation of these data by NDRS is the world's first example of linking cancer records with both germline and somatic molecular testing data in a real-world setting at population-level. Linkage of genomic data to the rich clinical phenotype, treatment and outcome data held within NDRS will enable the NHS to build up a comprehensive picture of genotype-phenotype correlations, facilitate genetic counselling of families with cancer, and monitor equity of access to molecular testing and targeted therapies. Through our collaboration with the UK Cancer Variant Interpretation Group (CanVIG-UK) [30], the datasets are also supporting national efforts to interpret germline variants of uncertain clinical significance (VUS).

CONCLUSION

The data presented here for 2019 diagnoses of colorectal cancer are the first of their kind to give a national picture of Lynch syndrome diagnostics across the entire cancer pathway, encompassing both germline and somatic testing. Only 44% of CRCs were screened for MMR deficiency; these figures varied over four-fold with respect to geography. These 2019 figures provide a

baseline level of tumour testing and indicate the level of underdiagnosis of LS at a point 2 years from when NICE recommended MMR testing in all colorectal cancers, but prior to the widespread disruption to NHS services caused by the SARS-CoV-2 pandemic. Now that the national data collection, processing, and analytical methodology is embedded within NDRS, it is possible to monitor improvements over time, and to benchmark the relative performance of individual NHS Trusts and Cancer Alliances.

DATA AVAILABILITY

Data are held within the National Disease Registration Service (NDRS), which is part of NHS England. Formal data requests may be made through the Data Access Request Service (DARS): https://digital.nhs.uk/services/data-access-request-service-dars.

CODE AVAILABILITY

Analytical code is available from the National Disease Registration Service (NDRS) upon reasonable request.

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AUTHOR CONTRIBUTIONS

Conceptualisation: FEM, JP, ML, GMB, CT, ACS, KJM, IMF, SH, and JB. Data curation: FEM, JP, FS, BS, OT, and SG. Formal analysis: FEM, JP, FS, BS, AT, OT, SG, ML, IMF, and SH. Funding acquisition: CT and JB. Investigation: FEM, JP, and SH. Methodology: FEM, JP, FS, BS, AT, OT, SG, and ML. Project administration: FEM and GMB. Resources: FS, BS, OT, and SG. Software: FS, BS, OT, and SG. Supervision: GMB, SH, and JB. Validation: FEM, JP, FS, BS, AT, SG, and ML. Visualisation: FEM, JP, AT, and ML. Writing—original draft: FEM, JP, ML, SH, and JB. Writing—review and editing: FEM, JP, FS, ML, GMB, CT, ACS, KJM, IMF, SH, and JB.

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COMPETING INTERESTS

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ETHICAL APPROVAL

Data used in this study were routinely collected by NDRS as part of NHS patient care and support. No human or animal subjects were used. Further ethical approval for this study was not required per the definition of research according to the UK Policy Framework for Health and Social Care Research.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41431-024-01550-w.

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Genes, Chromosomes and Cancer

WILEY

RESEARCH ARTICLE OPEN ACCESS

Hereditary Colorectal Cancer and Polyposis Syndromes Caused by Variants in Uncommon Genes

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ABSTRACT

A substantial number of hereditary colorectal cancer (CRC) and colonic polyposis cannot be explained by alteration in confirmed predisposition genes, such as mismatch repair (MMR) genes, *APC* and *MUTYH*. Recently, a certain number of potential predisposition genes have been suggested, involving each a small number of cases reported so far. Here, we describe the detection of rare variants in the *NTLH1*, *AXIN2*, *RNF43*, *BUB1*, and *TP53* genes in nine unrelated patients who were suspected for inherited CRC and/or colonic polyposis. Seven of them were classified as pathogenic or likely pathogenic variants (PV/LPV). Clinical manifestations of carriers were largely consistent with reported cases with, nevertheless, distinct characteristics. PV/LPV in these uncommon gene can be responsible for up to 2.7% of inherited CRC or colonic polyposis syndromes. Our findings provide supporting evidence for the role of these genes in cancer predisposition, and contribute to the determination of related cancer spectrum and cancer risk for carriers, allowing for the establishment of appropriate screening strategy and genetic counseling in affected families.

1 | Introduction

Hereditary colorectal cancer (CRC) and colonic polyposis are caused by different etiologies and associated with variable clinical phenotypes. Heterozygous pathogenic variants (PV) in mismatch repair (MMR) genes are the most frequent causes which are responsible for Lynch syndrome (LS) with deficient MMR (dMMR) tumor phenotype. Monoallelic PVs of *APC* and biallelic *MUTYH* inactivation are common causes for hereditary adenomatous polyposis. Less frequently, several other genes are involved, including *POLE/POLD1* for polymerase-proofreading-associated polyposis (PPAP) syndrome, *SMAD4* and *BMPR1A* for Juvenile polyposis syndrome, *STK11* for Peutz–Jeghers syndrome and *PTEN* for Cowden syndrome [1]. These confirmed cancer predisposition genes are routinely screened in patients with suspicion of gastrointestinal cancer syndromes following French recommendation [2]. However, genetic causes for a substantial proportion of cases still remain to be unveiled. Recent studies identified several potential susceptibility genes with growing evidence that strongly supports their role in hereditary CRC or colonic polyposis [1, 3]. Their related cancer risk needs to be evaluated with the accumulation of affected cases. Searching for germline inactivation of such genes presents thus important interests for the understanding of their roles and for genetic counseling of affected families.

In this report, we described the identification of germline variants in the *AXIN2*, *BUB1*, *NTHL1*, *RNF43*, and *TP53* genes in patient suspected for hereditary CRC or colonic polyposis. The *AXIN2* gene (Axis inhibition protein 2) is a component of Wnt-pathway in which it regulates the stability of β-catenin. The

BUB1 gene is a mitotic checkpoint protein kinase, playing a key role in mitotic spindle checkpoint. The *NTHL1* gene encodes Endonuclease III-like protein 1 and the *RNF43* gene encodes for Ring-finger protein 43 and is involved in the regulation of Wnt-pathway. Together with the *TP53* gene which plays a key role in cancer development, all these genes have been reported to be involved in CRC/polyposis susceptibility [1, 4].

2 | Materials and Methods

2.1 | Patients

Patients were identified through genetic consultation sessions and genetic testing was performed after the informed written consent was obtained. Patients suspected with predisposition to CRC or polyposis underwent germline variant screening using a 22-gene panel for digestive cancer predisposition (Table S1). Result assessment followed geneticists' prescription based on clinical indication, that is, either an oriented LS panel for suspected LS, or an expanded panel of 14 confirmed CRC predisposing genes for patient with proficient or unknown MMR tumor status, as well as for patients suspected of hereditary colonic polyposis. Eight potential CRC predisposition genes (called "research genes") were further explored, including *AXIN2*, *BUB1*, *GREM1*, *MSH3*, *NTHL1*, *RNF43*, *RPS20*, and *TP53* (Table S1). In total, 325 patients underwent an extensive genetic testing panel including all eight "research genes."

2.2 | Germline Variant Screening and Interpretation

Total genomic DNA was extracted from blood samples using automated STARlet platform (Hamilton Company, Reno, NV, USA). Next-generation sequencing (NGS) was performed using customized Agilent XTHS panel with capture-based target enrichment (Agilent, Santa Clara, CA, USA). Sequence alignment and variant calling were carried out using an in-house bioinformatics pipeline. Sanger sequencing was subsequently used for confirming variants of interest. The Human Genome Variation Society (HGVS) guidelines were used for variant nomenclature, with c.1 corresponding to the first nucleotide of the coding sequence (www.hgvs.org/varnomen). The sequence references are indicated in Table S2. Variant pathogenicity was determined using ACMG criteria [5]. General population data were referred to gnomAD database v.2.1.1 (European non-Finnish), and in silico prediction algorithms included Align GVGD [6], SIFT [7], Polyphen 2 [8], CADD V1.6 [9], SPIP [10], and SpliceAI [11].

2.3 | Somatic Variant Screening

Somatic analysis of the *RNF43* (NM_017763.5) gene was carried out using the method described previously [12].

3 | Results

Patients suspected with genetic predisposition to CRC or polyposis were screened for germline PVs using digestive cancer predisposition gene panel including 14 confirmed predisposition genes [2], as well as eight "research" genes which were previously reported to be involved in CRC susceptibility for further analysis in negative families. Among 880 patients tested between 2019 and 2023, 550 patients were tested by a LS-oriented panel with a diagnostic yield of pathogenic or likely pathogenic variant (PV/LPV) in MMR genes of 26.8% ($n = 149$) (Figure 1). For the remaining 325 patients with proficient or unknown tumor mismatch repair (p/uMMR) status, PV/LPV were detected in 28 patients (8.6%) in one of the confirmed CRC predisposing genes including MMR genes, *APC*, *MUTYH, BMPR1A*, *STK11*, and *POLD1* (Figure 1 and Table S2). In an attempt to find the genetic cause in a large proportion of negative patients with p/uMMR status, we further assessed "research" genes. In nine unrelated patients, rare variants were found in the *NTHL1* gene (two cases), the *AXIN2* gene (two cases), the *RNF43* gene (two cases), the *BUB1* gene (two cases), and the *TP53* gene (one case). These cases are described as follows and summarized in Table 1 and Figure 2.

3.1 | Cases With *NTHL1* Variant

Homozygous variant *NTHL1* c.268C>T, p.(Gln90*) was detected in probands of two families without familial consanguinity. This variant was previously reported as recurrent PV [13]. The proband of the Family 1 (Figure 2a) developed breast and endometrial cancers at 58 and 63 years old, in addition with more than 10 colonic adenomatous polyps. Two brothers were diagnosed, respectively, with a CRC at the age of 37 years and a pheochromocytoma at the age of 46 years. Her sister had a breast cancer at the age of 42 years. Her mother had colonic polyps with unknown number and histology. The variant was not carried by her cancer-free sister.

For the Family 2 (Figure 2b), the proband developed a meningioma at the age of 56 years and synchronous breast and endometrial cancers at the age of 68 years. Her father deceased from a CRC and her mother had a breast cancer. Three malignancies were diagnosed in her sibling: two sisters had respectively brain cancer at 18 and breast cancer at 62 and one brother had lung cancer at 59. Proband's mother and her cancer-free sister were both heterozygous carrier of the variant.

3.2 | Cases With *AXIN2* Variants

The proband of the Family 3 (Figure 2c) was diagnosed with a CRC at the age of 59 years associated with nine colonic adenomatous polyps. Genetic testing detected a heterozygous truncating variant in the *AXIN2* gene: c.2303_2306del, p.(Tyr768Phefs*13). In her family, three members (mother, one brother, and one niece) were diagnosed with CRCs at the age of 71, 50, and 56 years, respectively. Based on truncating nature of the variant and coherent family history, together with its absence in general population, we classified this variant as pathogenic.

For the Family 4 (Figure 2d), later-onset CRCs were diagnosed in the proband and two first-degree relatives in addition to adenomatous polyps found in the proband, his father, and six of his siblings with an age of 42 years for the earliest onset. The proband's tumor displayed MSS with normal expression of four MMR

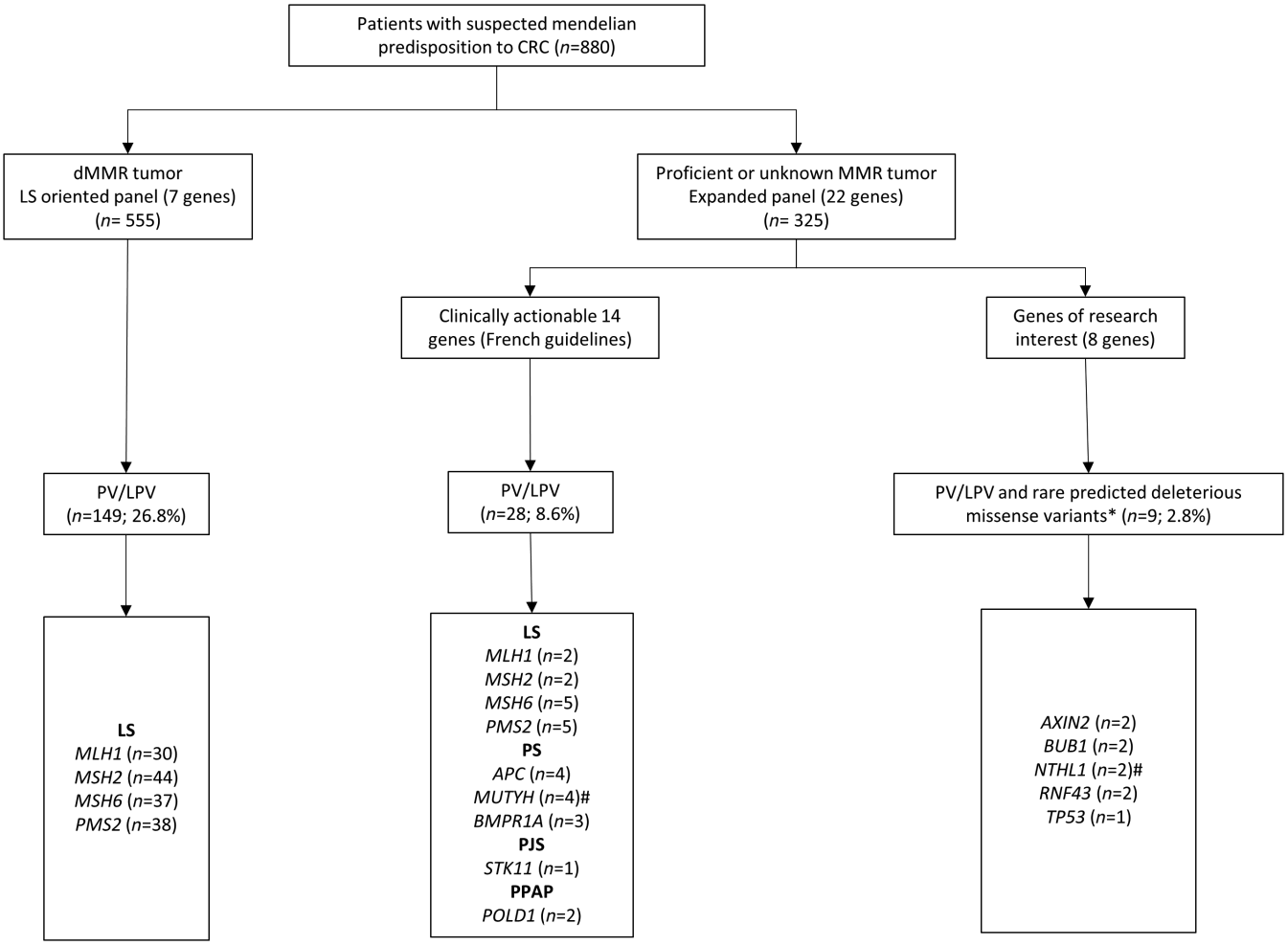

FIGURE 1 | Screening strategy for germline cancer susceptibility gene variants. Number sign (#) indicates homozygous carrier. Asterisk (*) indicates rare missense variant predicted to be deleterious with CADD Phred score ≥30. CRC, colorectal cancer; LS, Lynch syndrome; MMR, mismatch repair; PJS, Peutz–Jeghers Syndrome; PPAP, polymerase proofreading-associated polyposis; PS, polyposis syndromes; PV/LPV, pathogenic or likely pathogenic variant.

proteins. We found in this patient a heterozygous missense variant in the *AXIN2* gene: c.952A>G, p.(Ser318Gly). This variant is absent in the general population and is predicted to be deleterious by several in silico tools including Align GVGD (C55), SIFT, Polyphen 2, and CADD V1.6 (phred score = 31). Furthermore, this variant is predicted to alter splicing by the creation of a de novo splice donor site by both SPiP and SpliceAI software. Nevertheless, its pathogenicity could not be clearly determined at present until further analysis will be conducted, in particular on the transcription level. Co-segregation analysis in the family was also expected.

3.3 | Cases With *RNF43* Variants

The proband of the Family 5 (Figure 2e) was diagnosed with a CRC at the age of 48 years with a serrated polyp removed during his colonoscopy. Her mother developed synchronous colorectal and ovarian cancers at the age of 68 years. A heterozygous truncating variant in the *RNF43* gene was detected: c.394C>T, p.(Arg132*) and was classified as pathogenic, based on the interruption of protein synthesis, the absence in the general population, consistent clinical phenotype, and in addition, the loss of heterozygosity (LOH) of the wildtype allele revealed in the tumor (Figure S1).

For the Family 6 (Figure 2f), the proband was diagnosed with CRC associated with one serrated and three adenomatous polyps. The patient's tumor displayed MSS with normal expression of MMR proteins. A heterozygous missense variant in the *RNF43* gene was detected: c.655C>T p.(Arg219Cys). This variant is present with a low frequency (0.018%) in the European non-Finish population. It is predicted as deleterious by SIFT, Polyphen 2 and is highly scored by Align GVGD (C65) and CADD (phred score = 32) compatible with impaired function. Nevertheless, clinical and biological elements were still insufficient for pathogenicity determination, it remains as a variant of unknown significance (VUS). No co-segregation study was possible for this family as all affected members are deceased.

3.4 | Case With *TP53* Variant

The proband of the Family 7 (Figure 2g) developed a CRC associated with 20 adenomatous polyps at the age of 67 years. His

TABLE 1 | Summary of the clinicopathological characteristics of the patients carrying variants in recently proposed colorectal cancer susceptibility gene.

Nb. family	Gene	Variant	Proband's phenotype (age at diagnosis)	Phenotype in first- and second-degree relatives (age)	Variant classification
1	*NTHL1*[a]	c.268C>T, p.(Gln90*)	Br (58), En (63), >10 AP	Polyps, CRC (37), Br (42), pheochromocytoma (46)	5
2	*NTHL1*[a]	c.268C>T, p.(Gln90*)	Meningioma (56), Br (68), En (68)	Br (?), CRC (?), Br (62), brain (18), lung cancer (59)	5
3	*AXIN2*	c.2303_2306del, p.(Tyr768Phefs*13)	CRC (59), 9 AP	CRC (71), CRC (50), AP (41), CRC (56), 1 HP (36)	5
4	*AXIN2*	c.952A>G, p.(Ser318Gly)	CRC (69), 3 AP	CRC (80) + 17 AP, CRC (69), several polyps in the sibling	3
5	*RNF43*	c.394C>T, p.(Arg132*)	CRC (48), 1 SP (48)	CRC + OV (68)	5
6	*RNF43*	c.655C>T, p.(Arg219Cys)	CRC (48), 1 SP, 3 AP	GC (48), CRC (55)	3
7	*TP53*	c.845G>A, p.(Arg282Gln)	CRC (67), 20 AP	OV (43), gynecological cancer (?)	4
8	*BUB1*	c.2166G>A, (p.Trp722*)	15 AP (76)	CRC (67)	5
9	*BUB1*	c.625C>T, p.(Arg209*)	Chondrosarcoma (25), 15 AP (40–62)	CRC (?), Br (54), pancreas cancer (88)	5

Abbreviations: AP, adenomatous polyp(s); Br, tumors of breast; CRC, colorectum; En, endometrium; GC, stomach; HP, hyperplasic polyp(s); OV, ovary; SP, serrated polyp(s); (?), unknown age.
[a]Homozygous variant.

