Determining nail consistency by quantification of type I keratins

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SUMMARY

Nail consistency is a little studied characteristic of the nail plate. Numerous factors can influence its determination, including the anatomy of the nail apparatus, the structure of the nail plate, and the type and quantity of keratins present. To study the relationship between nail consistency and the expression level of type I keratins, a sample was chosen of 32 individuals in the same age group (49.94 ± 3.38 years), 18 with hard consistency nails and 14 with soft consistency nails, with the same number of individuals for each gender. Two buffers with different concentrations of reducing agent (50mM and 200mM) and two antibodies to various type I keratins were analyzed by immunoblotting. The mean extracted protein concentration at 50mM was significantly higher than the concentration at 200mM (p-value<0.001). The expression level obtained with the AE13 antibody did not vary with gender or nail consistency (p-values≥0.942). With the cytokeratin 17 antibody (CTK17), no differences were found by gender (p-value=0.341). However, significant differences were established between hard-consistency and soft-consistency nails (p-values≤0.007) for the two concentrations, and between concentrations for soft consistency nails (p-value=0.001). Hard-consistency nails had a higher expression level of K17. Adding to the elemental analysis between layers and the flexural behavior of the nail plate studied reported in previous studies, this work demonstrates that nail consistency also depends on the quantity of keratins expressed.

Key words: Epithelial keratin – Hair keratin – Immunoblot – Nail consistency – Nail plate

INTRODUCTION

The nail apparatus is a complex, versatile skin appendage (Haneke, 2006; Haneke, 2015) of epidermic origin (McCarthy, 2004). It is located dorsally in the distal phalanges of the fingers and toes, which it protects (Runne et al., 1981; McCarthy, 2004). In addition to epithelial components, the nail apparatus includes vascularization and innervation structures (Bas et al., 1999; Fleckman et al., 2001; de Berker, 2013; Haneke, 2015) with important sensorial and temperature regulating functions (Haneke, 2006; Haneke, 2015). Anatomically, it comprises the

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following principal elements: nail folds (proximal and lateral folds), nail matrix, nail bed, nail plate, eponychium and hyponychium (Fleckman et al., 2001; Haneke, 2006; de Berker, 2013; Fleckman et al., 2013; Haneke, 2015).The nail plate is made up of tightly packed, anucleate keratinized cells (Runne et al., 1981; Haneke, 2006) arranged in three layers: the dorsal, intermediate and ventral layers (Runne et al., 1981). The layers are differentiated by the orientation of the keratin filaments (Farren et al., 2004), the type of keratins present, and the relative thickness of each layer (Kobayachi et al., 1999). These and other factors influence the mechanical functions of the human nail plate and its physical characteristics of toughness, strength, and flexibility (Young et al., 1965; Finlay et al., 1980; Farren et al., 2004), and are also likely to influence nail consistency.

Nail consistency is a characteristic of the nail plate that is not routinely assessed during clinical examination. However, changes in the color, shape or thickness of the nail plate are important signs assessed in podiatry practice, because they can indicate systemic pathologies or nail disorders (Shemer et al., 2013; Baraldi et al., 2015; Zaiac et al 2015). We consider that when examining both normal and pathological nails, consistency should be one of the main factors assessed alongside color, shape, and other characteristics. Soft, medium, and hard consistency nails have been identified, and their relative frequencies have been found to vary with age, gender, and sports activity (Pérez Pico et al., 2017; Pérez Pico et al., 2019). Moreover, studies which develop a predictive mathematical model for nail consistency based primarily on the level of calcium in the dorsal layer of the nail plate (Mingorance Álvarez et al., 2021) and which analyze flexural behavior through fractional modeling (Traver et al., 2021) strengthen the hypothesis that nail consistency varies.

