IMMUNOGENETICS AND HPLC ANALYSES CONTRIBUTE TO UNDERSTANDING THE ETIOPATHOLOGY OF RHEUMATOID ARTHRITIS THROUGH STUDIES ON ANCIENT HUMAN REMAINS

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Genetic investigations on ancient human remains affected by rheumatological pathologies are a research field of particular interest for identifying origins and the etiopathology of diseases, especially those having an autoimmune background such as rheumatoid arthritis (RA). We wish to demonstrate how reliable studies concerning this topic require collaboration between multiple disciplines, usually starting from paleopathologic observations up to immunogenetic screening, even involving analytical chemistry. Here, we focused our investigation on the skeleton of Cardinal Carlo de'Medici (1595-1666) for whom RA and psoriatic arthritis (PsA) were postulated after paleopathologic examination. RA susceptibility is linked to specific HLA alleles belonging to DRB1*04 locus, such as DRB1*0401, while Cw*0602 and DRB1*07 predispose to PsA. Thus, we genotyped the Cardinal's remains to search for RA or PsA "risk genes". Ancient DNA is often subjected to hydrolysis followed by fragmentation. For this reason, all immunogenetic tests were preceded by an original RP-HPLC-FL method able to inform on the ancient DNA preservation and the extent of contamination, with the purpose of avoiding the risk of false positive results. After DNA isolation from a piece of bone from the Cardinal, PCR-SSP and reverse-SSO hybridization assays were applied to perform genomic HLA-typing. RP-HPLC-FL analysis revealed a good preservation of DNA without contamination by exogenous genomes. Molecular tests assigned to the Cardinal the genotype DRB1*0401/*1102 for HLA-DRB locus and Cw*04/*12 for HLA-C locus, data that support a genetic predisposition for RA but not for PsA. This multidisciplinary study has allowed us: (i) to ascertain that the remains undoubtledy belonged to the specific subject, Cardinal Carlo de'Medici; (ii) to sustain that the subject suffered from RA rather then that PsA, and (iii) to state that RA was already widespread in Europe at the Renaissance age, despite some authors claiming that the disease was introduced to the Old Continent from America after colonization during the 18th century

Rheumatological paleopathology is a research field that can provide a strong contribution to clarifying origins and the spreading of numerous diseases (1), especially when anthropological studies on remains investigate world-wide diffusion in former times of arthritic diseases which have an autoimmune background, such as rheumatoid arthritis (RA). The reconstruction of the history of RA is an extremely

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complex task, requiring a multidisciplinary approach, including paleopathological examinations of human remains and the study of archival documents when available. Macroscopic observations and diagnostic images cannot always yield a definite diagnosis. Ancient human remains are not always in the form of an intact tissue architecture or parts are missing. A reliable and complete study of this kind of specimen often needs information from molecular genetic testing, fundamentally based on genotyping assays, when some autoimmune rheumatic diseases (ARDs) associated to the HLA (Human Leukocytes Antigens) system are involved. We focused our attention on some ARDs proposed for Cardinal Carlo de' Medici (1595-1666) since previous paleopathological findings and radiological images of the Cardinal's remains revealed some skeletal abnormalities suggesting RA, although the hypothesis of psoriatic arthritis (PsA) has also been postulated (2). RA and PsA are both ARDs linked to the highest polymorphic human HLA genetic system codified by different HLA loci (3), while RA is a common ARD restricted to some alleles of the HLA-DRB1*04 gene family, e.g. DRB1*0401, and a few others (4). Conversely, HLA-Cw*0602 and DR*07 are considered the main risk genes for PsA (5). Our previous investigation on the origins of RA, carried out on an Italian mummy dating back to the 16th century, demonstrated that at that time RA was already present in Italy, (6) and not imported from America (7). The case of Cardinal Carlo (1595-1666) represents a further opportunity to strengthen our evidence that RA was already present in Italy in 1600 A.D.