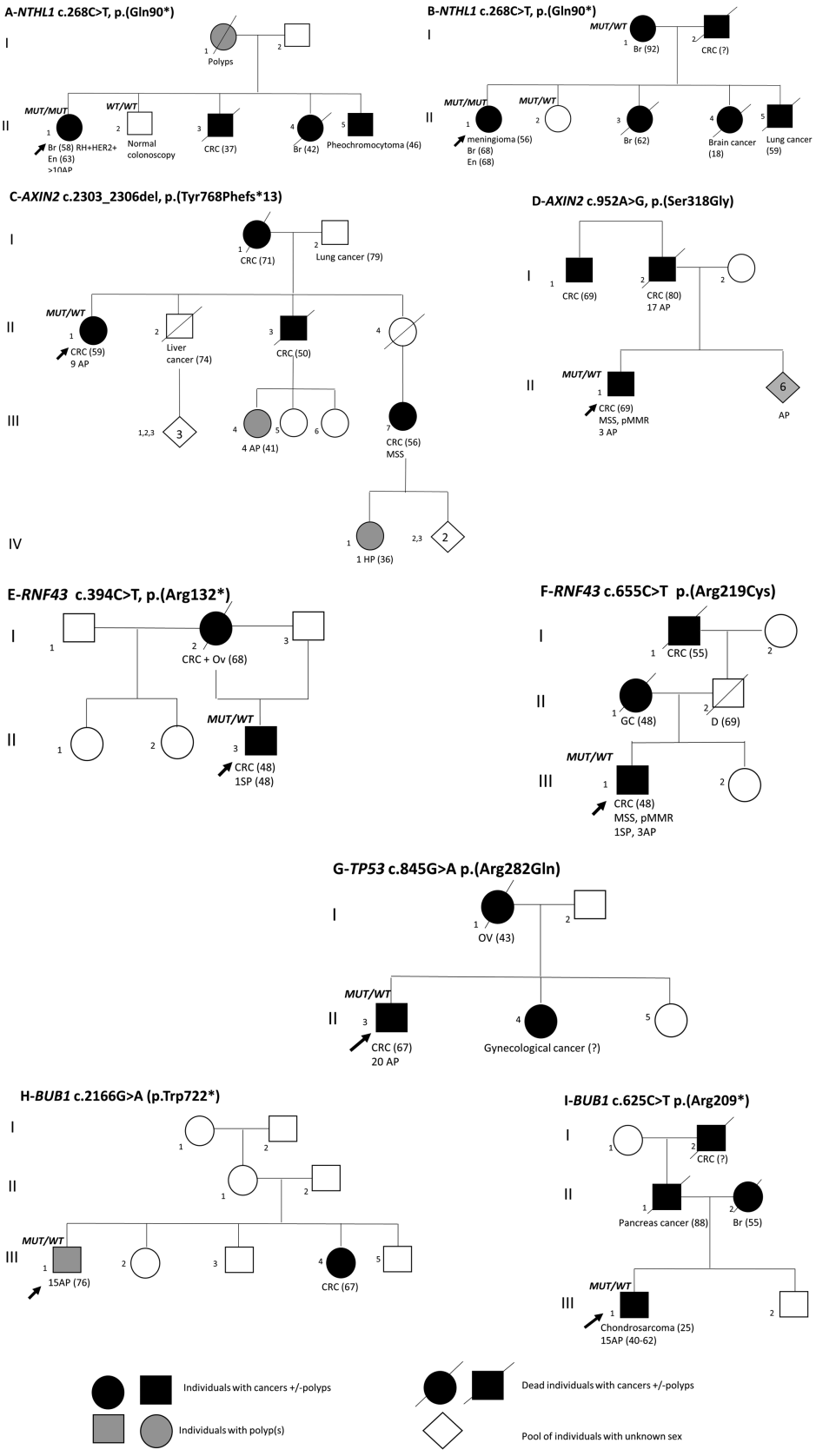

FIGURE 2 | Family 1–9 pedigrees. No consanguinity was reported. Black arrows indicate index cases. "MUT/MUT" and "MUT/WT" denote homozygous and heterozygous carriers, respectively. Age at diagnosis is indicated in brackets. AP, adenomatous polyp(s); Br, tumors of breast; CRC, colorectal; En, endometrium; GC, stomach; HP, hyperplasic polyp(s); MSS, microsatellite stable; OV, ovary; pMMR, proficient mismatch repair; SP, serrated polyp(s).

mother was diagnosed with ovarian cancer at the age of 43 years and his sister developed a gynecological cancer at an unknown age. A *TP53* gene variant was detected: c.845G>A p.(Arg-282Gln). This variant is absent in general population. It affects a highly conserved amino acid and is predicted as deleterious by SIFT and Polyphen 2. It is a hotspot somatic mutation in variable types of cancers [14]. Functional testing showed that it reduced transactivation activity [15]. On germline level, it was reported in families with childhood cancers or breast cancers compatible with Li–Fraumeni syndrome (LFS) with, interestingly, a CRC diagnosed in one of the carriers at the age of 47 years [16] [17]. Taken together, we classified it as likely pathogenic.

3.5 | Cases With *BUB1* Variants

The variant in the *BUB1* gene c.2166G>A p.(Trp722*) was identified in a patient from Family 8 (Figure 2h) who had 15 adenomatous polyps detected at the age of 76 years. His sister was diagnosed with CRC at the age of 67 years. Based on the truncating nature, the absence in the general population and consistent clinical manifestation, we classified it as pathogenic.

The proband of the Family 9 (Figure 2i) was diagnosed with a chondrosarcoma at the age of 25 years. He also had 15 colonic adenomatous polyps detected between the ages of 40 and 62 years. The father developed a pancreas cancer at the age of 88 years, and the mother was diagnosed with a breast cancer at the age of 55 years. The paternal grandfather had a colon cancer at an unknown age. A truncating variant of the *BUB1* gene: c.625C>T p.(Arg209*) was identified in the patient. The clinical feature, biological consequence, and a very low prevalence in the general population (0.004% in European non-Finish population) lead us to classify this variant as pathogenic.

4 | Discussion

We reported here nine variants detected in five uncommon genes: *NTHL1*, *AXIN2*, *RNF43*, *BUB1*, and *TP53* in patients suspected for hereditary CRCs and polyposis, among which seven were considered as PV/LPV. The pathogenicity of two other rare variants were not able to be determined although they were predicted to be deleterious or spliceogenic by in silico algorithms. Further investigations are needed for their classification. Co-segregation study presents an important interest in determining the role of such uncommon cancer predisposition genes in familial cancer syndromes and in establishing appropriate clinical surveillance for variant carriers. Unfortunately, it was not able to be carried out in these families partly because of their small size with few members affected with relevant cancers for testing. It will certainly be complemented when it is possible.

All five genes have been previously reported to be associated to hereditary CRC and/or polyposis susceptibility with apparently variable clinical or biological characteristics. *NTHL1* PVs cause recessively inherited multitumor syndrome [13, 18, 19]. So far, 50 biallelic PV carriers have been reported [20, 21] and many of them carried homozygous c.268C>T variant. To note, 0.38% of general European non-Finnish population are monoallelic carriers of this variant. Biallelic carriers developed mainly CRC, colonic polyps, breast cancer and less frequently, and endometrial cancer. A number of other cancers with lower frequency were reported. Regarding two female biallelic c.268C>T carriers in our series, both developed multiple-primary cancers. Consistent with reported cases, breast and endometrial cancers were diagnosed in both but at a later age (58 and 68 for breast and 63 and 68 for endometrial cancers) and one had more than 10 colonic polyps. One carrier had a meningioma which was one of the rare manifestations already found in *NTHL1* PV biallelic carriers [20]. Our findings, together with previous reports, suggested that female carriers have higher risk to develop breast and endometrial cancers, even in an advanced age. Other *NTHL1*-associated cancers were found in family numbers including early onset of CRC in male patient and early onset breast cancer in female patients as well as brain cancer but unfortunately their carrier status was not able to be confirmed. To note, a pheochromocytoma at the age of 46 years and a lung cancer at the age of 59 years were diagnosed in family numbers which were not described previously in *NTHL1* PV carriers.

Monoallelic germline PVs in the *AXIN2* gene were reported in less than 20 patients worldwide [22]. All were truncating variants leading to the synthesis of a protein lacking C-terminal functional disheveled and axin (DIX) domain and subjected to the degradation by nonsense mediated mRNA decay (NMD). The PV identified in the Family 3 was located within the DIX domain (Exon 10) thus impact doubtlessly protein function. On the contrary, the consequence on protein synthesis/function of the missense variant found in the Family 4 (Exon 3) required further investigation especially through splicing defect predicted by in silico algorithms. Reported *AXIN2* PV carriers manifested predominantly CRCs and colonic adenomatous polyposis. Extracolonic cancers seemed to be rare. Such observations were consistent with our finding in two families in which only colon cancer and polyps were diagnosed in affected members. It is reported that patients carrying *AXIN2* PV often present dental anomalies such as anodontia or oligodontia [22]. Unfortunately, no information about dental examination was available for carriers of these two families.

Monoallelic germline *RNF43* PVs are associated with inherited serrated polyposis syndrome [23–25]. To date, only seven PVs were reported in the literature involving 10 families including the Family 5 from this study. The variant c.394C>T, p.(Arg132*) identified in this family was likely recurrent since it was identified in three unrelated families. *RNF43* PV carriers commonly had serrated colorectal polyps susceptible to malignant transformation. Our carrier was diagnosed with a CRC at 48 with the detection of one serrated polyp, but not fulfilling clinical diagnosis of serrated polyposis syndrome. Serrated polyp was also detected in the patient carrying *RNF43* variant (VUS) c.655C>T, constituting a supporting element in its interpretation, although a definitive classification was not able to establish at present according to ACMG criteria.

It is well known that germline *TP53* PVs cause LFS, an aggressive condition predisposing carrier to different malignancies especially sarcomas, brain tumor, and breast cancer at young ages. CRC was not conventionally considered as a component of LFS tumor spectrum. However, recent study from Terradas

et al. reported the detection of *TP53* loss-of-function alterations in CRC patients with proficient tumor MMR (pMMR). Together with other similar findings, the role of *TP53* as a CRC predisposition gene was suggested which may be independent of LFS [26]. The novel *TP53* LPV found in this study provided an additional case supporting this hypothesis. However, the presence of >20 adenomatous polyps in our patient seemed to be uncommon which was described in only one family member of a carrier [26].

Germline monoallelic *BUB1* PVs were reportedly associated with hereditary early onset of CRC [4]. To date, a total of eight *BUB1* variants were reported in CRC patients, with only four being considered as functionally deleterious [27, 28]. Carriers manifested mainly early onset of CRC and colonic adenomatous polyps. We report here two additional carriers of *BUB1* truncating variants with colonic polyps (>15 for both) as predominant phenotype. However, the fact that carriers in our series had only colonic polyps detected at a later age seem to suggest that *BUB1* PVs predispose carriers rather to the development of polyps than to early-onset CRC. To note, one of the carriers developed a chondrosarcoma at the age of 25 years, but whether this tumor was in relation to *BUB1* inactivation could not be determined.

Apparently, these genes are responsible, each, for a small subgroup of patients with inherited pMMR colon cancer and polyposis. The prevalence of PV/LPV was shown to be low according to large series studies: 0.2% (8/3936) for *NTHL1* [13], 0.24% (8/3322) for *AXIN2* [22], 0.21% (1/473) for *TP53* [26], 0.14% (combined studies) for *BUB1* [27], as well as 1.3% (1/73) for *RNF43* in selected patients with serrated polyposis syndrome [29]. In our study, in 325 patients with pMMR or uMMR tumor status, the prevalence of PV/LPV in these genes were: 0.6% (2/325) for *NTLH1*, 0.3% (1/325) for *AXIN2*, 0.6% (2/325) for *BUB1*, 0.3% (1/325) for *TP53*, as well as 0.3% (1/325) in *RNF43*. Taken together, including two potential deleterious rare missense variants, these genes which were actually not considered as confirmed diagnostic predisposition genes and were not systematically screened were potentially implicated in 2.7% (9/325) of cases associated with (p/uMMR) tumor status. Thus, it seems important to include these genes in routine examination, possibly as a second intention when the investigation in diagnostic genes was negative, in particular for patients with pMMR tumor phenotype. Doubtlessly, accumulation of such rare cases is essential for evaluating precise related cancer risks for each of these genes in order to propose adapted clinical surveillance for affected families.

Limitations in this study included incomplete clinical data in some affected families. The inaccessibility to samples of affected family members hampered co-segregation analysis. Also, functional studies, especially spliceogenicity evaluation of two missense variants remain to be carried out in order to determine the pathogenicity.

In summary, our findings provided novel evidence showing that besides major confirmed digestive tract cancer predisposition genes, a number of other genes were shown to be involved in inherited CRC and polyposis susceptibility, although impacting each a small number of patients. Our observations further confirmed an etiological diversity for inherited CRC and polyposis. Indeed, additional genes are emerging with potential susceptibility to CRC/colonic polyps, such as *MBD4* [30]. It is still difficult to make a clear genetic/phenotype correlation, but data from described cases in the literature and this study appeared to be consistent, providing clues for the understanding of gene-related distinct cancer syndromes. We believe that a systematic screening in these uncommon genes should be recommended, allowing for the collection of related clinical and biologic characteristics, necessary for establishing propriate surveillance programs for carriers and their family.

Author Contributions

A.B. and Q.W. were responsible for designing the study, supervising the research, variant interpretation as well as the edition of the manuscript. A.F., H.Z., S.H., F.D., C.K., F.P., M.P., C.L., L.C., and J.-C.S. are the geneticists or genetic counselors who identified and consulted the probands. All authors approved the submitted version.

Acknowledgments

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Ethics Statement

Written informed consent was obtained for all patients who were tested and diagnosed within the frame of genetic counseling, in accordance with French law for diagnostic genetic testing. Samples were collected in the frame of care, from patients who consented to a research use of their samples. Testing was done in a hospital laboratory approved for genetic molecular diagnosis. The analyses were performed in accordance with French regulations and the principles of the Declaration of Helsinki.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets generated and/or analyzed during the current study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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RESEARCH

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Epidemiological characterization of rare diseases in Brazil: A retrospective study of the Brazilian Rare Diseases Network

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Abstract

Background The Brazilian Policy for Comprehensive Care for People with Rare Diseases was implemented in 2014; however, national epidemiological data on rare diseases (RDs) are scarce and mainly focused on specific disorders. To address this gap, University Hospitals, Reference Services for Neonatal Screening, and Reference Services for Rare Diseases, all of which are public health institutions, established the Brazilian Rare Diseases Network (RARAS) in 2020. The objective of this study was to perform a comprehensive nationwide epidemiological investigation of individuals with RDs in Brazil. This retrospective survey collected data from patients receiving care in 34 healthcare facilities affiliated with RARAS in 2018 and 2019.

Results The survey included 12,530 participants with a median age of 15.0 years, with women representing 50.5% of the cohort. Classification according to skin color demonstrated that 5044 (47.4%) participants were admixed. Most

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had a confirmed diagnosis (63.2%), with a predominance of phenylketonuria (PKU), cystic fibrosis (CF), and acromegaly. Common clinical manifestations included global developmental delay and seizures. The average duration of the diagnostic odyssey was 5.4 years (± 7.9 years). Among the confirmed diagnoses, 52.2% were etiological (biochemical: 42.5%; molecular: 30.9%), while 47.8% were clinical. Prenatal diagnoses accounted for 1.2%. Familial recurrence and consanguinity rates were 21.6% and 6.4%, respectively. Mainstay treatments included drug therapy (55.0%) and rehabilitation (15.6%). The Public Health System funded most diagnoses (84.2%) and treatments (86.7%). Hospitalizations were reported in 44.5% of cases, and the mortality rate was 1.5%, primarily due to motor neuron disease and CF.

Conclusion This study marks a pioneering national-level data collection effort for rare diseases in Brazil, offering novel insights to advance the understanding, management, and resource allocation for RDs. It unveils an average diagnostic odyssey of 5.4 years and a higher prevalence of PKU and CF, possibly associated with the specialized services network, which included newborn screening services.

Keywords Rare diseases , Public Health System, Brazil, Brazilian Rare Diseases Network

Introduction

Rare diseases (RDs) are individually rare but collectively affect a significant proportion of the population. Approximately 71.9% of RDs have a genetic cause, and there are over 6000 known RDs [1]. They represent a serious public health problem with major unmet needs since many are life-limiting or chronically debilitating. Patients and families with RDs often face long diagnostic journeys, while healthcare professionals struggle with identifying, managing, and obtaining accurate information about these conditions. RDs are often associated with early mortality and a considerable reduction in quality of life [1–5].

In Brazil, the Ministry of Health defines an RD as any disorder that affects up to 65 per 100,000 individuals [3, 4]. Previous international studies have reported an estimated population prevalence of RDs of 3.5–8.0%, suggesting that they have a substantial impact on public health [1, 5, 6]. Extrapolating these estimates to the Brazilian population [7] produces a corresponding figure of 7.0–16.2 million Brazilians affected by RDs, highlighting their significant burden and public health implications.

Brazil, the fifth-largest country worldwide, covers 8,510,417 square kilometers and is divided into five regions with 26 states, a Federal District, and 5570 municipalities [7]. The Brazilian Unified Health System (*Sistema Único de Saúde* [SUS]) was established in 1988 and aims to provide universal and equitable access to promotion, prevention, and health care services for all Brazilian citizens. Brazil has undergone an epidemiological transition in recent decades, marked by significant advancements in health indicators attributable to external factors. Notably, hereditary diseases and congenital anomalies contribute significantly to child mortality, ranking second among infant mortality causes since 2005 [8, 9].

In January 2014, the Brazilian Policy for Comprehensive Care for Persons with Rare Diseases was established within the scope of the SUS. This policy aims to reduce morbidity and mortality and improve the quality of life of individuals with RDs through promotion, prevention, early detection, timely treatment, disability reduction, and palliative care. It classifies RDs as genetic and non-genetic, with genetic RDs grouped into three categories: congenital anomalies and late-onset disorders, intellectual disability, and inborn errors of metabolism [10].

To date, over 30 reference services for RDs have been accredited. This is still insufficient to meet population demands. Most cases are treated in university hospitals (UHs), but whether their human and technological resources are adequate for RD care is unknown [10, 11]. Despite advances in diagnosis, mainly due to the development of new technologies and the recent organization of RD care in Brazil, the country lacks an established system for registering RDs. Except for a few infectious RDs that require mandatory reporting, epidemiological data on these conditions are scarce and, when available, are often restricted to specific RDs [2, 3].

High-quality epidemiological data on RDs are essential for understanding patient needs, enhancing healthcare management, and identifying the potential beneficiaries of clinical trials and novel therapies. However, epidemiological research encounters obstacles since many studies rely on limited national registries that often focus on specific disease groups [5]. Therefore, a coordinated effort to map the epidemiology of RDs in Brazil is needed. The Brazilian Rare Diseases Network (RARAS) was established in 2020 to bridge this gap, including UHs, RD reference services (RDRSs), and newborn screening reference services (NSRSs). This initiative encompasses a

national survey of the epidemiology, diagnosis, clinical presentation, and treatment of individuals with genetic and non-genetic RDs. It has two phases: retrospective and prospective. The retrospective phase involved data collection on RD cases treated at centers in 2018 and 2019, while data collection for the prospective phase has been going on since 2022 [2, 3]. This study presents the findings of the retrospective phase, undertaking a comparative analysis of distinct diagnostic status groups.

Materials and methods

A retrospective survey was conducted to collect data from patients under diagnostic investigation or with a diagnosis or suspicion of an RD who were evaluated between 2018 and 2019 at 34 centers participating in the RARAS. These centers include 15 UHs, 4 RDRSs, and 3 NSRSs, with the remaining centers having mixed roles: 8 are both an RDRS and a UH, 3 are both an RDRS and an NSRS, and 1 is both an NSRS and a UH. A map of the participating centers can be seen in Additional File 1.

This project's methodology has been previously published by Alves et al. [2] and Félix et al. [3]. All participating network services retrospectively searched for cases with genetic and non-genetic RDs and those under diagnostic investigation. Researchers collected data from each service by accessing medical records, using a standardized form in the Research Electronic Data Capture (REDCap) platform hosted at Ribeirao Preto Medical School, University of São Paulo [12]. The original survey is available at LattesData [13]. The form collected demographic, clinical, and therapeutic data. Given the different backgrounds of the data collectors, training was conducted for the participating centers. Initially, a pilot project was performed in five centers with different medical record management forms (paper or electronic). Two hundred and fifty cases were collected during the pilot phase from December 7, 2020, to January 15, 2021. The data were validated and curated. Based on this validation, retrospective data collection was initiated in the centers, which ended in March 2022.