Keratins form the type of intermediate filament characteristic of epithelial tissue (Woodcock-Mitchell et al., 1982). They are very stable filaments that provide integrity and mechanical support to the epithelial cells (Runne et al., 1981). Epithelial keratins are characteristic of epithelial tissue, while hair keratins are the majority type in cutaneous appendages (hair and nails). The structural unit of the fold is the heterodimer (Hatzfeld et al., 1990), a type I (acid) and a type II (basic or neutral) amino acid chain (Moll et al., 1982) assembled by the central α-helical rod domain. This domain is fundamental in the development of upper fold levels (coiled-coil conformation), together with the existence of disulfide bonds that keep the structure stable, particularly in hair keratins (Gniadecka et al., 1998). Human type I keratins are epithelial (K9-10, K12-K20, K23-28) and hair (K31-K40), and human type II keratins are epithelial (K1-K8, K71-K80) and hair (K81-K86) (Schweizer et al., 2006). Numerous studies have permitted characterization of epithelial and hair keratin expression patterns in the different elements of the nail apparatus (Baden et al., 1984; Heid et al., 1988; Moll et al., 1988; Westgate et al., 1997; Waseem et al. 1999; de Berker et al., 2000; McGowan et al., 2000; Perrin et al., 2004; Perrin, 2007; Perrin et al., 2011) some of them in human adults (Table 1). The nail plate contains both

Table 1. Keratin expression in human adult nail apparatus.

	Type I		Type II	
	Epithelial	**Hair**	**Epithelial**	**Hair**
Proximal nail fold	K10, K16, K17		K1, K6	
Eponychium	K10, K16, K17		K5, K6	
Matrix	K10, K14, K16, K17, K18,	K31, K34, K36, K38	K1, K5, K6, K7, K8,	K81, K85, K86
Nail bed	K10, K14, K16, K17		K5, K6, K7, K75	
Nail plate	K17			
Hyponychium	K10, K17		K5	
Digit pulp	K16, K17		K6	

Studies summarized from: de Berker, 2000; McGowan and Coulombe, 2000; Perrin et al., 2004; Perrin, 2007; Perrin et al., 2011.

epithelial and hair keratins (Lynch et al., 1986; Heid et al., 1988; Kitahara et al., 1991), although only 10-20% are epithelial, compared to a majority of hair keratins (Lynch et al., 1986; Heid et al., 1988).

Several factors may influence nail consistency, including the anatomy of the nail apparatus, the structure of the nail plate, and the type, quantity, and assembly of the keratins present. However, few studies have addressed nail consistency and its determining factors. The main objective of this work is to analyze the relationship between nail consistency and the quantification of two type I keratins present in the nail plate (the epithelial keratin K17 and a 44-46 kDa hair keratin doublet) and establish a new line of research to determine whether nail consistency depends on the pattern and/or relative quantity of the keratins expressed in the nail plate.

MATERIALS AND METHODS

Permission and sample description

Permission for the study was obtained from the University of Extremadura Bioethics Committee (Reg. 116/2016). All participants signed an informed consent form and after a physical examination of their feet, they filled in a questionnaire to provide the necessary health information. The inclusion criteria were: aged 40 to 55 years; no diagnosed disease capable of altering nail structure and/or composition; no prescribed medication described as altering nail structure and/or composition; no behaviors or treatments that alter the structure and/or composition of the nails; following a Mediterranean diet.

The final sample comprised 32 adult individuals (16 men and 16 women; 49.94 ± 3.38 years), 18 with hard consistency nails and 14 with soft consistency nails.

Nail consistency and sample collection

Trained personnel determined nail consistency *in vivo* by applying manual pressure to the nail edges in the lateral-medial and dorsal-ventral axis after the foot had been exposed to room temperature for 15 minutes, following the methodology described elsewhere (Pérez Pico et al., 2017). Nail clippers were used to collect samples on the free edge of the first toe, without exceeding the onychocorneal band. Nail samples were immersed in an ultrasound cleaning bath (Elma, Singen, Germany) to clean them and remove skin cells, fibers and nail plate debris, and samples were stored at -20ºC until analysis.

Antibodies

As representatives of type I keratins, AE13 and CTK17 antibodies were analyzed due to their traditional use in the study of nail apparatus keratins, and their relationship with symptoms in the nail plate associated to hereditary human diseases as paquioniquia congenita type 2, respectively.

Mouse monoclonal antibody AE13, kindly provided by Dr TT Sun (New York University), recognizes a 44-46 kDa type I human hair keratin doublet (Lynch et al., 1986). Mouse monoclonal antibody CTK17 (sc-393091, Santa Cruz Biotechnology, Heidelberg, Germany) recognizes 46 kDa type I human epithelial keratins. Mouse monoclonal antibody GAPDH (sc-32233, Santa Cruz Biotechnology, Heidelberg, Germany) was used in 50mM 2-mercaptoethanol immunoblots as loading control. Goat anti-mouse IgG (H+L) HRP conjugate secondary antibody was obtained from Advansta (San José, California, USA).