The achievement of reliable genetic results from anthropological material requires particular strategies to assure correct integrity and authenticity of ancient DNA (aDNA), to avoid false-positive results due to traces of contaminating modern DNA and to provide unquestionable genetic data (8-9). This information is obtained on the aDNA preservation and its contamination by measuring the D/L enantiomeric ratio of aspartic acid (Asp) and alanine (Ala) using HPLC methods before performing molecular studies on ancient human remains (10). To avoid any introduction of modern DNA, we subjected the Cardinal's bone to our own RP-HPLC-FL analysis (11). We used a compact bone, considered the most suited aDNA source for genetic assays thanks to bony lacunae osteocytes protecting against the introduction of exogenous DNA (12).

We started by analysing a piece of bone from Cardinal Carlo firstly by RP-HPLC-FL and then with a DNA isolation and genomic typing tests to find HLA predisposing genes for both RA and PsA.

MATERIALS AND METHODS

HPLC analysis of D/LAsp and D/LAla racemization

149 mg of the Cardinal's bone fragment were processed as previously discussed (11, 13). In short, this RP-HPLC method is carried out by on-line pre-column derivatization with *o*-phthalaldehyde-*N*-acetyl-*L*-cysteine system and fluorescence detection. *N*-acetyl-*L*-cysteine acts as optically active thiol, a derivative agent leading to formation of diastereomers, because the enantiomers are indistiguishable with column C_{18} used for reverse phase HPLC analysis. Five consecutive sample solutions of derivatized amino acids were injected for each sample. *o*-Phthalaldehyde improve sensitivity for fluorescence detection.

DNA extraction and purification

To prevent modern human contamination, all stages of molecular procedures were carried out under sterile conditions: working areas, reagents containers and tools were decontaminated with sodium hypochlorite (50%, 48 h) and then irradiated with UV light for one hour. Laboratory staff wore protective clothes. An "Olerup SSP HLA Wipe Test-Negative Control" (Olerup GmbH, Vienna, Austria) intended to monitor for introduction of recent DNA prior to and during molecular tests was performed according to the manufacturer's instructions. A whole bone piece of 480 mg was placed into a 15 mL conic tube, washed with a PBS solution for 10 min under vertical rotation and left under UV light 30 min for each side. The more external bone layer was removed with a scalpel and the remaining bone was powdered in a mortar. 200 mg of bone powder were transferred into a 15 mL tube, digested with 4.2 mL of a lysis buffer (Na_2EDTA 0.5 M, pH 8.0; Tris-HCl 10 mM, pH 7.4; NaCl 100 mM; SDS 0.1%, and Proteinase K at final concentration of 270 mg/mL) and then kept in a shaking water bath for 2 days at 37°C. Further 50 mg/mL of Proteinase K were added every 6 hours. Then, the debris was precipitated by centrifugation at 2000*g* for 15 min and the upper solution was recovered. Two different purification procedures were applied to two suspensions of the same digested solution of the Cardinal bone.

Method A. For the sample named CCEE (Cardinal Carlo Extraction with Epicentre kit), 600 mL of the

supernatant were transferred into a 15 mL tube and centrifuged at 7000*g* for 20 min to discard all debris. 350 mL of the suspension containing aDNA were placed into 1.5 mL tube and purified by means of the Masterpure™ DNA Purification Kit (EPICENTRE, Madison, WI, USA), based on salting-out/sodium-acetate/2-propanol extraction method. Some steps of the protocol were modified by us to increase the DNA extraction and purity, due to a weak brown colour in the recovered digested solution suggesting the presence of Maillard products (8). Afterwards, 200 mL of a specific protein precipitation reagent (saturated NaCl solution enriched with sodium acetate) were added to the digested solution, well mixed, and centrifuged at 11000*g* for 10 min. The supernatant was collected in a clean 1.5 mL microcentrifuge tube. This step was repeated twice in order to obtain a clearer aDNA suspension. aDNA was precipitated with pure isopropanol (previously kept at 80°C for 30 min) and centrifuged at 11000*g* for 10 min. The aDNA pellet was washed twice with ethanol 80%, dried and finally dissolved in 50 mL of Tris 10 mM, pH 7.4.