Skin color was described according to the Brazilian Institute of Geography and Statistics (IBGE) as *parda* (admixed), *branca* (white), *preta* (black), *amarela* (yellow), and *indígena* (indigenous). Phenotypic data were described according to the Human Phenotype Ontology (HPO) [14] and limited to five terms per case. Diagnostic information was recorded based on international ontologies (International Statistical Classification of Diseases and Related Health Problems, Tenth Revision [ICD-10] [15]; Orphanet [ORPHA] [16]; or Online Mendelian Inheritance in Man [OMIM] [17]), enabling comparison and aggregation with Orphadata. Reasons for hospitalization and causes of death were documented using ICD-10 [3].

Data analyses were performed using the IBM® SPSS Statistics software (version 26) and Python language (version 3.9.17), leveraging the Pandas (version 1.5.3), NumPy (version 1.24.3), and SciPy (version 1.10.1) libraries. In the descriptive analyses, each individual was evaluated independently. In the comparative analyses based on diagnostic status, each diagnosis was considered independently, as an individual might have more than one RD diagnosis. The chi-squared test was used to compare nominal variables, while the Kruskal–Wallis test was applied to compare continuous numerical variables. In both cases, the Bonferroni correction was utilized for multiple comparisons. The significance level was set at 0.05.

Results

Population

Data from 12,530 participants across 34 centers were collected. Most of the sample was female ($n=6331$; 50.6%), and 13 (0.1%) individuals had undetermined sex. The median age was 15.0 years (interquartile range [IQR]: 7–31; mean: 24.9 ± 20.4; range: 1–98) at the time of inclusion (Fig. 1a). The sample's characteristics are shown in Table 1.

Classification according to skin color demonstrated that 5044 (47.5%) individuals were admixed and 4881 (45.9%) were white. Most participants were born in the Southeast ($n=3765$; 33.6%) and Northeast ($n=3729$; 33.2%) regions. Individuals born in 1750 Brazilian municipalities were included. Twelve participants (0.1%) were born in other countries: two in Lebanon and one each in Egypt, Ecuador, Guinea-Bissau, Japan, Paraguay, Peru, Portugal, and Venezuela (Table 1). Most participants lived in the Southeast region ($n=3996$; 32.8%), followed by the Northeast region ($n=3950$; 32.5%).

The first evaluation at the participating centers occurred at a median age of 6.2 years (IQR: 0.9–20.7). The participants had a median follow-up duration of 2.8 years in the centers (IQR: 0.6–7.9) and 1.7 years in the medical specialty (IQR: 0.1–1.7). Of the total sample, 92 participants were followed up in more than one participating center.

Diagnosis

Regarding diagnosis status, 7931 (63.2%) participants had a confirmed diagnosis, while 2450 (19.5%) had a suspected diagnosis, and 2177 (17.3%) were considered undiagnosed. Sixty-seven participants had more than one confirmed RD diagnosis: 65 had two, and two had three.

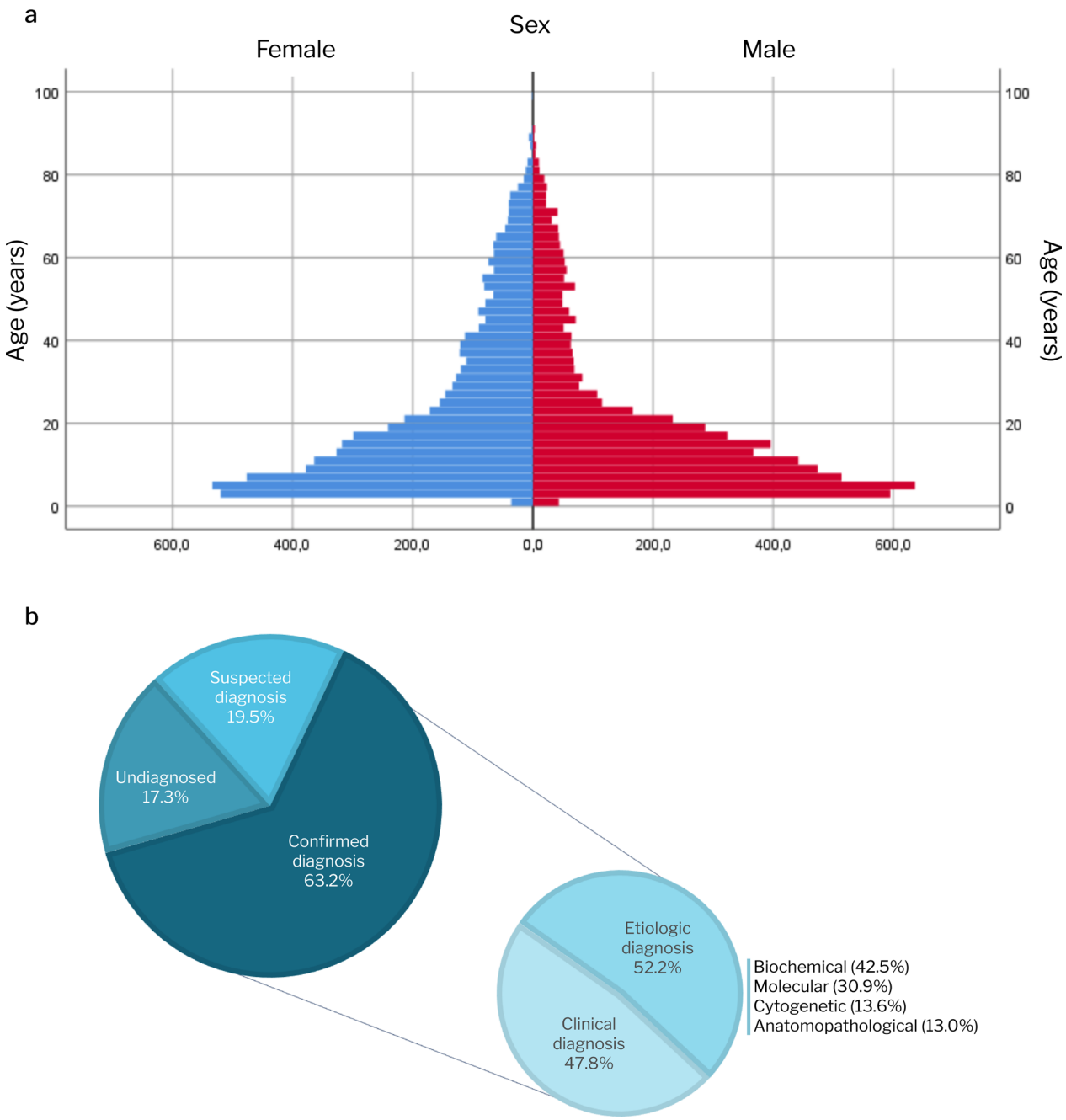

Fig. 1 **a** Histogram of participants' age and sex distribution ($n=12,502$) and **b** diagnostic status ($n=12,279$)

Regarding the diagnostic terminology, 6644 (64.7%) of the diagnoses were recorded using an ORPHA code, 2794 (27.2%) using an ICD-10 code, and 825 (8.0%) using an OMIM code. A total of 1778 different diagnostic codes were mentioned. The most frequent diseases were phenylketonuria (PKU; $n=623$), cystic fibrosis (CF; $n=506$), and acromegaly ($n=382$; Table 2). The diagnostic codes aggregated for the ten most prevalent conditions are detailed in Additional File 2. Upon excluding cases diagnosed through newborn screening, the most frequent diagnoses were CF ($n=389$), acromegaly ($n=381$), and osteogenesis imperfecta ($n=361$). The distribution of the most frequently reported diagnostic codes at each participating center is detailed in Additional file 3.

Most confirmed diagnoses were etiological ($n=5185$; 52.2%), with clinical diagnoses accounting for the

Table 1 Sample characteristics ($n = 12,530$)

	N	%
Color or race		
Admixed	5044	47.5
White	4881	45.9
Black	609	5.7
Yellow	68	0.6
Indigenous	30	0.3
Sex		
Female	6331	50.6
Male	6171	49.3
Undetermined	13	0.1
Region of birth		
Southeast	3765	33.6
Northeast	3729	33.2
South	1659	14.8
Midwest	1377	12.3
North	673	6.0
Born in other countries	12	0.1
Region of residence		
Southeast	3996	32.8
Northeast	3950	32.5
South	2081	17.1
Midwest	1497	12.3
North	642	5.3

remaining cases ($n=4743$; 47.8%). Among the cases with an etiological diagnosis, most were confirmed through biochemical ($n=2164$; 42.5%), molecular ($n=1574$; 30.9%), and cytogenetic ($n=691$; 13.6%) diagnostic methods (Fig. 1b). The primary funder for the diagnostic tests was the SUS (84.2%).

On average, 2.85 HPOs were reported per case. The most frequent signs and symptoms were global developmental delay (HP:0001263; $n=1246$), seizure (HP:0001250; $n=734$), and short stature (HP:0004322; $n=678$; Table 2). The median age at symptom onset was 0.8 years (IQR: 0–9; mean: 9.2), with a median age of 1 year for confirmed cases and 0.8 years for suspected diagnoses (Table 3). Only 17.8% of participants experienced symptom onset after the age of 18 years ($n=1638$).

The diagnosis was made prenatally in only 121 cases (1.2%) and via newborn screening in 979 (9.9%) cases. The median age at confirmatory diagnosis was 10.4 years (IQR: 2.1–33.1) upon excluding prenatal and newborn screening diagnoses (Table 3). The average time from the onset of the first symptom to the diagnostic confirmation was 5.4 ± 7.9 years ($n=4583$).

Family history

Family recurrence was reported in 2717 cases (21.6%) and consanguinity in 803 cases (6.4%). Consanguinity rates, expressed as percentages, were significantly higher in the Northeast region (14.0%), followed by the South (7.1%), North (6.5%), Southeast (6%), and Midwest (4.4%; $p<0.0001$). The mean maternal age at the patient's birth was 27.7 ± 7.0 years (range: 12–63), and the mean paternal age was 31.7 ± 8.4 years (range: 12–79).

Treatment

Regarding treatment, 6509 participants (54.3%) received specific therapy to treat their RD or manage its signs and symptoms. The most frequent therapies were drug therapy ($n=6108$; 55.0%), rehabilitation therapy ($n=1739$; 15.6%), and dietary therapy ($n=976$; 8.8%). Drug treatment was initiated at an average age of 22 ± 21.8 years, dietary treatment at 3.2 ± 8.3 years, and rehabilitation at 14.9 ± 19.4 years. The primary funding source for treatments was the SUS (86.7%), which supported 85.6% of the drug treatments, 83.2% of the dietary treatments, and 88.2% of the rehabilitative treatments.

Multi-specialty medical follow-up was reported in 84.0% ($n=9864$) of participants. Apart from medical genetics, the specialty where most data was collected, neurology was the most consulted specialty, representing 31% of consultations, followed by endocrinology (22.6%), neuropediatrics (21%), and ophthalmology (18.2%).

Hospitalization and death

A previous hospitalization was recorded for 4922 participants (44.5%). The mean number of hospitalizations was 4.12 ± 14.2 (range: 0–379), with 5% of participants undergoing at least 13 hospitalizations. The most frequent reasons for hospitalization were ICD-10 codes E22.0 (acromegaly and pituitary gigantism; $n=189$), Q78.0 (osteogenesis imperfecta; $n=161$), and E84 (CF; $n=125$; Table 2).

A mortality rate of 1.5% ($n=177$) was observed in the studied population during the evaluated period. The median age at death was 20.3 years (IQR: 1.6–55.7; mean: 30.3 ± 27.8; range: 0–87.7). The leading causes of death were ICD-10 codes G12.2 (motor neuron disease; $n=30$), E84 (CF; $n=10$), and I46 (cardiac arrest; $n=7$; Table 2). Autopsy was performed in 18 (10.3%) cases.

Table 3 presents comparative data on cases with confirmed diagnoses, suspected diagnoses, and undiagnosed cases based on the investigated characteristics. Details of the statistical results and pairwise comparisons with Bonferroni correction are available in Additional file 4.

Table 2 The ten most frequent disorders, signs and symptoms, causes of hospitalization, and causes of death

Most frequent diagnoses (N =12,261)*			
	Description	**N**	**%**
	Phenylketonuria	623	5.1
	Cystic Fibrosis	506	4.1
	Acromegaly	382	3.1
	Osteogenesis Imperfecta	360	2.9
	Dystrophinopathy	278	2.3
	Congenital adrenal hyperplasia	275	2.2
	Neurofibromatosis	271	2.2
	Mucopolysaccharidosis	225	1.8
	Amyotrophic lateral sclerosis	211	1.7
	Turner Syndrome	197	1.6
Most frequent signs and symptoms (N = 34,685)**			
HPO	**Description**	**N**	**%**
HP:0001263	Global developmental delay	1246	3.6
HP:0001250	Seizure	734	2.1
HP:0004322	Short stature	678	2.0
HP:0001249	Intellectual disability	514	1.5
HP:0001252	Hypotonia	451	1.3
HP:0005982	Reduced phenylalanine hydroxylase level	391	1.1
HP:0001324	Muscle weakness	390	1.1
HP:0002315	Headache	331	0.9
HP:0000252	Microcephaly	326	0.9
HP:0002015	Dysphagia	298	0.8
Most frequent causes of hospitalization (N = 4,922)***			
ICD-10	**Description**	**N**	**%**
E22.0	Acromegaly and pituitary gigantism	189	3.8
Q78.0	Osteogenesis imperfecta	161	3.3
E84	Cystic fibrosis	125	2.5
J18–J18.9	Pneumonia, organism unspecified	119	2.4
G12.2	Motor neuron disease	87	1.8
E25	Adrenogenital disorders	50	1.0
E84.0	Cystic fibrosis with pulmonary manifestations	46	0.9
R56	Convulsions, not elsewhere classified	38	0.8
G71.0	Muscular dystrophy	33	0.7
G40	Epilepsy and recurrent seizures	32	0.6
Most frequent causes of death (N = 177)			
ICD-10	**Description**	**N**	**%**
G12.2	Motor neuron disease	28	15.8
E84	Cystic fibrosis	10	5.6
I46	Cardiac arrest	7	3.9
R09.2	Respiratory arrest	3	1.7
J96.9	Respiratory failure, unspecified	3	1.7
J96.0	Acute respiratory failure	3	1.7
J96	Respiratory failure, not elsewhere classified	3	1.7
J38.4	Edema of larynx	2	1.1
E74.0	Glycogen storage disease	2	1.1
A41.9	Sepsis, unspecified organism	2	1.1
A41	Other sepsis	2	1.1

*Overall diagnoses. ** Total mentioned HPOs. ***Number of individuals with previous hospitalizations

Discussion

This study represents Brazil's first comprehensive evaluation of RD epidemiology, embodying an innovative approach based on collaborative efforts and a network-based framework. The specialized services network, including NSRSs, contributed to the higher prevalence of PKU and CF diagnoses in this epidemiological survey. Additionally, this study revealed the average duration of the diagnostic odyssey for individuals with RDs in Brazil (5.4 years). Moreover, a substantial portion of patients with RDs were found to remain undiagnosed.

The study population mainly comprised individuals born and residing in Brazil's Southeast, Northeast, and Southern regions, respectively, which are ranked as the most populous regions in the country [7]. Individuals born in 1750 Brazilian cities were included, representing 31.4% of all national municipalities [7]. Notably, São Paulo city, with 12.4 million inhabitants, has the highest population and contributed the most participants to this study. Higher rates of confirmed diagnoses were found among participants born and residing in the South and Southeast regions of the country compared to other regions, likely due to the greater availability of genetic testing and specialized resources for RDs in these areas, as reported in previous studies [8, 9, 11, 18].

The newborn screening program in Brazil encompasses PKU and CF, contributing to the high frequency of these conditions in this study. The screening also covers congenital hypothyroidism, hemoglobinopathies, congenital adrenal hyperplasia, and biotinidase deficiency [3]. Sickle cell disease was excluded due to its non-rare status in certain states of Brazil, especially among individuals with African ancestry [19]. Medical genetics services' prevalence may have influenced the lower frequency of congenital hypothyroidism. Upon excluding newborn screening cases, PKU was not the most common diagnosis. A considerable number of cases of CF were not identified through neonatal screening. This may be due to the inclusion of CF in the Brazilian neonatal screening program around 2001 [20] and its complete incorporation may not have occurred immediately. It is also important to consider the possibility of false negatives in the screening process.

Acromegaly emerged as a notable focal point in our study, standing out as one of the three most prevalent conditions in seven participating centers and the most frequent cause of hospitalization in the studied population. This prominence could be attributed to the specialized nature of at least four of these centers, which function as dedicated reference services for acromegaly treatment. This specialization can potentially cause selection bias, as individuals seeking care specifically for acromegaly may contribute disproportionately to the study population from these centers.

In our study, 67 participants had multiple confirmed RD diagnoses, which poses unique challenges and impacts patients physically, emotionally, and financially. With the advancing scope of genomic techniques, having multiple confirmed RD diagnoses is becoming increasingly common [21].

Compared to the 6.4% consanguinity rate observed in our study, previous research indicates variable consanguinity rates in different populations. Leutenegger et al. [22] found inbreeding in various populations around the world, with the highest levels in the Middle East, Central South Asia, and the Americas. A mean consanguinity rate of 0.96% was reported in South America, with higher rates in Venezuela (1.84%) and Brazil (1.60%) [23]. Previous studies have also indicated higher consanguinity rates in the Northeastern region [24]. Factors such as low paternal education and occupation levels were positively associated with consanguinity [23]. The higher consanguinity rates in our study compared to previous studies can be attributed to the population of participants with diagnosed or suspected RDs, including autosomal recessive disorders.

Many participants experienced numerous hospitalizations, especially those with confirmed RD diagnoses, suggesting that these hospitalizations may be related to therapeutic requirements. This observation underscores the complex, multidisciplinary specialized care that individuals with RDs uniquely need and emphasizes the importance of accordingly tailored accessible healthcare. Previous studies have reported the elevated economic burden of hospitalizations for RDs [6] and higher hospitalization rates among patients with metabolic and genitourinary system-related RDs [25]. Additionally, RDs have been previously associated with unfavorable inpatient outcomes, including in-hospital deaths, extended stays, intensive care unit admissions, and 30-day readmissions when compared to an inpatient population without RDs [26].