Extraction procedure, protein quantification and immunoblot

Keratins were extracted following the methodology described elsewhere (Kitahara et al., 1991) for clipped nails. Because it was a two-step sequential extraction procedure, two different buffers were used: buffer I (50mM Tris-HCl, 9M urea, 2mM phenylmethanesulfonyl fluoride and 50mM 2-mercaptoethanol, pH 9) and buffer II (200mM Tris-HCl, 9M urea, 2mM phenylmethanesulfonyl fluoride and 200mM 2-mercaptoethanol, pH 9). Two extracts were obtained from each sample with increasing concentrations of 2-mercaptoethanol, one containing 50mM, in which the epithelial keratins were soluble, and the other containing 200mM, in which the hair keratins were soluble (Kitahara

et al., 1991). Three independent repetitions of the extraction procedure were performed with each sample analyzed. The protein concentration present in the extracts was quantified by the Bradford colorimetric method (Bradford, 1976).

Because the molecular weight of the target keratins was very similar (44-46kDa: AE13 antibody; 46kDa: CTK17 antibody), it was necessary to perform independent immunoblots for each antibody. A total of 3 µg of protein per well was loaded in the immunoblots with the AE13 antibody and 10 µg with CTK17, to avoid problems of membrane saturation and perform the quantification in the linear range. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed under denaturing conditions (SDS-PAGE) (Laemmli, 1970) using gels with 10% acrylamide and electrophoresis buffer (25mM Tris, 192mM glycine, 0.1% w/v SDS). Separated proteins were transferred electrophoretically (40mV, for 4h, at 4ºC) to nitrocellulose membranes (GE Healthcare Life Sciences, Freiburg, Germany) with Mini Trans Blot System (Biorad Laboratories, Shanghai, China) using transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol).

Membranes were treated with blocking solution (50mM Tris-HCl, 75mM NaCl, 0.2% (v/v) Tween-20, pH 7.5, containing 10% (w/v) non-fat milk) for 2h at room temperature, then incubated overnight with primary antibodies AE13 and CTK17 (diluted 1:500 (v/v) in blocking solution) and GAPDH (diluted 1:2000 (v/v) in blocking solution) at 4ºC. Because the extraction conditions with buffer II (200mM) are incompatible with GAPDH protein extraction, Ponceau S stain was used as loading control rather than the GAPDH antibody (Fig. 1). The membranes were then washed (Tris-buffered saline buffer, pH 7.5, with 0.2% Tween-20) three times, for 10 minutes each, and incubated with goat anti-mouse IgG HRP conjugate secondary antibody (diluted 1:10000 in blocking solution) for 1h at room temperature. After four 10-minute washes, HRP chemiluminescent substrate Pierce ELC (Thermo Scientific, Rockford, Illinois, USA) was added for the membranes with the AE13 and CTK17 antibodies, and Clarity Max (Biorad Laboratories, Segrate, Italy) for GAPDH membranes. Membranes were exposed to Hyperfilm ELC (Amersham, Buckinghamshire, UK). Developed films were scanned and band signaling was measured using ImageJ software (http://rsbweb.nih.gov/ij/). The immunoblot results were obtained from three independent repetitions.

Statistics and variables

Statistical treatment was performed using IBM SPSS Statistics for Windows, Version 22.0 (IBM, Armonk, New York, USA). Normality was assessed by the Kolmogorov-Smirnov and Shapiro-Wilk tests. The paired sample T-test and the independent sample T-test were performed. For the two extraction conditions tested (50mM and 200mM), the variables analyzed were mean extracted protein concentration, mean quantification of the expression level with the AE13 antibody, and mean quantification of the expression level with the CTK17 antibody. To compare the extracted protein concentrations and the mean quantifications of the expression level for each antibody, the differences obtained under each extraction condition tested were analyzed. In all analyses the significance level was 0.05.

RESULTS

The three independent repetitions of protein extractions performed at 50mM showed a higher median (3.07, 3.81 and 4.01) and range (4.03, 3.95 and 4.40) than extractions performed at 200mM (2.49, 2.56 and 2.54) and (2.09, 2.39 and 2.17), respectively (Fig. 2). All the variables analyzed followed a normal distribution (p-values≥0.148). The comparison between the mean protein concentration extracted at 50mM and 200mM was independent of gender and nail consistency (p-values≥0.641). At 50mM, the mean extracted protein concentration was significantly higher (3.80 µg/µl) than the concentration obtained at 200mM (2.57 µg/µl) (p-value<0.001) (Fig. 2). Moreover, for each concentration analyzed, no differences were observed either by gender (p-values≥0.167) or nail consistency (p-values≥0.409) (Fig. 2).