Method B. 800 ml of the sample named CCEC (Cardinal Carlo Extraction with spin Columns) were centrifuged at 7000*g* for 20 min. Three aliquots of 200 mL of the digested aDNA suspension were separately processed using the QIAamp DNA Mini Kit spin columns, (QIAGEN GmbH, Hilden) and eluted with 100 mL of a Buffer Elution (BE: Tris-HCl 10 mM, pH 7.4, without EDTA), with a few modifications of the manufacturer's instructions with the aim to increase the DNA yield as follows: the elution step was realized by adding 50 mL of BE and incubated at 20-25°C for 10 min before adding other 50 mL of BE. To concentrate the aDNA, 100 mL of all of three aDNA eluted solutions were assembled and treated with vacuum centrifuge until a final volume of 50 mL.

HLA-genotyping from whole bones by PCR-SSP at "Low Resolution" (L.R.)

The PCR-SSP (PCR with Sequence-Specific Primers) genotyping assay discriminates all different HLA alleles: perfectly matched primers amplify only a single allele of group of alleles (positive result), while mismatched primer pairs give no amplification (negative result).

The HLA genotyping for DRB locus of CCEE sample obtained with method A was realized through Biotest DRB SSP kit Low Resolution (Biotest, Dreiech, Germany). Each 200 mL tube of a PCR block contained dried internal control represented by primers for the "housekeeping" gene Human Growth Hormone (HGH) along with specific or group-specific primers for different HLA alleles. The first tube acted as negative control where aDNA samples were replaced with ddH_2O. The PCR reaction mixture was slightly modified to facilitate aDNA amplification (enrichment in DNA polymerase and $MgCl_2$). For HLA-DRB test a 24-tube PCR block was employed. The aDNA samples were amplified in a PE 9700 (Perkin-Elmer, Foster city, CA, USA) with the amplification program recommended by Biotest. The PCR products are loaded on a 2% agarose gel stained with EtBr, electrophoresed and visualized at UV transilluminator.

HLA-genotyping from whole bones by reverse SSO hybridization assay

In order to confirm the PCR-SSP DRB results we repeated the genotyping test for HLA-DRB1 locus by using INNO-LiPA HLA-DRB1 Assay (Innogenetics, Ghent, Belgium) with manual procedure. The same LiPA (Line Probe Assay) method has also been used for identification of HLA-Cw genotype. Both loci were tested using CCEC aDNA sample obtained with method B. LiPA test is based on the PCR-SSO principle (PCR followed by reverse hybridization with Sequence-Specific Oligonucleotides) and requires PCR amplification with 5'-biotinilated primers. The probes, immobilized as parallel lines on a nitrocellulose membrane-strip, are designed to hybridize specifically only with complementary sequences, downright discriminating point mutations. For DRB1 alleles assignment, the aDNA target sequence of the exon 2nd, the site of DRB1 polymorphism, was amplified. Here, we describe the procedure for DRB1 analysis, because the protocol for Cw genotyping was the same except for the use of strips coated with SSO complementary to aDNA target sequences for the 2nd and 3rd exons. Therefore, six aDNA samples were simultaneously amplified for screening HLA-DRB1 locus: one positive control represented by a modern DNA sample, one negative control (all reagents without aDNA) and four aDNA solutions of the Cardinal's bone were added to PCR master mix at different concentrations, i.e. 6, 5, 4 and 3 mL instead of 5 mL suggested by the manufacturer's instructions. In fact, the observed impurities co-extracted with nucleic acids can inhibit PCR and the dilution of aDNA solutions is a strategy to improve the amplification. 50 mL of PCR amplification mix were prepared in accordance with the manufacturer's instructions for the positive and negative controls, while for the remaining ancient samples an amount of sterile ddH_2O was varied depending on the volume of the aDNA solution utilized for each PCR mix. Amplification of diluted aDNA solutions helps to choose the better PCR optimized conditions in terms of aDNA amount and size after gel visualization. The cycle parameters were set according to the Biotest SSP program. At the gel electrophoresis only one amplicon of the Cardinal's aDNA was visualized – that corresponding to 3 mL of aDNA diluted solution.