Some form of instituted therapy was identified more frequently among individuals with confirmed RD diagnoses. Participants with confirmed RD diagnoses may have received more frequent therapy due to selection bias, reflecting possibly more severe symptoms and referrals to specialized centers. Disease severity may have also driven immediate therapy initiation for improved management and outcomes. Ninety-two participants received care from multiple centers, illustrating co-management challenges in complex, multisystem RDs [10, 25]. Our study also emphasized the importance of multidisciplinary care for individuals with RDs. However, it is essential to acknowledge that medical genetics data

Table 3 Comparative analysis based on diagnostic status

	Confirmed diagnosis (N = 7931)	Suspected diagnosis (N = 2450)	Undiagnosed (N = 2177)	Significance
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	***P* value**
Age (years) (N = 12,159)	18 (9–37)	13 (6–26)	11 (6–18)	< 0.0001*
Age of symptom onset (years) (N = 9328)	1 (0–14)	0.8 (0–8)	0.2 (0–2)	< 0.0001*
Age at first evaluation at the center (years) (N = 11,546)	7.3 (0.7–26.8)	6.5 (1.3–17.4)	3.8 (0.9–10.8)	< 0.0001*
Age at first evaluation in the specialty (years) (N = 11,277)	8.1 (1.1–27.3)	7.6 (1.9–18.5)	5.6 (1.7–12.6)	< 0.0001*
Length of follow-up at the center (years) (N = 11,592)	3.7 (1–9.4)	1.3 (0.2–4.6)	1.8 (0.3–5.5)	< 0.0001*
Length of follow-up in the specialty (years) (N = 11,317)	2.7 (0.6–7.2)	0.6 (0–2.6)	0.5 (0–2.6)	< 0.0001*
Age at confirmatory diagnosis (years) (N=4944)	10.4 (2.1–33.1)	NA	NA	–
Number of previous hospitalizations (N = 4294)	2 (1–4)	1 (1–3)	1 (1–2)	< 0.0001*
Maternal age at birth (years) (N = 4837)	27 (22–33)	27 (22–32)	27 (22–33)	0.332
Paternal age at birth (years) (N = 3996)	31 (25–37)	30 (25.7–37)	31 (25–37)	0.995
	N (%)	**N (%)**	**N (%)**	***P* value**
Color or race				
White	3330 (66.6)	763 (15.3)	907 (18.1)	< 0.0001*
Admixed	3054 (61.2)	1072 (21.5)	863 (17.3)	
Black	425 (69.2)	103 (16.8)	86 (14.0)	
Yellow	45 (64.3)	11 (15.7)	14 (20.0)	
Indigenous	21 (70.0)	5 (16.7)	4 (13.3)	
Sex				
Female	4254 (67.4)	1085 (17.2)	971 (15.4)	< 0.0001*
Male	3687 (60.7)	1200 (19.8)	1184 (19.5)	
Undetermined	7 (53.8)	5 (38.5)	1 (7.7)	
Region of birth				
Southeast	2516 (66.3)	582 (15.3)	700 (18.4)	< 0.0001*
Northeast	2200 (59.6)	739 (20.0)	753 (20.4)	
South	1258 (72.1)	213 (12.2)	275 (15.7)	
Midwest	828 (61.2)	325 (24.0)	201 (14.8)	
North	321 (47.8)	254 (37.9)	96 (14.3)	
Born in other countries	7 (63.6)	2 (18.2)	2 (18.2)	
Region of residence				
Southeast	2688 (66.5)	610 (15.1)	744 (18.4)	< 0.0001*
Northeast	2339 (60.0)	800 (20.5)	758 (19.5)	
South	1658 (76.2)	234 (10.7)	286 (13.1)	
Midwest	906 (61.5)	355 (24.1)	213 (14.4)	
North	299 (46.8)	246 (38.6)	93 (14.6)	
Family recurrence				
No	4953 (62.9)	1418 (18.0)	1503 (19.1)	0.030
Yes	1713 (63.5)	531 (19.7)	452 (16.8)	
Consanguinity				
No	5487 (61.5)	1697 (19.1)	1734 (19.4)	< 0.0001*
Yes	440 (55.1)	158 (19.8)	200 (25.1)	

Table 3 (continued)

	N (%)	N (%)	N (%)	*P* value
Previous hospitalization				
No	3789 (62.8)	1143 (19.0)	1099 (18.2)	< 0.0001*
Yes	3583 (70.4)	809 (15.9)	697 (13.7)	
Death				
No	7688 (65.0)	2113 (17.9)	2021 (17.1)	0.094
Yes	127 (71.8)	30 (16.9)	20 (11.3)	
Treatment related to rare disease				
Yes	5317 (83.9)	620 (9.8)	397 (6.3)	< 0.0001*
No	134 (40.9)	73 (22.3)	121 (36.8)	

Each row corresponds to the total number of valid data, i.e., without considering missing values. In this analysis, each diagnosis was evaluated independently, considering that a participant may have more than one RD diagnosis

P-values marked with * represent statistical significance (P < 0.05)

were not separately collected as a distinct medical specialty. Instead, this specialty was encompassed within the primary care for most cases, where data collection and treatment were conducted.

The SUS plays a vital role in RD diagnosis and treatment. It serves as the primary funder for therapies and diagnostic methods related to RDs. The SUS enables the availability of genetic testing [11], specialized consultations, and treatment options that incorporate the National Committee for Health Technology Incorporation recommendations and enable the subsequent development of clinical guidelines [10, 27]. Working as a network becomes essential to optimize the use of resources and enhance collaboration between institutions.

Five of the 34 participating centers exclusively care for pediatric patients, while the remaining centers offer care to both pediatric and adult patients. This distribution reflects the prevalence of RDs affecting individuals across the age spectrum. Interestingly, our data revealed a median age at symptom onset of 0.8 years, indicating that symptoms typically manifest early in life. Additionally, our findings show that over 80% of individuals experienced symptoms before the age of 18 years, surpassing the figure of 70% reported in a previous study [1]. This difference could be attributed to the participation of dedicated pediatric care centers in our study. Our findings suggest that RD symptoms often present at a younger age, highlighting the need for early diagnosis and intervention, especially in pediatric patients, but continue to pose challenges into adulthood.

The diagnostic odyssey, defined as the time from symptom recognition to a definitive diagnosis [28], averaged 5.4 years, consistent with the figure of 4.8–7.6 years reported in other studies worldwide [29, 30]. Notably, a previous study in Brazil reported that the diagnostic odyssey for mucopolysaccharidosis lasted 4.8 years [31]. Prolonged diagnostic odysseys for RDs often involve disease progression, incorrect diagnoses, invasive procedures, delayed treatment initiation, financial burden, and inappropriate interventions [32].

Despite thousands of described RDs, many remain undiagnosed, subjecting individuals to prolonged, costly diagnostic odysseys across multiple healthcare centers [32]. However, even after such efforts, around 6% and 7% of patients with RDs in the United States and Australia, respectively, remained undiagnosed even in expert clinical settings [32, 33]. Factors that may explain the higher rates of undiagnosed cases (exceeding 17%) in our study include poor access to molecular diagnostic techniques. A recent study by RARAS reported that molecular diagnostic tests were available in just over half of the participating centers [11]. Most cases with an etiological diagnosis were confirmed through biochemical and molecular methods. Interestingly, while not the primary confirmatory method, cytogenetic testing was the most accessible diagnostic method in the participating centers, according to the same study.

In the comparative analysis, individuals with a confirmed RD diagnosis showed a higher age, longer follow-up duration in specialized centers, and higher number of previous hospitalizations. Specifically, the undiagnosed group may include individuals who are in the diagnostic journey or odyssey and have not yet obtained a confirmed diagnosis. Subsequent investigations within the RARAS initiative will aim to prospectively assess such cases, establishing a national registry of RDs.

The average age at death was 30.3 years, representing a 47-year reduction compared to the Brazilian population's 2021 life expectancy [34]. In our study, 25% of deaths occurred within the first 1.6 years of life, indicating that RDs significantly impact life expectancy. Previous data

suggested that 22% of infant deaths were due to confirmed genetic disorders [35]. Causes of death related to RDs vary and are often documented as complications rather than the underlying disease. Cardiac and respiratory arrests were frequently recorded causes that did not fully represent the primary cause. The accurate documentation of complications and comorbidities is crucial in RDs, offering insights into disease progression and leading to the development of targeted interventions to improve patient care and reduce RD-related mortality [36]. It is important to recognize that undiagnosed cases might also contribute to mortality figures since some individuals may miss the opportunity to receive care in specialized healthcare facilities, leading to an unrealized suspicion of an RD.

While our study provides valuable information, it has limitations, including sample size and potential bias. The estimated population prevalence for RDs ranged from 3.5 to 8.0% [1, 5, 6], suggesting a significantly larger affected population. Considering the Brazilian population, the country's total number of individuals with RDs would be 550–1200 times larger than the population studied in this project phase [7]. It is essential to note that this study did not include all national healthcare centers, potentially missing patients not evaluated during the study or not receiving care at participating centers. Moreover, the predominance of genetic RDs may have resulted from the specialized expertise and diagnostic resources in genetic centers, leading to selection bias.

This study faced operational limitations related to data sources, including finding, accessing, sharing, and reusing information. A "data quality culture" was promoted to address these issues, emphasizing the need for reliable and comprehensive data. Collectors had diverse backgrounds and digital literacy levels, which could have introduced errors and affected data reliability. Tools, training, support materials, and dedicated channels were provided to mitigate their effects. The complex RD domain made case identification and classification challenging, potentially leading to underreporting and underdiagnosis. Awareness efforts, feedback sessions, outlier identification, case discussions, and standardized data collection protocols were implemented to address this issue [2, 37].

This study revealed appreciable missing data in medical records, which can introduce record-keeping, memory, and registration biases. Missing data in medical records can limit retrospective research, potentially due to registration bias. However, data collection directly from participants in the prospective project phase aims to fill these gaps. A potential contribution of our study is the enhancement of registration methods. By identifying and addressing limitations in data collection and diagnostic terminology classification, we lay the groundwork for more accurate and comprehensive RD registration. This enhancement improves our understanding of RD epidemiology and supports the development of effective public health policies and resource allocation strategies. Standardized data collection protocols and advanced information systems will ensure that future studies and registries capture vital data points, facilitating ongoing RD monitoring and research [2].

Diagnosis data in our study came from three different ontologies, each with limitations regarding disease terminology. While this study's protocol allowed centers to select RD terminology, including ICD-10, it had limitations in RD classification [38, 39]. Accurate RD classification is crucial for efficient healthcare resource allocation and improved analysis for differential diagnosis and clinical decision support. While data were aggregated from the Orphadata database designed for RDs, this database does not encompass all described RDs. In Brazil, ICD-10 remains the classification used by the SUS for diagnosis, hospitalization, and death registration [10, 39]. In the context of HPO terminology, it is noteworthy that the number of HPO terms may have been underestimated due to the limitation of five terms per case.

Future research within the RARAS will encompass the diagnostic and treatment journey of participants with multiple confirmed diagnoses, explore specific therapies and the duration of hospitalizations, investigate the correlation between diagnostic ontologies, and examine population genetics. Other research avenues include exploring the relationship between parental age and RDs and examining the correlations of diagnoses with available diagnostic methods at each center.

We also identified challenges in finding a minimal data set (MDS) that applied to Brazilian patients with RDs. To address this issue, we conducted a systematic review to create a comprehensive MDS for future project phases [40, 41]. Standardizing data collection through an MDS is critical for accurately identifying RDs and optimizing diagnostic and treatment processes, particularly in resource-limited settings. Validating it as a national tool for epidemiological tracking and analysis is essential for structuring health information systems and guiding more effective public health policies. Further research phases are required to refine prevalence estimates and comprehensively understand specific RDs and their impact on the Brazilian population by including a broader range of healthcare facilities. This retrospective analysis did not address factors such as participants' socioeconomic status, referral sources, or willingness to participate in other studies. However, these variables became part of the data collection protocol and will be examined in forthcoming studies.

The perspectives presented here shed light on the future research directions derived from our study, fostering further advancements in the field. These data can support future studies and ultimately lead to improvements in RD diagnosis, treatment, and management. Understanding the magnitude of RDs is crucial for effective resource allocation, policy development, and the provision of appropriate healthcare services for affected individuals [3, 5].

This multicenter study presents the initial nationwide data on the care provided to individuals with RDs in Brazil, highlighting the importance of collaboration between specialized services. Reliable epidemiological data will support public health approaches, including population impact assessment, cost evaluation, and improved RD management, and facilitate clinical trial development [5]. This study also emphasizes the vital role of the collected information in shaping public policies while identifying limitations such as data gaps and constrained terminologies for disease classification. Until this study was performed, our understanding of RDs in Brazil, except for specific disorders, was limited by a lack of comprehensive evidence. Establishing a national network, including data collection infrastructure, marked a significant step towards advancing the understanding of RDs in Brazil and addressing this gap.

The longitudinal and prospective continuation of this study is necessary and currently underway, with the expectation that it will impact health policy for RDs regarding resource allocation and improving the quality of life of affected individuals. The results of our study also provide valuable guidance for the refinement of data collection forms and instruments, thereby enhancing the effectiveness and accuracy of information related to RDs in Brazil.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13023-024-03392-7.

Additional file 1. Map of participating centers

Additional file 2. The ten most frequent RD diagnoses in RARAS and the applied coding

Additional file 3. Top three diagnostic codes and their corresponding counts and percentages at each participating center

Additional file 4. Post-test analysis of demographic factors and medical outcomes across distinct diagnostic statuses

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Author contributions
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Availability of data and materials
Data analyzed in this study are available interactively through the Brazilian Online Atlas of RD (RARASBR; https://doi.org/10.25504/FAIRsharing.d7b6c8) [42] and LattesData [13]. For any further inquiries, please contact the corresponding author.

Declarations

Ethics approval and consent to participate
This study protocol was reviewed and approved by the Research Ethics Committee (REC) of Hospital de Clínicas de Porto Alegre (approval number: 33970820.0.1001.5327), the coordinator center for the study, and in all participating centers. Written informed consent was dispensed by the respective RECs for this project phase.

Consent for publication
All authors have given final permission to submit for publication.

Competing interests
The authors declare that they have no competing interests.

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Population-specific facial traits and diagnosis accuracy of genetic and rare diseases in an admixed Colombian population

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Up to 40% of rare disorders (RD) present facial dysmorphologies, and visual assessment is commonly used for clinical diagnosis. Quantitative approaches are more objective, but mostly rely on European descent populations, disregarding diverse population ancestry. Here, we assessed the facial phenotypes of Down (DS), Morquio (MS), Noonan (NS) and Neurofibromatosis type 1 (NF1) syndromes in a Latino-American population, recording the coordinates of 18 landmarks in 2D images from 79 controls and 51 patients. We quantified facial differences using Euclidean Distance Matrix Analysis, and assessed the diagnostic accuracy of Face2Gene, an automatic deep-learning algorithm. Individuals diagnosed with DS and MS presented severe phenotypes, with 58.2% and 65.4% of significantly different facial traits. The phenotype was milder in NS (47.7%) and non-significant in NF1 (11.4%). Each syndrome presented a characteristic dysmorphology pattern, supporting the diagnostic potential of facial biomarkers. However, population-specific traits were detected in the Colombian population. Diagnostic accuracy was 100% in DS, moderate in NS (66.7%) but lower in comparison to a European population (100%), and below 10% in MS and NF1. Moreover, admixed individuals showed lower facial gestalt similarities. Our results underscore that incorporating populations with Amerindian, African and European ancestry is crucial to improve diagnostic methods of rare disorders.

According to the Online Mendelian Inheritance in Man (OMIM) databank, there are more than 10,000 genetic and rare diseases (RD) affecting 7% of the world's population[1,2]. This corresponds to approximately 500 million people. Although as a whole genetic and RD are a significant cause of morbidity and mortality in the pediatric population[3], by separate each disorder affects a very reduced number of people. Depending on the country, the prevalence to consider a disease as rare ranges from 1 affected individual in 50,000 people to 1 in 200,000. This low prevalence has limited the research on rare disorders.

Currently, there is limited knowledge on the etiology of these disorders. A reduced percentage of diseases (20%) presents a known molecular basis associated to a detailed phenotype description, and treatment is only available for 0.04% of RD[3]. As orphan diseases, many RD are chronic and incurable, representing severe and debilitating conditions[4]. The diagnosis and management of genetic RD is currently a clinical challenge[5]. Precise and early diagnosis is crucial for individuals and their families to get effective care and to reduce disease progression. However, due to the limited knowledge and complexity of these pathologies, diagnosis may take several years[6]. People often suffer during a long diagnostic odyssey, with delays in their correct treatment and management[7]. For most rare diseases, there are no reliable biomarkers for early diagnosis[8].

Among the wide constellation of clinical symptoms associated to genetic and rare disorders, craniofacial dysmorphologies emerge as potential biomarkers[9,10]. These phenotypes are highly prevalent[2,6] and are commonly used for diagnosis, management and treatment monitoring of genetic and RD[6]. Up to 40% of these

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disorders present characteristic craniofacial phenotypes, including Down, Morquio, Noonan, Apert, Rett, Fragile X, Williams-Beuren and Treacher-Collins and velocardiofacial syndromes, as well as other conditions such as microcephaly, holoprosencephaly, palate/lip cleft, and other 2,000 rare genetic disorders[10,11].

The genetic and environmental factors causing these disorders alter the complex process that orchestrates facial morphogenesis during pre- and postnatal development, inducing facial dysmorphologies. Facial development is highly regulated by multiple signaling pathways[12–14], including Fibroblast Growth Factor (*FGF*), Hedgehog (*HH*), Wingless (*WNT*) and Transforming Growth Factor Beta (*TGF-β*) and Bone Morphogenetic Proteins (*BPMs*). Disruptions in the regulation of any of these signaling pathways can lead to facial dysmorphogenesis[15].

The facial patterns associated with each disorder are unique, but vary within and among diagnostics, ranging from subtle facial anomalies to severe malformations[16]. In the clinical practice, craniofacial dysmorphology is commonly assessed through qualitative visual assessment and basic anthropometric measurements. However, this approach may not capture with optimal precision the anatomical complexity of the facial dysmorphologies associated with these disorders. Qualitative descriptions of facial phenotypes are sometimes based on general terms such as coarse face, large and bulging head; saddle-like, flat bridged nose with broad, fleshy tip; or malformed teeth[17–19]. Accurate identification of dysmorphic features for diagnosis thus depends on the clinician's expertise, and only highly trained dysmorphologists are able to recognize the facial "gestalt" characteristic of the rarest disorders[19].

Recent research seeks to incorporate into the clinical diagnosis of RD the use of objective and quantitative tools to assess facial phenotypes[20–25]. Automated systems have been developed to improve and accelerate the diagnostic process[9,10,26]. Within the clinical practice, Face2Gene is the most commonly used system (FDNA Inc., https://www.face2gene.com/), a community-driven phenotyping platform trained over 17,000 people representing more than 200 syndromes[9]. Face recognition is performed on 2D images that can be collected with any type of digital camera or phone, without previous training. Syndrome classification is achieved using DeepGestalt, a cascade Deep Convolutional Neural Network (DCNN)-based method that achieved an average 91% top-10 accuracy in identifying the correct syndrome[9].

Other diagnostic approaches based on 3D photogrammetry have been developed more recently[10,20,21]. The advantage of 3D facial models is that they are more efficient than 2D images in capturing the complexity of facial phenotypes, but their widespread use is limited because the photographic equipment required for generating 3D models is not commonly available in the clinical practice. Hallgrímsson et al. (2020)[10] analyzed 3D facial models from 7,057 subjects including subjects with 396 different syndromes, relatives and unrelated unaffected subjects (https://www.facebase.org/). Deep phenotyping based on quantitative 3D facial imaging and machine learning presented a balanced accuracy of 73% for syndrome diagnosis[20].

Automated methods have thus demonstrated high potential to facilitate the diagnosis of facial dysmorphic syndromes[6,9,10,26]. These tools present high accuracy diagnosis in European and North American populations, that are the populations in which the machine learning algorithms have been trained and validated. However, these tools have not been thoroughly tested in populations with different ancestries, and it is not well understood the how facial phenotypes associated with genetic and RD might be influenced by the complex patterns of population ancestry characterizing human populations.

Population ancestry in facial dysmorphologies: a long-disregarded factor. Facial shape shows wide variation across world-wide human populations[27]. Facial differences between populations are detected in the shape of the forehead, brow ridges, eyes, nose, cheeks, mouth and jaw[28]. These facial phenotypes result from divergent evolutionary and adaptive histories of human populations occurred during the evolution of *Homo sapiens* over the last 200,000 years. Nowadays, continuous migration and admixture keep shaping the facial phenotypes of human populations. Depending on dominance and epistatic interactions between alleles fixed or predominant in each parental group[30], admixed populations can display a variety of craniofacial morphologies, ranging from resemblance to one of the parental groups to a combination of both parental phenotypes and the evolution of novel phenotypes[29]. Therefore, the evolutionary and population dynamics of human populations result in genetic and phenotypic patterns that surrogate population ancestry[30–32], and can modulate the facial phenotypes associated to disease.

Few studies to date have analyzed the craniofacial phenotypes associated with genetic and RD in populations of non-European descent[33–36], leaving African, Asian and Latin-American populations often disregarded and underrepresented. Unfortunately, there are no reliable representations of facial phenotypes in genetic and rare diseases in populations of non-European descent. However, it is crucial to account for the influence of population ancestry on facial variation to develop quantitative approaches that efficiently diagnose these disorders in populations from all over the world.

To cover this gap, here we assessed the facial dysmorphologies associated to prevalent genetic and RD in a Latin-American population from the Southwest of Colombia. Latin-Americans are fascinating cases of hybrid/admixed populations that evolved over relatively short periods of time[30,37]. Peopling of the Americas likely started 12–18,000 years ago[38,39] by migration waves coming from North and South East Asia[30], following coastal and continental routes[41]. Amerindian populations established all over the continent and adapted to a variety of environments over thousands of years. During the last 600 years, admixture with European and African populations further shaped the genetic ancestry of Latin-American populations[42,43]. In particular, the population from the region of Cali is the result of diverse migratory processes[44]. Admixture with the indigenous Amerindian population began in the sixteenth century with the arrival of Spanish colonizers. In the eighteenth century, large colonial settlements of slaves brought from Africa were established in Cali for the exploitation of sugar cane that significantly changed the population structure of Valle del Cauca. Nowadays, the population of Cali

is characterized by indigenous and mestizo communities, with Amerindian and African ancestry components predominating over the European ancestry contribution[44].

In this study, we compared the facial phenotypes associated to four genetic and RD, including Down syndrome (DS), Mucopolysaccharidosis type IVA metabolic disorder known as Morquio syndrome (MS), and two types of RASopathies, Noonan syndrome (NS) and Neurofibromatosis type 1 (NF1). The facial phenotype of these syndromes has not been previously characterized in Latin-American populations, and differences between populations with different ancestry backgrounds have not been assessed[34–36]. Here, we quantitatively assessed the facial phenotypes associated to these syndromes, and compared our results in a Colombian admixed population with those reported in European descent populations. We also assessed the diagnostic accuracy of automatic methods currently used in the clinical practice, and detected evidence suggesting that further research is needed to optimize these methods in admixed populations of non-European descent.

Materials and methods

Participant recruitment for photographic sessions. The Colombian sample comprised 130 individuals from Valle del Cauca, a Southwest region in Colombia (Table 1). The cohort included 79 age matched controls and 51 individuals diagnosed with Down, Morquio, Noonan and Neurofibromatosis type 1 syndromes that were recruited from the clinical genetics consultation at Hospital-Fundación Valle del Lili in Cali (Colombia), a tertiary health reference center for these genetic and rare disorders. In most cases, clinical diagnoses were confirmed by molecular genetic testing.

Down syndrome (DS, OMIM 190685), caused by trisomy of chromosome 21, was selected because it is one of the most common genetic disorders, and previous studies have shown that the clinical manifestations associated with DS vary across ethnicities[35]. Within RD, we included Morquio syndrome type A (MS, OMIM 253000) because Colombia presents one of the highest prevalence of MS in the world, probably as a result of founder effects[45]. Morquio syndrome is a subtype of Mucopolysaccharidosis disorders caused by more than 180 autosomal recessive mutations in the *GALNS* gene[46] that alter the metabolism of the extracellular matrix glycosaminoglycans[47]. Individuals with MS show coarse facies with an excessively rapid growth of the head[48].

Finally, we also included in the analyses two RASopathies, Noonan syndrome (NS, OMIM 163950) and Neurofibromatosis type 1 (NF1, OMIM 162200), which are prevalent in Valle del Cauca and present altered craniofacial development by genetic mutations that cause Ras/MAPK pathway dysregulation[49].

To assess the facial phenotypes associated with these disorders, individuals diagnosed with DS, MS, NS and NF1 and age matched controls were recruited for photographic sessions at educational and research centers in Cali (Colombia) in 2021. The photographic material was taken under the protocol approved by ethics committee "Human Research Ethics Committee of the Icesi University" with Approval Act No. 309. To photograph the participants and to record relevant clinical information, we obtained informed consent from the participants or from their parents or legal guardians in the case of minor children, in accordance with national guidelines and regulations.

Facial image acquisition and anatomical landmark collection. Facial shape was captured from 2D images taken using a professional digital camera (SONY Alpha 58 + 18–55) that was attached to a tripod and placed at one-meter distance in front of the participants. To capture a natural facial gesture, the images were acquired in an upright position with facial neutral expression. Participants were asked to sit still, looking towards the front, with open eyes and closed mouth. Although this was challenging in children with Down syndrome, who usually show hyperactivity and tongue protrusion due to hypotonia, several photographs were taken until a neutral facial expression was achieved.

To measure facial shape of each individual and to detect the traits associated with each disorder, we recorded the 2D coordinates of a set of 18 anatomical facial landmarks (Fig. 1 and Supplementary Table 1). Landmarks were acquired using an automatic facial landmark detection procedure adapted from the open-source software library Dlib[50]. The automatic landmarking process is explained in detail in Supplementary Information. In brief, from the set of 68 landmarks registered by Dlib, 15 landmarks directly matched our configuration of 18 facial landmarks (Fig. 1, Fig. S1, Table S1). Three additional landmarks were approximated through direct computations between the landmarks coordinates automatically returned by Dlib: the glabella was computed as the midpoint point between the innermost points located in the eyebrows, and the palpebrale inferius landmarks of the right and left eyes were computed as the midpoint between the two central lower eyelid landmarks.

Diagnosis	M	F	Total	Age (years old)
Control	32	47	79	4–59 ($\bar{x}$ = 23.5)
Down syndrome	8	11	19	3–28 ($\bar{x}$ = 12.7)
Morquio syndrome	6	5	11	9–28 ($\bar{x}$ = 17.9)
Noonan syndrome	4	5	9	5–39 ($\bar{x}$ = 16.4)
Neurofibromatosis type 1	6	6	12	6–52 ($\bar{x}$ = 17.5)
Total	56	74	130	

Table 1. Sample composition by diagnosis. The table provides the number of male (M) and female (F) participants, as well as the total sample size for each syndrome. The age range within each diagnostic group is also provided, where $\bar{x}$ represents the average age.

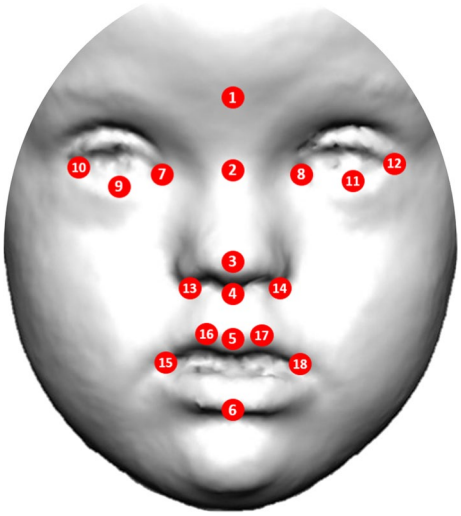

Figure 1. Anatomical position of facial landmarks used in morphometric and statistical analyses to quantify dysmorphologies associated to genetic and rare disorders Down, Morquio, Noonan syndromes and Neurofibromatosis type 1 in a Colombian population.

The validity of the data was assessed by comparing the coordinates of landmarks automatically detected by Dlib with the coordinates of landmarks manually collected by an expert facial morphologist. Manual and automatic measurement differences were assessed for each individual landmark using the root mean square error (RMSE) (Fig. S2). This method was first validated with the 2D facial images of 20 control subjects, and the average RMSE was 1.75 mm. To validate the automatic landmarking method with images of syndromic patients, we manually landmarked 20 patients, including 5 individuals diagnosed with each syndrome represented in our sample. The RMSE for syndromic patients was slightly higher (RMSE = 1.96 mm), but below 2 mm (Fig. S2). Considering that this error threshold is widely accepted in studies of biological anthropology for craniometric measurements[51], the precision of the automatic detection method of anatomical points was validated on both control and syndromic samples.

Quantification of facial phenotypes. We used Euclidean distance matrix analysis (EDMA) to describe the facial phenotype associated to each syndrome. EDMA is a robust morphometric method for assessing local differences between samples[52] by detecting linear distances that significantly differ between pairwise sample contrasts and comparing patterns of significant differences across samples.

To account for size differences between subjects, the 2D coordinates of the facial landmarks of each subject were scaled by their centroid size, estimated as the square root of the sum of squared distances of all the landmarks from their centroid[53]. After scaling, as EDMA represents shape as a matrix of linear distances between all possible pairs of landmarks, a total of 153 unique facial measurements were calculated for each individual. Linear distances were compared for each group of DS, MS, NS and NF1 syndromes with control individuals by performing a two-tailed two-sample shape contrasts on all unique inter-landmark linear distances from each sample. Relative differences between patients and controls were computed as (mean distance in controls—mean distance in patients) / mean distance in controls.

Statistical significance was assessed using a non-parametric bootstrap test with 10,000 resamples. EDMA statistically evaluated the number of significant local linear distances in each two-sample comparison based on confidence interval testing. We used the default α level in EDMA ($\alpha = 0.10$), and a 90% confidence interval was calculated for each linear distance. The shape differences were sorted in increasing order, and the first 5% and the last 5% differences were discarded. The resulting minimum and maximum differences were used to set up the lower and upper confidence limits for each linear distance. Interlandmark distances were considered non-significantly different between controls and patients when the resulting interval contained the value zero. Otherwise, the equality null hypothesis was not accepted, and we assumed that a significant shape difference existed at the α level[54]. To pinpoint specific local shape differences and to reveal the unique morphological pattern of variation associated with each disorder, the ten longest and shortest significant relative differences were plotted on facial figures.

Facial dysmorphology score. To confirm that results were not random due to the small sample sizes available in rare diseases, we combined the results from EDMA with an iterative bootstrapping method that further assessed whether the facial dysmorphologies associated to each syndrome were statistically significant[55]. First, we estimated from the EDMA results a facial dysmorphology score (FDS) as the percentage of significantly different distances between patient and control groups. Then, we ran simulations with random samples of controls and patients generated by iterative bootstrapping to assess the statistical significance of the patterns revealed by EDMA. For each disorder, we first created subsamples of N randomly chosen controls (where N is

the total number of patients available in the sample). Then, using a subsampling approach, we automatically generated random pseudo-subsamples containing a known number of patients (namely M). This procedure was repeated with increasing numbers of patients and resulted in a series of staggered pseudo sub-samples that contained from M = 0 to M = N patients. A total of 150 simulations were run in each round, and in each of these simulations, we computed an EDMA analysis and an FDS score.

The results from each round of random groups were separately represented in histograms. The first round of simulations contained no patients (M = 0) and only included control individuals, representing facial differences that can be found randomly in the general population. To assess whether the FDS value obtained using the complete patient dataset was significantly different or similar to the FDS resulting from a random sample, we compared the distribution of FDS random values with the FDS observed in the whole sample. The *P*-value assessing the statistical significance of the comparison was computed as the ratio between the number of simulations containing no patients that provided a higher FDS than the observed FDS divided by the total number of simulations. *P*-values below 0.05 indicated that the FDS obtained using the real dataset was higher that the FDS obtained randomly in a sample of control subjects.

Face2Gene diagnostic assessment. To assess the accuracy of automated diagnostic methods in the Colombian sample, we compared the clinical diagnosis based on clinical and genetic testing with the diagnosis estimated from the frontal facial 2D images of the patients using the Face2Gene technology (FDNA Inc., Boston, MA, USA; https://www.face2gene.com). Following Gurovich[9], we assessed the top-one and top-five accuracies for each disorder, estimated as the percentage of cases where the Face2Gene model predicted the correct syndrome as the first result or within the five first results from the sorted list of probable diagnoses. We also calculated these accuracies expanding the diagnostic range to the disorder family.

Moreover, we evaluated the similarity between the Colombian patients and the facial gestalt models used by Face2Gene for syndrome classification. For each individual, we selected the first diagnostic prediction that matched their clinical and genetic diagnosis and recorded the gestalt similarity. We classified the level of similarity between the individual and the corresponding gestalt model into seven categories, including "very low", "low", "low-medium", "medium", "medium–high", "high", and "very high" gestalt similarity, using the "gestalt level" barplot provided by Face2Gene.

Finally, to further test the influence of population ancestry on the diagnostic accuracy of Face2Gene, and to directly compare the results with individuals from European descent populations, we performed an extensive search of public image databases to obtain 2D photos of European subjects diagnosed with DS, NS, MS and NF1 syndromes. We collected the images of 45 subjects with DS[56]; and 24 diagnosed with NS[57]. Unfortunately, no 2D images of European individuals diagnosed with MS and NF1 were found publicly available. Using these images, we tested the accuracy of Face2Gene in DS and NS employing the same method previously described for the Colombian population. However, we could not use these publicly available images to perform EDMA and FDS analyses on the European samples, because the pictures were not taken under controlled conditions[56], and diverse facial expression and head position would lead to bias in results of quantitative shape comparisons.

Results

EDMA analyses showed that each syndrome presented a characteristic facial phenotype.

In individuals with Down syndrome, all facial structures including the eyes, nose and mouth presented significant differences as compared to controls. Overall, DS was associated with wider but shorter facial traits (Fig. 2A).

Results showed a 6.5% increase of relative distance between the midpoint between the eyebrows (glabella) and the most inferior medial point of the lower right eyelid (palpelabre inferius), and a 7.5% increase between the right palpelabre inferius and the outer commissure of the right eyes (exocanthion), indicating hypertelorism. Additionally, in this Colombian sample, people with DS exhibited longer measurements in the buccal portion, with a 6–8% increase of mouth width as measured from the crista philtri to the chelions (Fig. 2A). However, the midfacial and nasal regions were reduced (Fig. 2A). People with DS presented a 6–8% reduction in measurements of midfacial height, with the largest difference detected as a 9.7% reduction of the distance between the tip and the root of the nose (Fig. 2A). The facial dysmorphology score (FDS) indicated that up to 58.2% of facial traits were significantly different in people with DS (Fig. 2B).

The facial pattern associated with Morquio syndrome was also characterized by wider and shorter midfacial traits, as observed in Down syndrome. However, facial dysmorphologies were more abundant and severe in MS than in DS, with 65.4% of facial traits significantly different in diagnosed individuals and higher percentages of relative change (Fig. 3 A, B). The most affected regions were the midface and the nose, whereas the mouth was the least affected. Individuals with MS presented hypertelorism, with 14% increase in the distance between the midpoint between the eyebrows (glabella) and the inner commissures of the left and right eyes (endocanthions). Individuals with MS also showed larger distances in the base of the nose, with a 14–19% increase in the distance from the tip of the nose to the insertion of the right and left alar bases (subalare) as compared to controls. Mouth width was also increased in MS; whereas midfacial heights measuring the distance between the eyes and the nose were significantly reduced from 10 to 16% in individuals with MS (Fig. 3A).

In Noonan syndrome, facial dysmorphologies were abundant and concentrated in the orbital and nasal regions. EDMA detected significantly increased distances in the upper face, but decreased distances in the midface (Fig. 4A).

Patients presented a lower position of the eyes, with 9 to 13% increased distances between the glabella or sellion and the landmarks located in the eyes. The mouth also showed a more inferior position, with 8–10% increased relative distance between the tip of the nose and the superior lip, but the shape of the mouth did not show large differences between patients and controls. The reduction of midfacial heights in individuals with NS

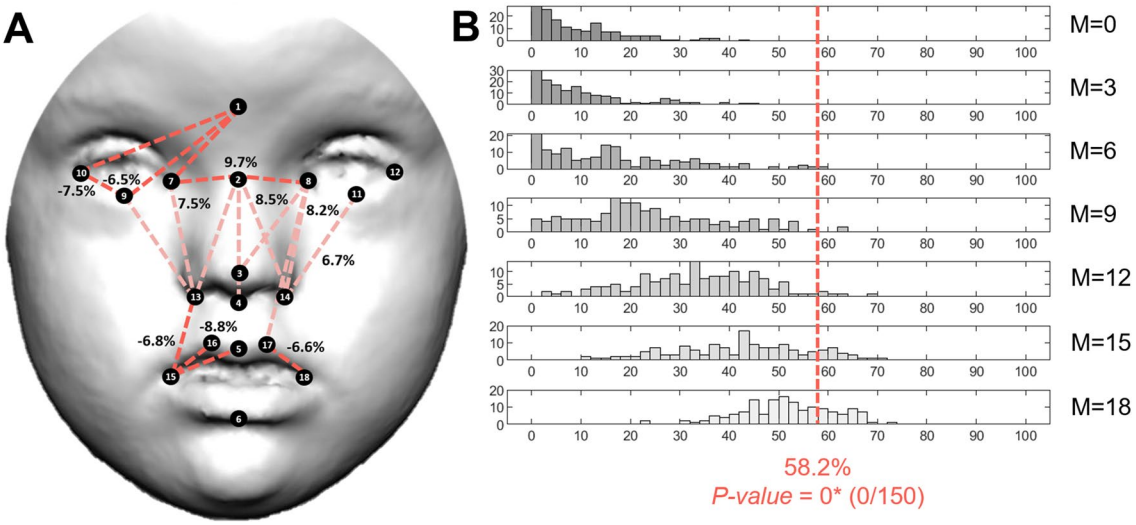

Figure 2. Localized Euclidean Distance Matrix Analysis facial shape pairwise contrasts and iterative bootstrapping tests of facial dysmorphology between controls and individuals diagnosed with Down syndrome. (**A**) EDMA results. Dotted lines represent facial measurements significantly different in control and patient groups. Lines in light tones indicate measurements that are shorter in patients as compared to controls, whereas lines in dark tones represent measurements that are longer in patients. (**B**) Iterative bootstrapping tests based on facial dysmorphology scores (FDS). Histograms represent the simulation results for each random group separately, which contain an increasing number of patients, from no patients (M = 0) to all patients (M = N). From top to bottom histograms, the simulations included 0, 3, 6, 9, 12, 15 and 18 patients. The dotted red line shows the FDS score obtained with the complete sample of control and patients (Table 1). * Statistically significant *P*-value.

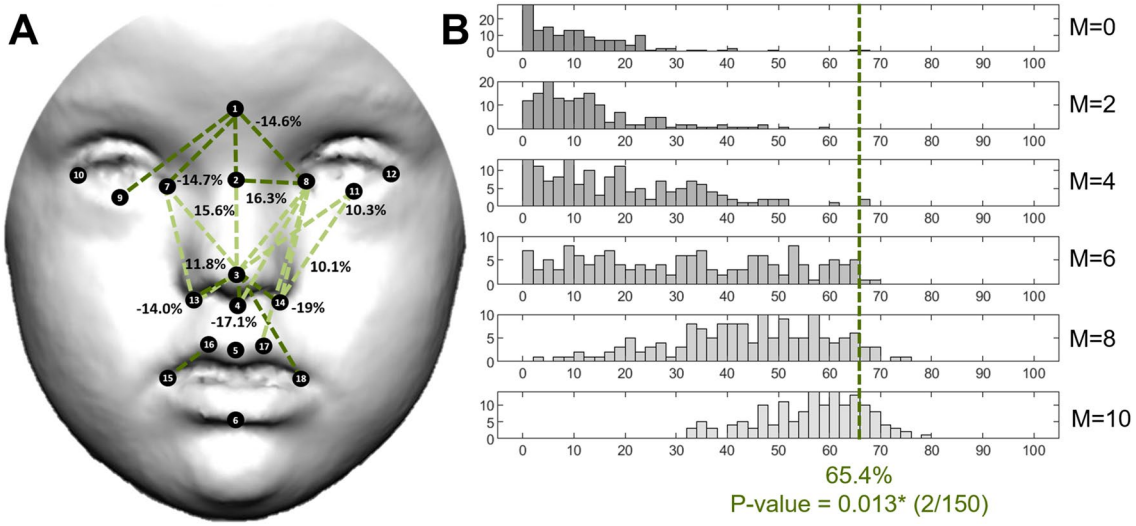

Figure 3. Localized Euclidean Distance Matrix Analysis facial shape pairwise contrasts and iterative bootstrapping tests of facial dysmorphology between controls and individuals diagnosed with Morquio syndrome. From top to bottom histograms, the simulations included 0, 2, 4, 6, 8 and 10 patients. For more details see legend in Fig. 2.

ranged from 5 to 11%, with a similar magnitude as in DS (Fig. 4A). FDS indicated that 47.7% of facial traits were significantly different in NS (Fig. 4B).