An HLA-DRB1 strip for each amplified sample (positive control, negative control and the 3 mL amplified aDNA) were placed into a different trough of a tray by adding 10 mL of amplification product for positive and negative controls and 15 mL of amplified aDNA. The chemical denaturation of all amplicons was obtained by adding an equal volume of Denaturing Solution (alkaline solution containing EDTA). The denaturation step proceeded for 5 min at 20-25°C. Thereafter, 2 mL of hybridization solution pre-warmed at 56°C were added in each trough and the tray was incubated for 30 min in a shaking water bath at 56°C. During this step, the single strands of DNA target hybridizes only with those SSOPs having complementary sequences. After having taken away the liquid, an incubation with Stringent Wash Solution (buffer containing 0.1% SDS) was protracted for 10 min at 56°C to remove any mismatched amplified material. The strips were then rinsed with 2 mL of Rinsed Wash Solution (RWS) for 1 min. For the color development, a solution of streptavidin conjugated with alkaline phosphatase solution (St-AP) was added for binding to biotinylated hybrids previously formed. After incubation for 30 min at 25°C, all strips were rinsed with RWS and the liquid discarded. This step was followed by adding 2 mL of a substrate consisting of Nitroblue Tetrazolium-5-Bromo-4-Chloro-3-Indolyl Phosphate p-toluidine (NBT-BCIP, pre-diluted 1:100 with Tris buffer) to all troughs which were left for 30 min at 25°C on the shaker. The incubation with the chromogen NBT-BCIP resulted in a purple-brown precipitate where a positive reaction took place. This reaction was stopped by washing with ddH_2O. The genomic assignment was read manually and then confirmed through a "Liras for LiPA" dedicated software which generates automatic allele interpretation after having scanned a "reading template" chart carrying the sticked strips. All typing assays, as well as the DNA extraction and purification, was repeated twice on different days and by different operators, with the same results.

RESULTS

Data from RP-HPLC-FL analysis reported a D/L Asp ratio average of 0.055 (< 0.1) for Cardinal Carlo's bone, a value indicative of the presence of DNA sequences with sufficient length to be extracted and amplified by PCR, and a D/L Ala ratio average of 0.016 indicating no contamination from modern DNA.

The chromatogram of the Cardinal's bone sample and analysis conditions is shown in Fig. 1. Therefore, three spectrophotometer measurement of double-strand aDNA performed on CCEE sample revealed an average concentration of 112 mg/mL with an $A_{260/280}$ nm ratio of 1.44. Gel electrophoresis demonstrated a good yield and quality (no signs of fragmentation) of the aDNA (Fig. 2). For the CCEC sample a similar aDNA yield was achieved but with an A_{260}/A_{280} nm ratio < 1.30, a sign of protein impurities presence (figure not shown). The PCR products were electrophoresed and photographed by UV transilluminator (BIO-RAD, Hercules, CA, USA). The allele interpretation was acquired by reading the positive reaction pattern on a worksheet

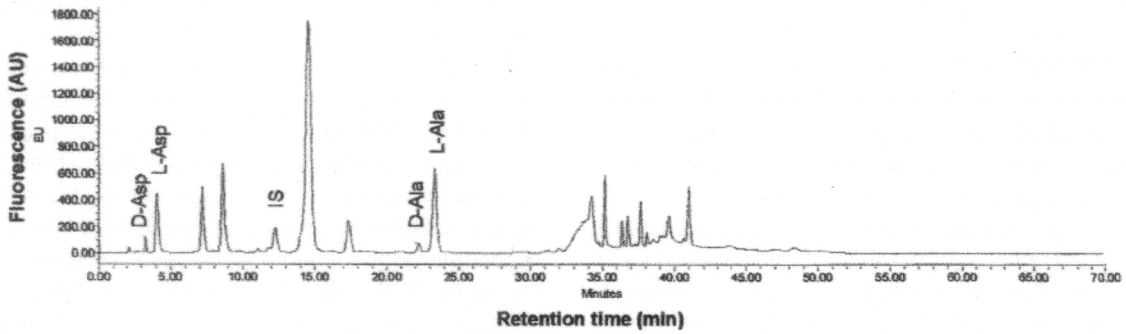

Fig. 1. *Representative chromatogram from RP-HPLC-FL amino acids racemization analysis of Cardinal Carlo. D-homoserine is used as internal standard (IS). Peaks: D-Asp, L-Asp, IS, D-Ala and L-Ala. Conditions: Atlantis dC18 column (150 x 4.6 mm); mobile phase: methanol and acetate buffer (10 mM; pH 5.5) in gradient elution; column temperature: 20°C; flow rate: 1.0 mL/min; fluorescence detection:* $l_{Ex/Em}$ *= 325/434.*