Neurofibromatosis type 1 was associated with minor facial dysmorphologies, which were less abundant and less severe than in the previous syndromes (Fig. 5A). Individuals with NS only presented 11.4% of significantly different facial traits as compared to controls, and the percentages of relative change were low, mostly ranging from 1 to 5% (Fig. 5A,B). The largest difference was a 10% increase in facial distance between the glabella and the labiale superius (Fig. 5A). Along with larger distances in the midline of the face, EDMA detected reduced distances on the right and left sides of the face, with shorter distances from the right and left chelion to the eye landmarks, the endocanthion and the palpebrale inferius. Hypertelorism was not present in individuals with NF1 (Fig. 5A). In NF1, the FDS score was not significant (Fig. 5B), indicating that the facial dysmorphology

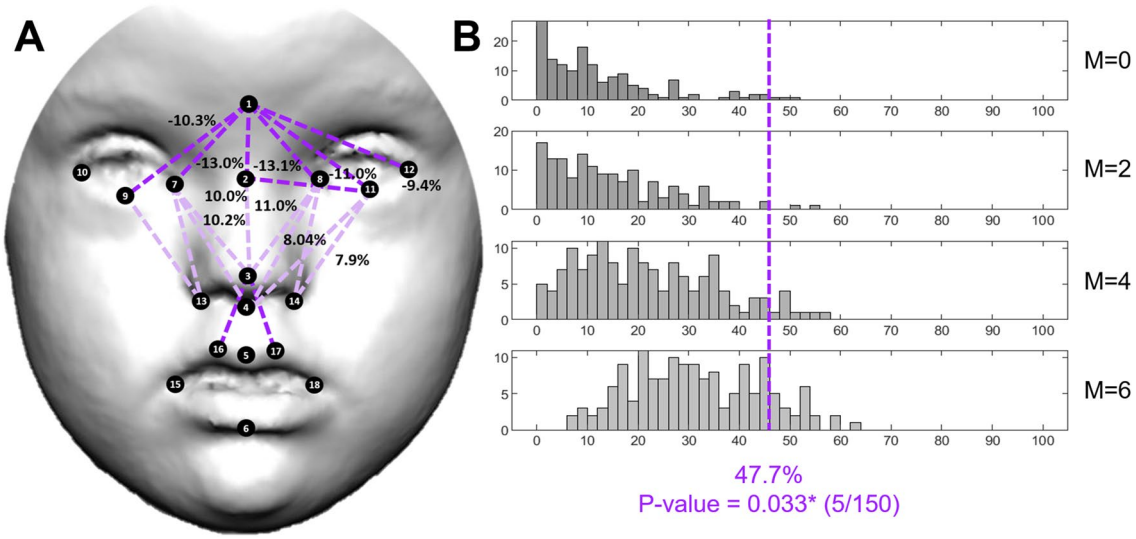

Figure 4. Localized Euclidean Distance Matrix Analysis facial shape pairwise contrasts and iterative bootstrapping tests of facial dysmorphology between controls and individuals diagnosed with Noonan syndrome. From top to bottom histograms, the simulations included 0, 2, 4 and 6 patients. For more details see legend in Fig. 2.

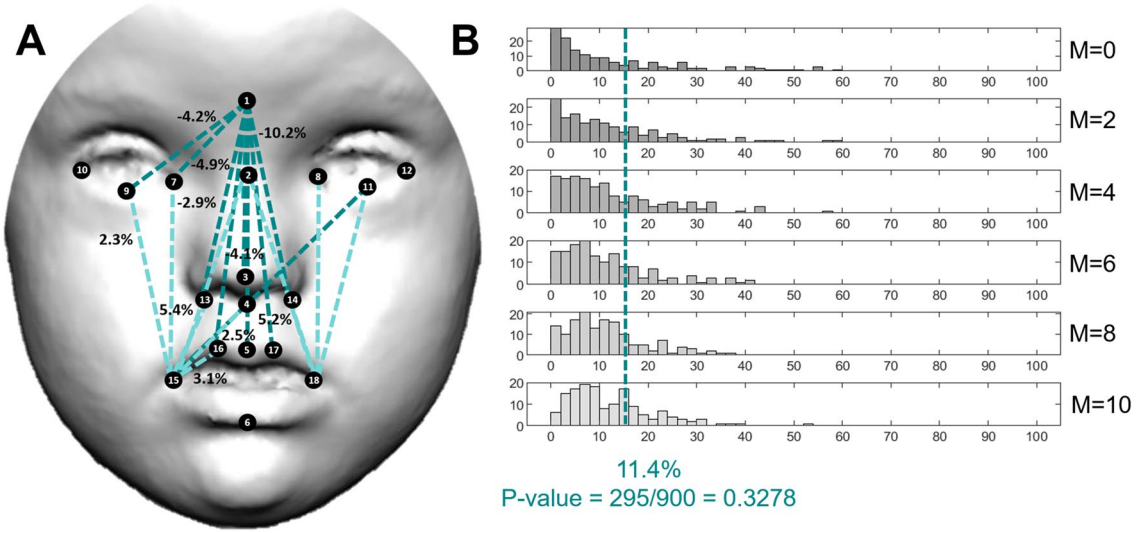

Figure 5. Localized Euclidean Distance Matrix Analysis facial shape pairwise contrasts and iterative bootstrapping tests of facial dysmorphology between controls and individuals diagnosed with Neurofibromatosis type 1. From top to bottom histograms, the simulations included 0, 2, 4, 6, 8 and 10 patients. For more details see legend Fig. 2.

pattern associated with NF1 is so subtle that overall is not larger than facial differences that could be randomly detected using a sample of control subjects.

For the other syndromes, the simulation tests confirmed that the facial dysmorphologies associated with Down, Morquio and Noonan syndromes were significant and different from random comparisons in control subjects. Few simulations resulted in a higher FDS than the FDS obtained with the complete real sample (Figs. 2B, 3B, 4B, first row and blue line). Moreover, in DS, MS and NS, facial dysmorphology scores increased as larger numbers of diagnosed individuals were included in the simulations (Figs. 2B, 3B, 4B, middle rows), confirming the severity of the facial dysmorphologies associated to these syndromes. Finally, the simulations comparing all recruited diagnosed individuals (last row) with random subsamples of control subjects (first row) indicated that FDS scores can range widely from 10 to 80%, underscoring the biasing effects of small sample sizes.

Face2Gene accuracy in Colombian and European populations. After quantifying the facial dysmorphologies associated to DS, MS, NS and NF1 in the Colombian sample, we tested the accuracy of the diag-

nosis provided by the automatic diagnostic algorithms of Face2Gene. We assessed the correspondence between the estimated Face2Gene diagnosis based on facial frontal 2D images with the diagnosis based on clinical and genetic testing.

Face2Gene estimated Down syndrome diagnosis with top-1 accuracy of 100%, as DS diagnosis was listed as the first diagnosis in all individuals, with an average gestalt similarity of 6.2 (Table 2, Fig. 6). When comparing the gestalt similarities in Colombian and European populations, a Wilcoxon test did not find a significant difference between the average gestalt similarity ($P=0.4$). However, a Levene test detected a significant difference in the variance of gestalt similarity scores ($P=0.01$). Whereas in the Colombian population the gestalt similarity in DS ranged from very high to very low; in the European population the range of variation was limited from very high to medium (Fig. 7).

In Morquio syndrome, the top-1 accuracy of Face2Gene was 0%, as the specific diagnostic of mucopolysaccharidosis type IVA (MPSIVA) was never listed as a first prediction (Table 2). Although Face2Gene could not identify the specific type of MS, the automatic diagnostic algorithms associated the facial dysmorphologies with a diagnosis related with mucopolysaccharidosis disorders in 36.4% of cases, with a medium–high average gestalt similarity of 5.6 (Table 2). When the first 5 diagnostic predictions were considered, the top-5 accuracy raised to 45.4% for exact MPSIVA diagnosis and to 100% for mucopolysaccharidosis disorders, but with a low-medium gestalt similarity (Table 2, Fig. 6). In our sample, we detected four genetic variants (p.Gly301Cys, p.Arg386Cys, p.Arg94Cys, p.Gly333Asp, and p.Ser80Leu) that are missense mutations commonly found in the Colombian population[45] (Table S2). Due to the small sample size and genetic heterogeneity of the patients, it was not possible to test whether different genetic variants were associated to different facial phenotypes. Comparative European samples were not available.

The top-1 accuracy of Face2Gene for Noonan syndrome was 66.7%, with a medium–high average gestalt similarity of 5.2 when considering subjects in which the diagnosis was successful (Table 2). Top-5 accuracy increased to 77.8% for exact NS diagnosis, and to 88.9% when considering Noonan Syndrome-Like Disorder diagnoses,

	Top-1 accuracy				Top-5 accuracy			
	Exact diagnosis		Within disorder family		Exact diagnosis		Within disorder family	
	% cases	Gestalt similarity	% cases	Gestalt similarity	% cases	Gestalt similarity	% cases	Gestalt similarity
DS	100	6.2	100	6.2	100	6.2	100	6.2
MS	0	0	36.4	5.6	45.4	3	100	3.4
NS	66.7	5.2	66.7	5.2	77.8	4.7	88.9	4.4
NF1	8.3	1	50	1.7	66.6	1.2	66.6	1.6

Table 2. Accuracy of Face2Gene diagnosis based on 2D facial images in Down, Morquio, Noonan and Neurofibromatosis type 1 syndromes in a Colombian population. Percentage of cases matching the genetic diagnosis are provided for each syndrome, as well as gestalt similarity values.

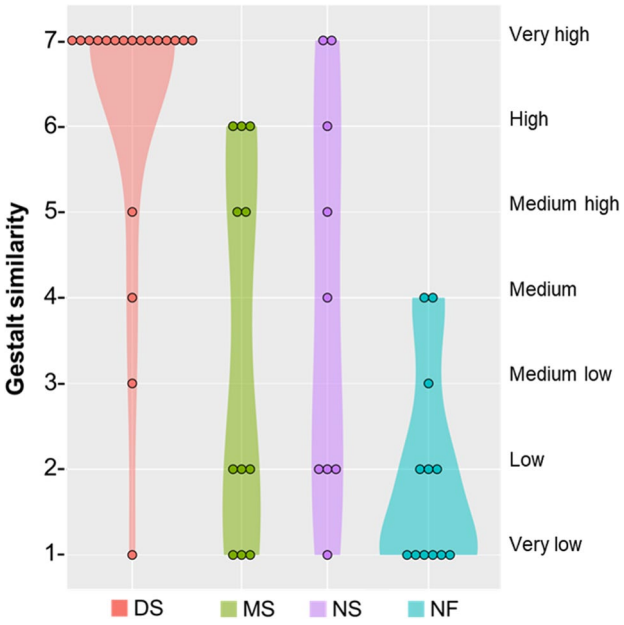

Figure 6. Gestalt similarity scores between Colombian individuals and Face2Gene models of Down, Morquio, Noonan and Neurofibromatosis type 1 syndromes. Violin plots are based on top-5 accuracy Face2Gene predictions within family disorder. Each plot shows the number of individuals scored at each gestalt similarity level, from very high to very low.

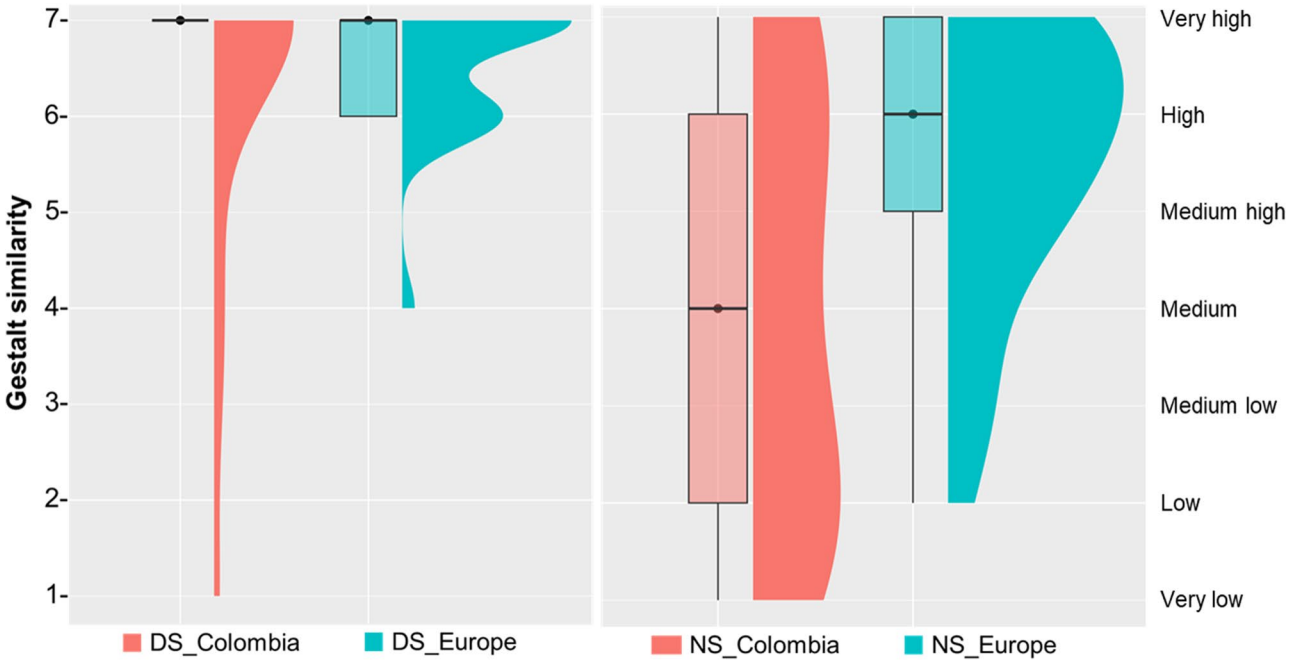

Figure 7. Comparison of gestalt similarity scores between Colombian and European populations in Down and Noonan syndromes. Raincloud plots are based on top-5 accuracy Face2Gene predictions within family disorder, and show the corresponding average gestalt similarity score, the range of variation, and the distribution within each disorder and population.

with a medium gestalt similarity of 4.4 and wide variation among individuals (Table 2, Fig. 6). Although differences did not reach statistical significance probably due to small sample sizes ($P = 0.09$), the comparison between populations showed that in Europe, both the diagnostic accuracy and the gestalt similarity were higher than in Colombia. Using 2D images of patients from European origin, the Face2Gene top-1 accuracy for NS was 100% and the average gestalt similarity was 5.5 (Fig. 7).

Finally, in Neurofibromatosis type 1, Face2Gene presented a top-1 accuracy of 8.3% associated with a very low gestalt similarity of 1 (Table 2). When diagnoses within the RASopathies disorder family were considered, 5 out of 12 individuals were diagnosed as Noonan syndrome and the top-1 accuracy raised to 50% (Table 2). The top-5 diagnostic accuracy was 66.6% and was associated with low gestalt similarity values of between 1 and 2 in 87.5% of individuals (Table 2, Fig. 6). Comparative European samples were not available for NF1.

Discussion

Our analyses provided an accurate quantitative comparison of facial dysmorphologies in Down, Morquio Noonan and Neurofibromatosis type 1 syndromes in a Latin-American population from Colombia. An objective and highly detailed description of the facial phenotype is a major improvement over qualitative descriptions of the complex facial dysmorphologies associated with these genetic disorders. We quantified local facial trait differences presented in people diagnosed with these disorders as compared with age matched controls of the same population, localizing the largest statistically significant facial dysmorphologies.

Our results indicated differential facial patterns associated with each disorder, with major significant dysmorphologies in DS, MS and NS, and minor facial dysmorphologies associated with NF1. Different types of genetic alterations, which ranged from aneuploidy and overall genetic imbalance in DS; to point genetic mutations affecting different processes or signaling pathways, such as the metabolism of mucopolysaccharides in MS, and the *RAS/MAPK* pathway in NS and NF1, significantly affected the facial phenotypes. These genetic alterations deviate the signaling pathways regulating normal facial development[16,58], and alter normal morphogenesis and growth during pre- and postnatal development[15] of individuals with genetic and rare disorders.

Population-specific facial traits in Colombian individuals with genetic and rare disorders. Overall, the facial patterns observed in the Colombian Latin-American population coincide with the descriptions reported in the literature for each syndrome [48,59–61]. However, there are specific local traits that differ, suggesting that facial traits associated to genetic and rare diseases might be modulated by population ancestry, as a result of different evolutionary and adaptive histories of human populations[33–35].

Down syndrome. Down syndrome presents a worldwide prevalence of 14 per 10,000 live births, with life expectancy increasing from 25 to 60 years in developing countries[62–65]. In most Latino-American regions, the real incidence of patients with DS remains unknown, and is usually underreported. A cross-sectional study in Brazil reported a DS birth rate of 4 cases per 10,000 live births[66]; whereas in Colombia several studies have reported a prevalence rate between 1 per 1,000 to 5 per 10,000 live births[67,68]. DS is an aneuploidy caused by trisomy of

chromosome 21, and is the leading genetic cause of intellectual disability[63]. Moreover, DS is associated with craniofacial dysmorphologies that impair vital functions such as breathing, eating, and speaking. In the literature, the DS craniofacial phenotype is mostly based on the analysis of European descent populations, and the characteristic traits include brachycephalic heads with maxillary hypoplasia leading to facial flatness; depressed nasal bridge and reduced airway passages[59]; dysplastic ears with lobe absence; eyes with oblique palpebral fissures, epicanthal folds, strabismus and nystagmus[16,69]; and oral alterations including open mouth, cleft lip, lingual furrows and protrusion, macroglossia, micrognathia, and narrow palate[70,71].

In the Colombian population, we found facial dysmorphologies that are consistent with the craniofacial patterns reported in the literature. For instance, our analyses detected differences in linear facial measurements that correspond to typical DS traits such as hypertelorism, maxillary hypoplasia, and shorter and wider faces associated to a brachycephalic head[16,72]. Results also suggested other characteristic traits of DS, such as midfacial retrusion, and depressed nasal bridge[59]. Open mouth and macroglossia[70,71] were also observed during the photographic sessions in the participants of our study.

However, in contrast to European and North American populations[55], in the Colombian population we detected that the mouth was wider in individuals diagnosed with DS as compared to euploid controls. This difference could be caused by unnatural facial gestures of the participants when asked to close the mouth during the photo shoot, or by facial differences associated to ancestry. In fact, Kruszka et al.[33–35] analyzed individuals diagnosed with DS in diverse populations, and showed craniofacial differences between individuals from different populations (Africans, Asians, and Latin Americans), demonstrating that ancestry is a relevant factor when assessing craniofacial variation associated to rare disorders.

Morquio syndrome. In Morquio syndrome, the worldwide prevalence ranges from 1 case per 75,000 to 1 per 200,000 live births; whereas in Colombia the prevalence rises up to 0.68 per 100,000 live births[45]. As a mucopolysaccharidosis syndrome, the typical alterations of MS involve the supporting tissue and the osteoarticular system[73]. Individuals with MS display abnormalities such as skeletal dysplasia, short stature and trunk, kyphoscoliosis, pectus carinatum, genu valgum, and joint hyperlaxity[74]. Oral diseases often include periodontal disease, malocclusions, caries, and premature tooth loss[46]. Individuals with MS show coarse facies, with an excessively rapid growth of the head[48]. Craniofacial features include a prominent forehead, hypertelorism, prognathism, wide mouth and nose, depressed nasal bridge, plump cheeks, and lips with an oversized tongue[48]. In the Colombian population, the facial dysmorphologies observed were consistent with traits reported in the literature, which included hypertelorism, prognathism, wide nose, and wide mouth[46,48].

In the Colombian sample, Morquio syndrome was associated with the most severe facial dysmorphologies. Considering that keratan and chondroitin sulfate alterations associated with MS cause irreparable damage to leukocytes and fibroblasts, and accumulate over life inducing extreme deformations of the osteoarticular system, facial dysmorphologies associated with MS are expected to increase with age, becoming more severe in adult individuals[46]. Further research is required to test this hypothesis and to assess whether pharmacological treatments can slow down the progression of the disease and reduce the facial dysmorphologies associated with MS. This is especially relevant in Colombia, which is a country with one of the highest prevalence of MS in the world[45].

Moreover, dysmorphologies associated with MS vary among individuals. Typically, MS patients present severe phenotypes, although less severe forms have been described as mild or attenuated phenotypes[73]. There is no consistent evidence regarding the genotype–phenotype correlation in MS, and whether different *GALNS* mutations are associated with the degree of severity in facial dysmorphology. In our Colombian sample, we detected four genetic variants (*p.Gly301Cys*, *p.Arg386Cys*, *p.Arg94Cys*, *p.Gly333Asp*, and *p.Ser80Leu*). Two of these genetic variants, *p.Gly301Cys* and *p.Arg386Cys*, that are the most frequently reported mutations in cases of Morquio syndrome; specifically in Colombia, but also in other American (Brazil, Chile, Argentina, Canada), and European countries (Spain, Portugal, Italy, Poland)[45,75–77]. The high prevalence of the *p.Gly301Cys* mutation in the Colombian population could result from founder and migration effects[45]. The *p.Arg386Cys* variant has been further detected in China and Turkey[75–77]; whereas the *p.Arg94Cys* allele has been previously reported in Middle East, Brazil, and Italy[76,77]. Other genetic variants, such as *p.Ile113Phe*, which are more frequently reported in British and Irish populations[45,75–77], were not detected in our Colombian sample. Further tests with larger samples associated to each genotype are needed to test whether the population-specific genetic variants can be associated to different facial phenotypes in Morquio syndrome.

RASopaties: Noonan and NF1 syndromes. Regarding Noonan syndrome, the worldwide prevalence of NS is 1 per 1,000 to 1 per 2,500 live births[49]. NS is the most common type of RASopathy, and is a rare genetically heterogeneous autosomal dominant disorder caused by mutations in either the *PTPN11*, *SOS 1*, *KRAS*, *BRAF* or *RAF1* genes. Individuals with NS display facial features such as hypertelorism, epicanthic folds, strabismus, downward slanting palpebral fissures, ptosis, high arched palate, deeply grooved philtrum with high peaks of upper lip vermillion border, midfacial hypoplasia and micrognathia, broad flat nose, low-set posteriorly rotated ears, curly/sparse/coarse hair, and short webbed neck[60]. In the Colombian population, we detected hypertelorism, downward slanting palpebral fissures, and midfacial hypoplasia in cases of NS, as reported in populations of European descent[60]. In addition, our results quantified relative changes in the position of the mouth in Colombian individuals diagnosed with NS not reported before[78].

In Neurofibromatosis type 1, the worldwide incidence is 1 per 2,500 to 1 per 3,000 individuals[49]. NF1 is an autosomal dominantly inherited neurocutaneous disorder caused by a mutation in the *neurofibromin* gene. The clinical manifestations of NF1 are variable, and the timing of the onset has a major influence[49]. Regarding craniofacial traits, individuals with NF1 present macrocephaly, facial asymmetry caused by dysplasia of the sphenoid wings[61], as well as bone deformities caused by plexiform neurofibromas, enlarged mandibular canal,

retrognathic mandible and maxilla, and short cranial base[79]. The facial pattern associated with NF1 in individuals from Colombia was also compatible with typical traits of NF1, such as midface hypoplasia[49]. However, our results did not detect facial asymmetry or hypertelorism as prominent facial differences between diagnosed individuals and controls in the Colombian population[49].

Overall, our results support previous evidence demonstrating that rare disorders present distinctive facial traits that are population specific, with clinical features that are significantly different in Africans, Asians, and Latin Americans[34–36]. However, comparative facial quantitative analyses including subjects from different world regions are not usually available for most genetic and rare disorders, and reference data for diagnosis is mainly based on phenotypes defined on populations of European descent. In fact, almost no images of individuals of Latin American origin are included in reference medical texts[16]. Our results underscore the need to extend the analyses to populations from all over the world to achieve a complete and more accurate phenotypic representation of genetic and RD to optimize the diagnostic potential of facial biomarkers in the clinical practice.

Variable accuracy diagnosis in a Colombian population with diverse ancestry. Deep learning algorithms such as Face2Gene have shown potential as a reliable and precise tool for genetic diagnosis by image recognition[9,26,80,81]. In the Colombian sample analyzed here, Face2Gene diagnosed Down syndrome with 100% accuracy, with the same accuracy as in the European sample. This result suggests that in a relatively common genetic disorder such as DS, in which the machine learning algorithm is likely trained in a large sample of individuals with a distinctive and well-represented facial phenotype, Face2Gene shows high diagnostic accuracy, independently from the genetic ancestry.

However, we found that this result cannot be extrapolated to other rare disorders. For instance, we detected a lower accuracy in the diagnosis of Noonan syndrome in the Colombian sample as compared with the European sample. Although Face2Gene correctly identified the disorder in most Colombian subjects, especially when considering the top5-accuracy within Noonan syndrome-like disorders (88.9%), the percentage of top1-accuracy was reduced from 100% to 66.7% in the Colombian sample. We hypothesize that when machine learning algorithms are trained in a relatively small sample of individuals with homogeneous European ancestry, the accuracy of diagnosing rare disorders might be more sensitive to population ancestry. Individuals from diverse populations may show lower gestalt similarity scores when assessed with predictive models that are trained on a population with different genetic and facial variation, and this may lead to reduced diagnostic accuracy.

Unfortunately, no data was publicly available on European samples to compare the diagnostic accuracy of Face2Gene in Morquio and Neurofibromatosis type 1 syndromes. Our results showed that the top1-accuracy for exact diagnosis of Mucopolysaccharidosis type IVA was 0% in the Colombian sample, despite Morquio syndrome was associated with the most severe facial dysmorphologies. Only a low percentage of cases (36.4%) were identified as a mucopolysaccharidosis-like syndrome in the first prediction. In the case of NF1, the top1-accuracy was also very low (8.3%), although the facial dysmorphologies in this disorder were less abundant and severe, and this result could just reflect the difficulty to diagnose NF1 from facial traits.

Finally, in the Colombian sample we detected a wide range of variation in gestalt similarity scores for most disorders, even for Down syndrome. In European subjects, the gestalt similarity for DS was high or very high in 95.5% of cases, and only 5% of subjects showed a medium gestalt score, even when the images included in Ferry et al. (2020)[56] were ordinary photos with uncontrolled lighting, pose, and image quality. In Colombia, 79% of individuals diagnosed with DS were associated with very high gestalt similarity values, but in 21% of subjects the gestalt similarity was lower, and ranged from medium–high to very low values. Specifically, individuals with the lowest scores exhibited traits that suggested an admixed ancestry, a hypothesis that needs further assessment.

The potential of facial biomarkers to diagnose genetic and rare disorders. Qualitative visual assessment of facial dysmorphologies is frequently employed for diagnosis, clinical management and treatment monitoring of RD[16]. Experts in dysmorphologies can identify the facial "gestalt" distinctive of many dysmorphic syndromes[16]. However, this facial assessment relies on the expertise of the clinician, and is very challenging because there is no clear one-to-one correspondence between disorders and facial dysmorphologies. Different genetic mutations can cause the same syndrome or similar phenotypes, whereas the same mutation can induce different phenotypes[12,82]. In addition, within the same rare disease there may be several subtypes, and symptoms may vary even within individuals of the same genetic disorder and the same family[3]. This complex biology generates confusion at the time of diagnosis and warrants the development of efficient, objective and reliable diagnostic methods.

Computer-assisted phenotyping can overcome these pitfalls and provide widely accessible technologies for quick syndrome screening[6]. In this automated approach, methods can be based on 2D or 3D images[9,10,26]. The advantage of 2D methods is that data collection is easy and can be readily translated into the clinical practice, as physicians can take facial images even with simple digital cameras or smartphones. The collection of 3D models is more sophisticated and requires specialized equipment but provides more accurate phenotype descriptions by incorporating the depth dimension.

To further improve the methods of craniofacial assessment to diagnose individuals with genetic syndromes and RD that exhibit facial dysmorphologies, it is crucial to assess the large morphological variation displayed by human populations in facial phenotypes. Factors such as age, sex and ancestry should be accounted for in diagnostic methods. Clinical manifestations in some genetic disorders usually begin at an early age, with two thirds of patients expressing symptoms before the second year of birth[3]; although in other disorders facial dysmorphologies develop later, during postnatal development. Male and female faces present sexual dimorphism at adulthood[83], and diseases can differently affect the facial phenotype depending on sex differences[84].

The role of population ancestry in the facial phenotype associated with genetic and rare disorders also needs to be further investigated in future analyses, assessing the reliability and validity of automatic diagnostic tools in admixed populations with diverse contributions of Amerindian, African and European ancestry components. This is critical in rare disorders with heterogenous clinical presentation and phenotype, where clinical diagnosis is a challenging process[5,6] that may take several years, leading to the so-called diagnostic odyssey[7].

Accurate and early diagnosis of genetic and rare disorders are crucial for adequate health care and clinical management. Without a diagnosis, individuals and their families must proceed without basic information regarding their health and future developmental outcomes[6]. Even though gene-based technologies have greatly improved diagnostic procedures[25], the mutations causing many rare diseases are still not known and access to genetic testing is limited[3]. Genetic consultations may become a long process, and broad molecular testing such as exome and genome sequencing represent a high expense that is not affordable for all families and health care systems, especially in low-medium income countries[7]. In this context, faster, non-invasive and low-cost diagnostic methods based on facial phenotypes emerge as complementary tools for providing earlier first reliable diagnoses[9,10,25,26].

Therefore, in future research the recruitment of participants must be expanded to include as many individuals with RD as possible, together with large comparative samples of age-matched controls, from both sexes, and from diverse world regions that faithfully represent the complex craniofacial variation and evolutionary histories of human populations. For instance, the population in Southwestern Colombia is characterized by high levels of admixture from people with Native American, African, and European ancestry[44,85]. Including the morphological variation of faces from such different ancestry backgrounds is key to pinpoint the facial dysmorphologies associated with diseases in worldwide diverse populations[86]. Our simulation analyses further highlight the importance of maximizing the recruitment of diagnosed and control individuals, as results considerably change depending on the cohort and sample sizes.

Conclusions

Facial phenotypes associated with genetic and rare disorders can be influenced by population ancestry[34–36]. Our ancestry comparisons highlight that diverse genetic background variation can modulate the phenotypic response to disease, affecting the accuracy of current tools of clinical diagnosis. In the future, deep learning algorithms including a high variety of populations with different ancestry backgrounds will optimize the precision and accuracy of diagnosis in an unbiased approach. Such predictive models will support clinicians in decision-making across the world.

Data availability

Raw phenotype data from the Colombian population cannot be made available due to restrictions imposed by the ethics approval. Images from publicly available sources can be accessed from the original publications[56,57]. Anonymized landmark data and Matlab code for computing Facial Dysmorphology Score (FDS) is available at https://github.com/xaviersevillano/EDMA_FDS_analysis_2D.

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Author contributions

L.M.E., E.C., H.P. and N.M.A. designed the study and wrote the manuscript; L.M.E., E.C., E.G., P.S., D.R., D.O, J.C.C., H.P. and N.M.A. organized and performed data collection; L.M.E., E.C., A.G, X.S. and N.M.A. performed data analysis and prepared the figures. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

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ORIGINAL ARTICLE

Objective differential diagnosis of Noonan and Williams–Beuren syndromes in diverse populations using quantitative facial phenotyping

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Abstract

Introduction: Patients with Noonan and Williams–Beuren syndrome present similar facial phenotypes modulated by their ethnic background. Although distinctive facial features have been reported, studies show a variable incidence of those characteristics in populations with diverse ancestry. Hence, a differential diagnosis based on reported facial features can be challenging. Although accurate diagnoses are possible with genetic testing, they are not available in developing and remote regions.

Methods: We used a facial analysis technology to identify the most discriminative facial metrics between 286 patients with Noonan and 161 with Williams-Beuren syndrome with diverse ethnic background. We quantified the most discriminative metrics, and their ranges both globally and in different ethnic groups. We also created population-based appearance images that are useful not only as clinical references but also for training purposes. Finally, we trained both global and ethnic-specific machine learning models with previous metrics to distinguish between patients with Noonan and Williams–Beuren syndromes.

Results: We obtained a classification accuracy of 85.68% in the global population evaluated using cross-validation, which improved to 90.38% when we adapted the facial metrics to the ethnicity of the patients ($p = 0.024$).

Conclusion: Our facial analysis provided for the first time quantitative reference facial metrics for the differential diagnosis Noonan and Williams–Beuren syndromes in diverse populations.

KEYWORDS

facial analysis, facial phenotyping, machine learning, Noonan, Williams–Beuren

1 | INTRODUCTION

Noonan syndrome is a congenital genetic disorder that affects between 1 per 1000 and 1 per 2500 live births (Noonan, 1994; Nora, 1974), and it is caused by different mutations in several genes (OMIM #163950, #605275, #609942, #610733, #611553, #613224, #613706, #615355, #616559, #616564, #618499, #618624 or #619087). Subjects with Noonan syndrome typically present characteristic facial features and short stature (Allanson et al., 2010; van der Burgt

et al., 1999), and about half have congenital cardiac abnormalities (Noonan, 1994). Although it is generally diagnosed based on the observation of key features, molecular testing can provide a confirmation of diagnosis in about 70% of the cases (Allanson & Roberts, 1993; Bhambhani et al., 2014). An early diagnosis is not only important for a prompt treatment but also to provide genetic counseling to the family. However, early diagnosis of Noonan syndrome is challenging and late diagnoses are frequent, with reports showing an average age of diagnosis of 9 years (Sharland et al., 1992).

The differential diagnosis of Noonan syndrome includes Williams–Beuren syndrome (OMIM #194050) (Allanson, 1987; Morris, 1993), among other disorders. Williams–Beuren syndrome has a prevalence of about 1 in 7500 live births (Strømme et al., 2002)[,] and patients with this condition present similar characteristics to patients with Noonan syndrome, including facial dysmorphology and short stature (Allanson, 1987; Cassidy & Allanson, 2010; Morris, 1993). Williams–Beuren syndrome is also associated with congenital heart disease (Morris, 1993, 2010). As both the physical manifestations and their severity are variable, individuals with Williams–Beuren syndrome are often undetected during early childhood, with an average diagnostic age of 3.66 years (Huang et al., 2002). Diagnostic confirmation of Williams–Beuren syndrome is often attained using fluorescence in situ hybridization, but it can also be established using other techniques such as array comparative genomic hybridization (Pober, 2010).

Diagnostic tests are typically requested after the identification of signs and symptoms associated with either Noonan or Williams–Beuren syndrome, and they are often not available in developing countries. In many cases, the examination is made based only on phenotypical observations and symptoms, which may lead to errors and delays in the correct diagnosis. Although several studies have reported independently similar facial phenotypes among patients with Noonan and Williams–Beuren syndrome, there are also studies reporting distinctive facial features specific to each syndrome (Allanson, 1987; Castelo-Branco et al., 2007; Digilio & Marino, 2001; Morris & Mervis, 2000; Noonan, 1994; Romano et al., 2010; Winter et al., 2018; Wu et al., 1999). However, even though these distinctive observations are often found in patients presenting either Noonan or Williams–Beuren syndromes, they are not always present and they are modulated by the ethnic background of the patients(Kruszka, Porras, Addissie, et al., 2017; Kruszka et al., 2018). An objective and accurate way to differentiate between these two genetic syndromes can significantly improve the clinical management of these patients and their outcomes.

In this work, we use a digital facial analysis technology to objectively quantify and illustrate facial phenotypical differences between patients with Noonan and Williams–Beuren syndrome. We use our technology to determine a set of objective metrics that can be used as a reference to help differentiating between these two syndromes. As the phenotype of genetic syndromes is modulated by the ethnic background of the patients (Kruszka, Addissie, et al., 2017; Kruszka, Porras, Addissie, et al., 2017; Kruszka et al., 2018; Kruszka, Porras, Sobering, et al., 2017), we also present the metrics that are relevant for patient populations from four different ethnic groups: African descent, Asian, Caucasian, and Latin American.

1.1 | State of the art

The phenotypical observations of patients with Williams–Beuren and Noonan syndromes have been studied independently in the literature (Allanson, 1987, 2016; Kruszka, Porras, Addissie, et al., 2017; Kruszka et al., 2018; Morris, 1993, 2010; Noonan, 1994; Roberts et al., 2013). Some studies have reported similar facial observations among patients with either of those syndromes: hypertelorism (Allanson, 1987; Levin & Enzenauer, 2017; Noonan, 1994; Wu et al., 1999), telecanthus (Castelo-Branco et al., 2007; Chen, 2012; Morris & Mervis, 2000; Romano et al., 2010), ptosis (Allanson, 2016; Digilio & Marino, 2001; Winter et al., 2018), epicanthal folds (Allanson, 2016; Kruszka et al., 2018; Morris, 1993; Roberts et al., 2013), and short nose (Allanson, 2016; Kruszka et al., 2018; Morris, 1993; Roberts et al., 2013). However, other studies have reported distinctive facial features between patients with Williams–Beuren and Noonan syndromes. Patients with Noonan syndrome are often described as presenting low-set ears and widely spaced eyes (Bertola et al., 2006; Essawi et al., 2013; Kruszka, Porras, Addissie, et al., 2017; Rokhaya et al., 2014; Şimşek-Kiper et al., 2013), whereas patients with Williams–Beuren syndrome are described as presenting a short nose and a wide mouth (Kruszka et al., 2018; Patil et al., 2012; Pérez Jurado et al., 1996). Other discriminative facial features reported include down-slanted palpebral fissures in patients with Noonan syndrome (Bertola et al., 2006; Essawi et al., 2013; Hung et al., 2007; Kruszka, Porras, Addissie, et al., 2017; Şimşek-Kiper et al., 2013) and a long philtrum in patients with Williams–Beuren syndrome (Kruszka et al., 2018; Patil et al., 2012; Pérez Jurado et al., 1996). However, as given in Table 1, variable reports on the incidence of these observations suggest that those characteristics are not discriminative for an accurate differential diagnosis based on physical observations between Noonan and Williams–Beuren syndromes. Only 17% of the patients with Noonan syndrome from Senegal study (Rokhaya et al., 2014) and 58% of the patients from Turkey study (Şimşek-Kiper et al., 2013) were reported as presenting low-set ears. When patients with Noonan syndrome were stratified based on the ethnic background (Kruszka, Porras, Addissie, et al., 2017), 82% of

TABLE 1 Reported incidence of discriminative facial features between patients with Noonan and Williams–Beuren syndromes in different studies and populations

Noonan syndrome					
Study	**Population**	**Low ears**	**Down-slanted eyes**	**Widely spaced eyes**	**Epicanthal folds**
Rokhaya et al. (2014)	Senegal	17%	Not reported	100%	Not reported
Şimşek-Kiper et al. (2013)	Turkey	58%	73%	85%	Not reported
Essawi et al. (2013)	Egypt	57%	100%	100%	Not reported
Hung et al. (2007)	Taiwan	Not reported	59%	Not reported	56%
Bertola et al. (2006)	Brazil	Not reported	66%	44%	Not reported
Yoshida et al. (2004)	Japan	Not reported	Not reported	100%	Not reported
Kruszka, Porras, Addissie, et al. (2017)	African	82%	87%	80%	70%
	Asian	94%	86%	96%	64%
	Latin American	88%	73%	94%	55%
Williams–Beuren syndrome					
Study	**Population**	**Wide mouth**	**Short nose**	**Long philtrum**	**Epicanthal folds**
Patil et al. (2012)	India	100%	100%	85%	52%
Pérez Jurado et al. (1996)	Mixed	Not reported	90%	83%	71%
Kruszka et al. (2018)	African	88%	88%	88%	13%
	Asian	78%	75%	79%	63%
	Latin American	91%	74%	93%	73%

African descent, 94% of Asian, and 88% of Latin American patients presented low-set ears. Similarly, the incidence reports of widely spaced eyes in patients with Noonan syndrome ranged from the 44% reported (Bertola et al., 2006) in a Brazilian population to the 100% reported (Rokhaya et al., 2014) for a patient population from Senegal, and (Hung et al., 2007) for a population from Taiwan.