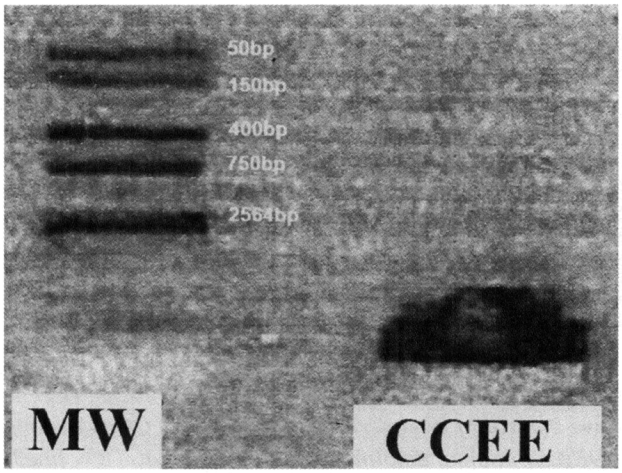

Fig. 2. *aDNA band of Cardinal Carlo's bone isolated by method A (CCEE sample). 10 mL of the digested solution from the bone tissue, purified through acetate/2-propanol method, are loaded and electrophoresed on a 2% agarose gel with 1X TBE Buffer containing 4 mL of ethidium bromide (EtBr, 10 mg/mL), and then visualized under UV light. Molecular Weight size markers (MW, One Lamba, Inc, Canoga Park, CA, USA) establish the aDNA length which is far greater than 2564 bp and without evident signs of fragmentation. The band sizes of MW from bottom to up are: 2564bp, 750bp, 400bp, 150 bp and 50 bp.*

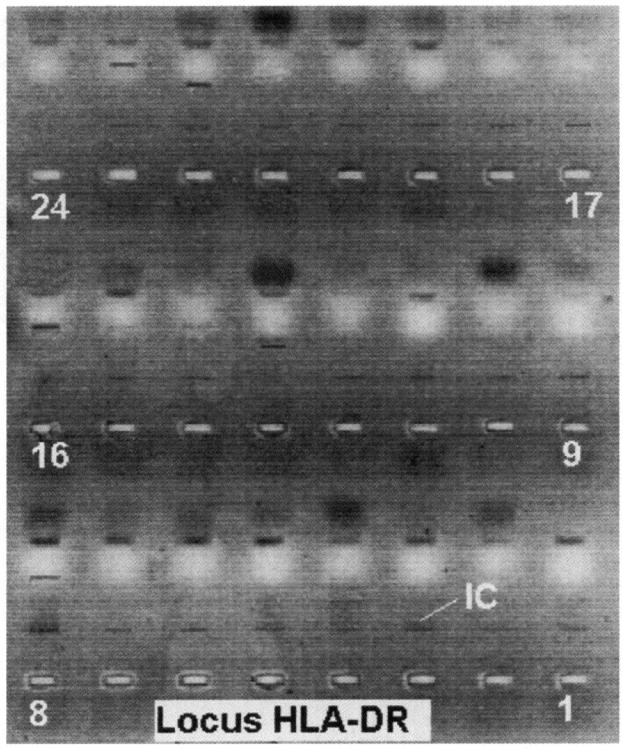

Fig. 3. *Result of the Cardinal genotyping test for HLA-DRB locus through PCR-SSP L.R. technique. CCEE aDNA sample is used. The bands at lines 8 correspond to DRB1*04 (phenotipically DR4) and the band at line 5 represents an example of internal control (IC). Line 1 is the negative control. The other positive bands establish the second allele of the Cardinal, i.e. DRB1*11 (serotype DR11, lines 13 and 16). The DRB1*04 allele amplification is confirmed by the presence of the DRB4 gene (line 23) notoriously linked with DR4 in the DR haplotype. This test also reveals the positivity for DRB3 locus (phenotype DR52, line 22) which is always associated as haplotype with the DRB1*11 gene on the HLA-DRB sub-region.*

supplied with the Biotest kit. The results of the HLA-DRB at Low Resolution test assigned to the Cardinal the genotype DRB1*04/*11 (Fig. 3).

We underline that LiPA Assay performed on CCEC sample (Fig. 4) needed further aDNA dilution for achieving a PCR product, an effect related to the dilution also of Maillard products impairing the amplification process. Moreover, aDNA purification through acetate-isopropanol purification seems to be the better methodology minimizing the co-extraction of PCR inhibitors, in agreement with several literature data. LiPA Assay defined the genotypes DRB1*0401/*11 for DRB1 locus (data not shown) and Cw*04/*12 for the HLA-Cw locus (data not shown). The negative control strip showed no positive reaction.

Worthy of note, both the aDNA isolation methods (A and B) have given reproducible typing data, as demonstrated by the same results achieved for the DRB locus by means of PCR-SSP and LiPA tests. All the laboratory staff had been previously typed and none of them had the alleles detected in the analyzed sample.

DISCUSSION

Our RP-HPLC-FL analysis has proved to be a reliable system for evaluating aDNA in ancient bones that is suitable for amplification prior to performing expensive immunogenetic tests, and above all to exclude any bone contamination of foreign genomic

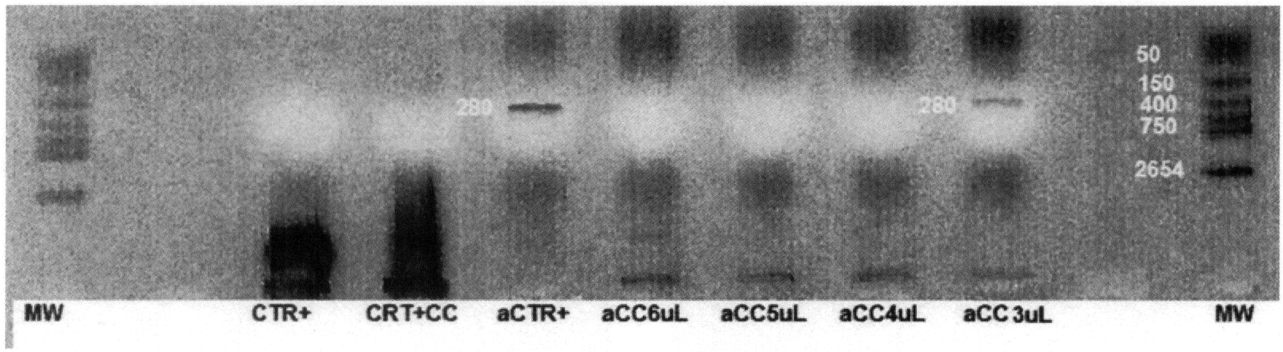

Fig. 4. *Example of PCR amplification at several aDNA dilutions before performing LiPA Assay for DRB1 locus. Here is shown a comparison between non-amplified DNA (CTR+: positive control of a modern DNA, and CTR+CC: Cardinal Carlo DNA) and amplicons obtained after PCR performed with DNA solution of amplified modern DNA (aCTR+, line3) and aDNA of Cardinal Carlo at different aDNA volumes: 6 mL (aCC 6 mL, line 4), 5 mL (aCC 5 mL, line5), 4 mL (aCC4 mL, line 6) and 3mL (aCC 3 mL, line 7). The positivity of the DNA amplification with LiPA kit is demonstrated by the appearance of a PCR product, visible as a single band with a length of ~280 bp after loading 10 mL each sample on a 2% agarose gel. Amplicons are visible only for aCTR+ and aCC 3 mL samples. The sizes of all DNA samples has been compared with Molecular Weights the length of which are the same reported in Fig. 2.*

material. Moreover, the HLA screening technology is a powerful tool when the aDNA sequences are of reduced size as the primers are projected to amplify only short amplicons (range about 110-370 bp).