On the other hand, only 78% of the Asian population with Williams–Beuren syndrome (Kruszka et al., 2018) presented a wide mouth, as compared to the 100% reported (Patil et al., 2012) for an Indian population. When looking at the nose size, 100% of patients from India presented a short nose (Patil et al., 2012), compared with 74% of Latin American (Kruszka et al., 2018).

To the best of our knowledge, quantitative methods to distinguish between patients with Noonan and Williams–Beuren syndrome have been explored only in the study by Preus (Preus, 2008). In that study, a clustering analysis showed that patients with Noonan and Williams–Beuren syndrome are clinically distinguishable. However, that study focused on many clinical observations that are not easily observable. For instance, cardiac abnormalities cannot be observed without the specialized equipment, which may not be available in in rural areas and developing countries. Similarly, although family history information is essential for an early diagnosis, it is sometimes unknown to the clinical team. In addition, that previous study analyzed a small population of patients, it did not provide objective metrics that can be translated into direct clinical use, and it did not consider the ethnic variability of the patients.

In the current study, we provide reference facial metrics adapted to the ethnic background of the patients that can be used directly at any clinic. In addition, we illustrate facial appearance features that can be quantified by computer methods, but only qualitatively assessed by the human eye, and which are relevant to differentiate between Noonan and Williams–Beuren syndrome. To the best of our knowledge, this is the first time that facial analysis technology is used to quantify and illustrate graphically on population-based computer-generated images the specific facial features that allow for the distinction of these two genetic syndromes in diverse populations, in addition to providing reference geometric measurements.

2 | METHODS

2.1 | Data

We evaluated the face photographs of 286 (49 infants, 47 toddlers, 71 children, 28 adolescents, and 91 adults; 150 male and 136 female) individuals with Williams–Beuren

syndrome from 19 countries, and 161 (45 infants, 29 toddlers, 47 children, 18 adolescents, and 22 adults; 93 male and 68 female) patients with Noonan syndrome from 14 countries. All participants were diagnosed with molecular testing and/or clinical evaluation by local expert geneticists. Verbal or written formal consent from the parent/guardian was obtained by local institutional review boards and the protocol #7134 at the Children's National Hospital. A subset of these dataset is publicly available through the "Atlas of Human Malformation Syndromes in Diverse Populations" of the National Human Genome Research Institute – National Institutes of Health (Muenke et al., 2016). Clinical findings and additional details on these data can be found in previous studies (Kruszka, Porras, Addissie, et al., 2017; Kruszka et al., 2018). We categorized the patients into four groups: African descent (28 patients with Williams–Beuren and 35 with Noonan syndrome), Asian (26 patients with Williams–Beuren and 40 with Noonan syndrome), Caucasian (121 patients with Williams–Beuren and 40 with Noonan syndrome), or Latin American (111 patients with Williams–Beuren and 46 with Noonan syndrome). In this study, we only included those patients whose face photographs were frontal, with eyes open, and with even illumination conditions. We discarded all pictures with illumination artifacts or shadows that could affect the appearance of the face. We also discarded pictures in which any part of the face was not totally visible (e.g., glasses, hair over the eyes).

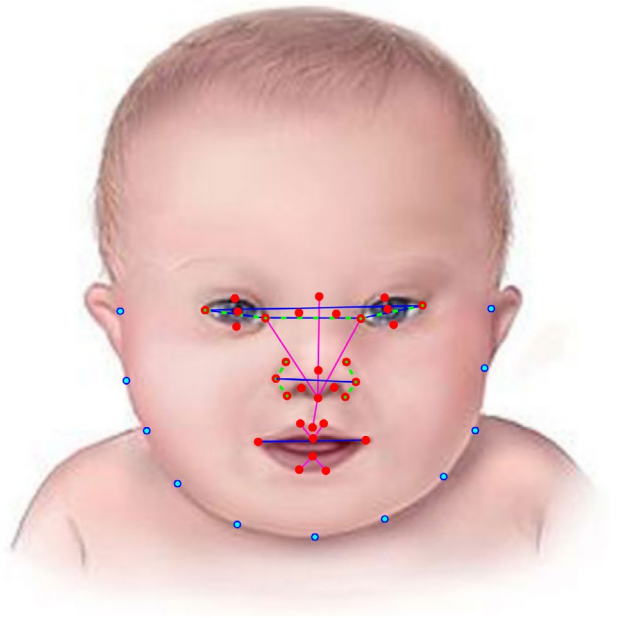

FIGURE 1 Representation of the facial landmarks and geometric metrics. Inner facial landmarks are represented as red circles. Horizontal distances between these landmarks are represented as blue lines. Vertical distances are represented as magenta lines. Angles are represented with green dashed lines, with the center of the angle represented as a green circle around the landmark, and the extremes represented with a green dot inside the landmark

2.2 | Facial analysis

The facial analysis methods used in this study are based on the technology previously described (Cerrolaza et al., 2016; Ojala et al., 1996). We have used that technology to identify Down (Kruszka, Porras, Sobering, et al., 2017), 22q11.2 deletion (Kruszka, Addissie, et al., 2017), Noonan (Kruszka, Porras, Addissie, et al., 2017), and Williams–Beuren syndromes (Kruszka et al., 2018) from healthy individuals in diverse populations.

2.2.1 | Quantification of facial features

Our face analysis technology quantifies a set of geometric measurements (i.e., distances and angles) from 44 anatomical facial landmarks (e.g., lateral canthi, oral commissures…). The location of each of the landmarks and the geometric measurements is represented in Figure 1. We estimated the average of the measurements on the right and left sides of the face to obtain symmetric metrics that are easier to interpret and to use as clinical references, and their absolute differences to quantify asymmetry. All horizontal measurements were normalized with respect to the ear-to-ear distance, and all vertical measurements were normalized to the distance between the mid-point between the oral commissures and the nose root. Asymmetry measurements were normalized with respect to the average value from the measurements at the left and right sides. In addition, our technology quantifies the appearance around each of a subset of 33 inner facial landmarks using texture descriptors based on local binary patterns (LBP) as represented in Figure 2 (Cerrolaza et al., 2016; Ye et al., 2005), which are sensitive to lines, shadows, and local intensity contrast.

2.2.2 | Feature selection and classification

Once all geometric and appearance metrics were calculated, we selected the most discriminative ones between Noonan and Williams–Beuren syndrome using recursive feature elimination (Guyon et al., 2002) based on a support vector machine (SVM) classifier (Cortes & Vapnik, 1995). To compensate for the different number of patients with Noonan and Williams–Beuren syndromes, we used a weighting scheme (Du & Chen, 2005) that balanced the contribution of each individual to the SVM classifier, therefore the total weight of the patients with Noonan and Williams–Beuren syndrome was the same. We evaluated our classifier using leave-one-out cross-validation

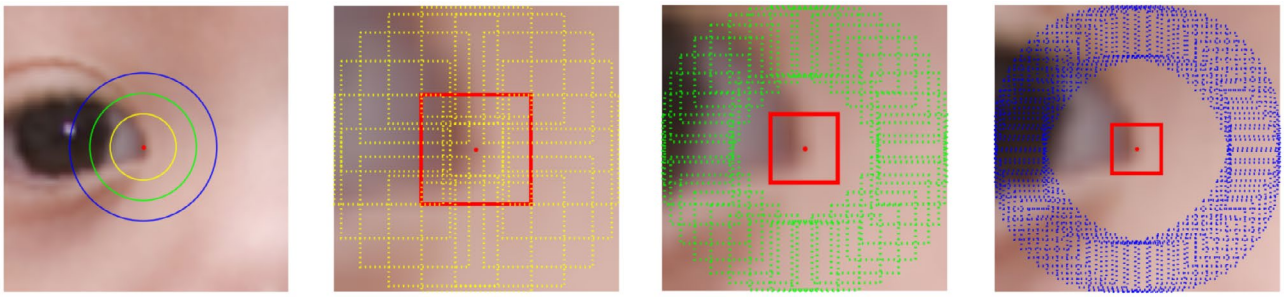

FIGURE 2 Representation of the image patches used to calculate the local binary patterns (LBP) around the medial canthi of the right eye. (a) the area around the landmark that is involved in the calculation of the LBPs at the three resolutions, in yellow for the highest resolution (R1), green for a medium resolution (R2), and blue for the lowest resolution (R3). (b), (c), and (d) illustrate the image patches involved in the calculation of the LBP at resolution levels R1, R2, and R3, respectively. At each level, the LBPs are calculated by comparing the image patch around the landmark (in red) with the patches in their neighborhood (in yellow for R1, green for R2, and blue for R3)

TABLE 2 Interpretation of the quantitative results in the global population

	Significant differences		Relevant differences	
	Noonan	**Williams–Beuren**	**Noonan**	**Williams–Beuren**
Eyes	• More pronounced hypertelorism and telecanthus	• More pronounced down-slanted palpebral fissures	• Higher orbital rim	• Smaller palpebral fissures
Nose		• Longer nasal alas • Shorter nose	• More asymmetric nasal bridge	
Mouth		• Thicker lower lip • Wider mouth		

(Devijver & Kittler, 1982) for increasing numbers of features, and we selected the optimal as the minimum number of features at which the area of the receiving operator characteristic curve converged (Bradley, 1997). In addition to the optimal list of features obtained, we also estimated the individual discriminative power of each feature using the non-parametric Mann–Whitney U test (Mann & Whitney, 1947).

We performed the above process to obtain the optimal list of features that are discriminative in the global population, regardless of the ethnic background of the patients. Then, we repeated it for each different population, thus obtaining a list of optimal discriminant features adapted to the ethnicity of the patients. Finally, we compared the performance of the global and the ethnic-specific models in discriminating between Williams–Beuren and Noonan syndromes.

3 | RESULTS

We obtained an average accuracy of 85.68% in the discrimination of patients with Noonan syndrome and Williams–Beuren syndrome in the global population using the list of 14 optimal facial features identified by our face analysis technology. Specifically, we obtained accuracies of 87.58% and 84.62% in the correct identification of Noonan and Williams–Beuren syndrome, respectively. The list of optimal geometric and appearance features, their distribution, and individual p-value in the global population can be consulted in our supplementary material. The clinical interpretation of those features is given in Table 2, organized according to the region of the face at which they were observed: eyes, nose, and mouth.

We obtained average accuracies of 93.65%, 87.88%, 91.30%, and 89.17% in the African descent, Asian, Caucasian, and Latin American populations, respectively, when using population-specific models. As with the global population, the details of the geometric and appearance facial features can be consulted in our supplementary material. Table 3 gives our interpretation of the optimal features identified for each population.

Table 4 gives the accuracy in differentiating between Noonan and Williams–Beuren syndromes of the models created both for the global population and for each population included in this study. Similar to our previous works identifying genetic syndromes from a healthy population(Cerrolaza et al., 2016; Kruszka, Addissie, et al., 2017; Kruszka, Porras, Addissie, et al., 2017; Kruszka et al., 2018; Kruszka, Porras, Sobering, et al., 2017; Zhao et al., 2014), we obtained improved results when we adapted our technology to specific ethnic groups. In average, we obtained an improvement of 5.49% when using specific models for each ethnicity, with

TABLE 3 Interpretation of the quantitative results in the African descent, Asian, Caucasian, and Latin American populations. Characteristics not observed in the global population are indicated in green

	Significant differences		Relevant differences	
	Noonan	**Williams–Beuren**	**Noonan**	**Williams–Beuren**
African descent population				
Eyes	• More pronounced hypertelorism	• Smaller palpebral fissures with more significant ptosis		• Smaller palpebral fissures • More asymmetric palpebral fissures
Nose				• Thicker/more rounded nasal lobe • More asymmetric nasal alas
Mouth		• Thicker lower lip • Wider mouth		
Asian population				
Eyes		• More pronounced down-slanted palpebral fissures		• Smaller palpebral fissures • More asymmetric palpebral fissures
Nose				• Longer nasal alas
Mouth		• Thicker lower lip • Wider mouth		• More asymmetric philtrum and cupid's bow
Caucasian population				
Eyes	• More pronounced hypertelorism and telecanthus	• More pronounced down-slanted palpebral fissures	• Higher orbital rim	• More pronounced ptosis
Nose		• More asymmetric nasal alas and lobe		• Shorter nose
Mouth		• Thicker lower lip • More asymmetric upper lip thickness • Wider mouth		
Latin American population				
Eyes	• More pronounced hypertelorism • Higher orbital rim			• Smaller palpebral fissures
Nose		• Shorter nose		
Mouth		• Thicker lower lip • Wider mouth		• More asymmetric lips • Flatter philtrum and cupid's bow

TABLE 4 Comparison of the accuracy obtained with the global model (trained with all ethnic groups) and with the specific model trained with a specific ethnic group on each population

Ethnicity	Global model	Ethnicity-specific model	Improvement	*p*-value*
African descent	87.30%	93.65%	7.27%	0.363
Asian	84.85%	87.88%	3.57%	0.800
Caucasian	83.23%	91.30%	9.70%	0.044
Latin American	86.62%	89.17%	1.91%	0.727
Global population	85.68%	90.38%	5.49%	0.024

**p*-value calculated using a Fisher's exact test.

a p-value of 0.024 estimated using a Fisher's exact test. However, our results also show that the improvement is only statistically significant ($p < 0.05$) on the Caucasian population, with a p-value of 0.044.

4 | DISCUSSION

Despite many phenotypical similarities reported in the literature between patients with Noonan and Williams–Beuren

syndrome (e.g., short stature, ptosis, down-slanted palpebral fissures, cardiac abnormalities) (Allanson, 1987; Morris, 1993, 2010; Noonan, 1994; Roberts et al., 2013), our facial analysis demonstrated that these two genetic conditions can be distinguished in the global population with accuracy higher than 85% based only on facial observations. Patients with Noonan syndrome present significantly more pronounced hypertelorism and telecanthus, whereas patients with Williams–Beuren syndrome present significantly more down-slanted palpebral fissures, shorter nose with longer alas, and a wider mouth with a thicker lower lip. In addition, patients with Noonan syndrome are likely to have higher orbital rim and a more asymmetric nasal bridge, and patients with Williams–Beuren syndrome often present smaller and less rounded palpebral fissures, although differences between the two populations in these observations were not found to be statistically significant when evaluated individually.

Our results also indicate that the physical manifestations are modulated by the ethnic background of the patients. Similar to previous works classifying individuals with genetic syndromes from healthy subjects (Kruszka, Addissie, et al., 2017; Kruszka, Porras, Addissie, et al., 2017; Kruszka et al., 2018; Kruszka, Porras, Sobering, et al., 2017), we obtained a higher classification accuracy when we adapted the list of relevant discriminative facial features to specific ethnic groups. Our results show that, although the features described above are discriminative between Noonan and Williams–Beuren syndromes in the global population, there are other features that can be more discriminant on specific populations, either individually or combined with previous features.

In the African-descent population, unlike the global population, the palpebral slanting angle is not essential to discriminate Williams–Beuren and Noonan syndrome. Patients of this ethnic group with Williams–Beuren syndrome often present a more rounded nasal lobe and asymmetric nasal alas, and more asymmetric palpebral fissures. Importantly, although these features combined were relevant to identify patients with Williams–Beuren syndrome from Noonan syndrome, they were not found to be significantly different between the two populations when evaluated individually.

In the Asian population, a wider mouth with a thicker lower lip and more down-slanted palpebral fissures were significant to distinguish patients with Williams–Beuren syndrome from patients with Noonan syndrome. Moreover, patients with Williams–Beuren syndrome often showed more asymmetry in the palpebral fissures and in the cupid's bow and philtrum, in addition to smaller palpebral fissures and longer nasal alas. Differences in these features were not statistically significant when compared individually with patients with Noonan syndrome.

We identified similar discriminative features in the Caucasian population that those found in the general population except for the nasal observations. Moreover, in this population, patients with Williams–Beuren syndrome presented significantly more asymmetric nasal alas and lobe than patients with Noonan syndrome, and a significantly more asymmetric upper lip. They often presented shorter nose as well, although differences with respect to patients with Noonan syndrome were not found to be statistically significant.

The Latin American population with Noonan syndrome showed a significantly higher orbital rim and more pronounced hypertelorism. Patients with Williams–Beuren syndrome presented a significantly wider mouth with a thicker lower lip, and a shorter nose. They often presented smaller palpebral fissures and a flatter philtrum and cupid's bow, but these features were not found to be significantly different between the two populations when evaluated individually.

Although ethnic-specific classification models provided a higher accuracy compared with the model created from the global population, this improvement was statistically significant only for patients from the Caucasian population. One possible explanation for this is a lower phenotypical variability of the Caucasian population used in this work compared with the other ethnic groups. To categorize patients, we followed the racial and ethnic categories used by the National Institutes of Health. However, the Asian population analyzed in this work includes patients from China, India, and Malaysia, thus introducing a high ethnic variability in the Asian group. This higher variability makes it difficult to find ethnic-specific features, which translate into a classification model with an accuracy that is higher in average but not significantly different to the model built from the global population. As more data become available, it will be possible to focus on the study of more specific populations.

Although many of the discriminant facial observations between Noonan and Williams–Beuren syndromes found are consistent among ethnicities (i.e., more significant hypertelorism in patients with Noonan syndrome and wider mouth in patients with Williams–Beuren syndrome), there are a few observations that are specific to each ethnic group and that can be subtle to the human eye. However, they can be quantified using a systematic analysis as presented in this work. Our facial analysis technology uses an objective and quantitative approach to identify and stratify facial phenotypes, which is essential to detect those subtle facial features that are indicators of genetic conditions. In this work, we used this technology not only to distinguish patients with Noonan and Williams–Beuren syndromes, but also to provide reference metrics that can be used in any clinic. Moreover, these metrics were objectively defined for different ethnic groups, which resulted in improved accuracy for the potential diagnosis of the syndromes from phenotypical observations. Our results show the potential

of our facial analysis technology to support the assessment of patients with genetic syndromes in areas of the world with diverse populations and where access to specialists is sometimes limited.

Finally, we also used our technology to create population-based computer-generated images that illustrate the specific appearance of relevant facial features for the differential diagnosis of Noonan and Williams–Beuren syndromes. These images can be used as a reference for the identification of these syndromes in populations with different ethnic background, both for training and diagnostic purposes. However, other observations from clinical evaluation as well as family history or behavioral observations, if they are available, provide additional information that needs to be considered for a clinical diagnosis.

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CONFLICT OF INTEREST

The authors do not have any conflicts of interest that are relevant to this manuscript.

AUTHOR CONTRIBUTIONS

All authors conceptualized this work together. A.R.P and M.G.L. designed the methods. A.R.P. implemented the methods, performed the experiments, and wrote the initial draft of the manuscript. M.G.L. reviewed the results and revised the manuscript. M.S. provided the clinical perspective and revised the results and manuscript.

DATA AVAILABILITY STATEMENT

A subset of the facial photographs used in this study are available through the "Atlas of Human Malformation Syndromes in Diverse Populations" of the National Human Genome Research Institute – National Institutes of Health (Muenke et al., 2016). The discriminative facial metrics between Noonan and Williams-Beuren syndromes and their ranges in diverse population are available as supplementary material of this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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