The positivity of Cardinal Carlo to the DRB1*0401 certainly indicates that he bore the causative HLA genetic variant responsible for RA onset (14). On the contrary, his negativity for Cw*06 and DR*07 genes excludes a genetic susceptibility for PsA. It is ascertained that DRB1*11, Cw*04 and Cw*12 genes are not involved in RA, PsA or other ARD's onset. Although a multifactorial origin has been proposed for PsA, and other not well-identified non-HLA genes might contribute to the development of this pathology (15), the primary predisposing genes remain undoubtedly Cw*0602 and DRB1*07 alleles (16, 17), always associated as haplotype Cw*06-DR*07 owing to the strong LD (Linkage Disequilibrium) between these two HLA genes (17). HLA-DRB1*0401 is considered one of the most spread risk alleles among the Caucasian population, particularly in Northern Italy where the gene frequency is 0.03 (18). On account of this, it is not unlikely that Cardinal Carlo had DRB1*0401, since he belonged to a Florentine dynasty. Indeed, all marriages of the de' Medici family were contracted with Italian or European individuals, implying the transmission of typical European genetic HLA markers throughout the generations. Most members of the de'Medici family were reported to be affected by arthritic diseases including the Cardinal (19).

Predisposed individuals carrying RA or PsA susceptibility genes can manifest the disease only after contact with various triggering environmental factors, such as allergens, dietary components, and in particular viruses and bacteria.

In fact, *Proteus mirabilis* haemolysin (20), *Epstein-Barr virus* glycoprotein gp100 (21), *Escherichia coli* heat shock protein DnaK and DnaJ (22) *Cytomegalovirus* (23) and above all *M. tuberculosis* 65-kDa protein (24) are infective agents whose epitopes can evoke a remarkable autoimmune reaction in people bearing specific DR4 gene variants. This autoimmune response stimulates autoreactive TCD4+-cell clones which, once they have lost their tolerogenicity, initiate a massive auto-antibody production against self-determinants via a mechanism called "molecular mimicry" (25). The existence of the so-called "shared epitopes" (SEs) is well ascertained, characterized by the amino acid sequence ^{70}QK/RRAA^{74} and common to all RA-predisposing DRB1*04 allelic variants of DRB1*0401 (26). All these DRB1*04 RA-related alleles possess the SE in the 3rd hypervariable

region of the DRb chain. Each of these alleles shows different frequencies among different ethnic groups (26). Parts of Type II collagen seem to be the strongest candidate for arthritogenic peptides in DR4 positive patients and the autoantibody attack arises specifically in the joints evoking a tissue-specific inflammation and damages, characteristic of RA (29).

Slight changes of the nucleotide sequence can codify for peptide motifs conferring protective effects against RA, as it occurs for DRB1*0402 allele having the ^{70}DERAA^{74} motif with an aspartic acid residue encoded at position 70 of the DRb chain. Other protective alleles also include DRB1*1301 and *1302 (30). HLA- DRB1*07 is considered neutral or even protective for RA (31). It is important to highlight that DRB1*0402, *1301, *1302 and DRB1*07 protective alleles against RA were not found in the Cardinal's bone.

Cardinal Carlo was infected with tuberculosis bacteria at the age of 9. It is remarkable that some epitopes of *M. tuberculosis* are common to specific amino acid sequences of cartilage proteoglycans (25), whereas a sequence homology between a KDLL peptide of a 65-kDa mycobacterial protein and some HLA Class II molecules, among which DR1 and DR4, is reported (24). Furthermore, T-cells reacting against mycobacterial 65-kDa hsp have also been isolated from rheumatoid synovial fluid of patients affected by RA (32).

In conclusion, although HLA genes *per se* have no diagnostic value, the positivity for RA-susceptibility DRB1*0401 allele, the absence of the *Cw6 gene, the lack of a protective effect of DRB1*0402, *07, *1301 and *1302 against RA, and the tuberculosis infection, combined in the paleopathological examination of the Cardinal's skeleton, suggest an RA diagnosis rather than a PsA one.

Some authors have localized the origin of RA in the Tennessee area among American Indians (7). We have, however, demonstrated pathogenetic signs and genomic predisposition to RA in two ancient human samples prior to American colonialism: an Italian Mummy and Cardinal Carlo de' Medici of Florence, both of the 15th century (6).

In short, the main implication of a multidisciplinary research like this is to contribute to clarifying the history of RA or, at least, to add further chemical, genetic and pathological information useful for understanding more about the origins, etiopathology and diffusion of this disease.

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