Comparison of the mean quantification of the expression level for the AE13 antibody between the two concentrations tested was also

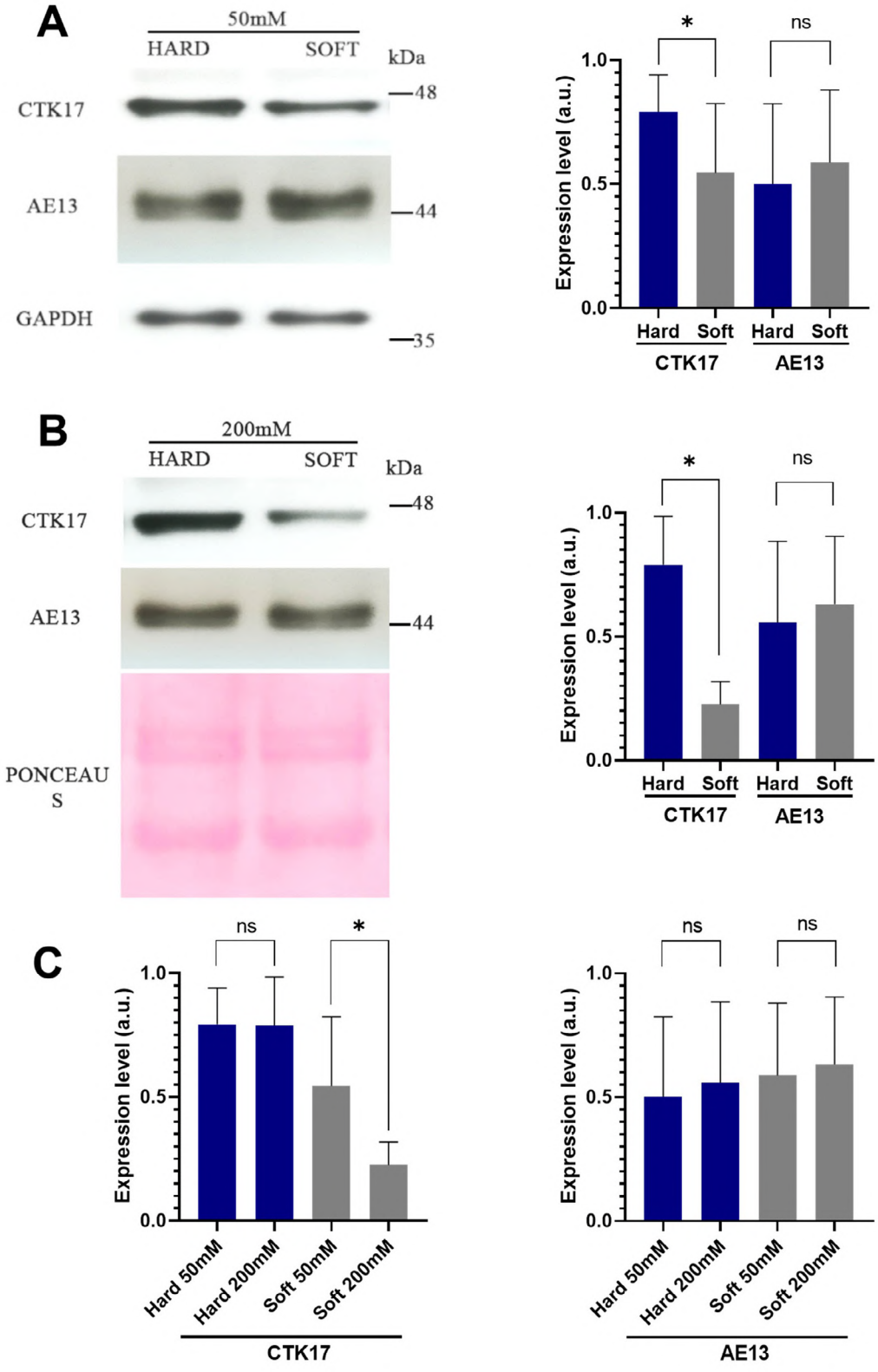

Fig. 1.- Expression level detected with the AE13 and CTK17 antibodies under the two extraction conditions tested (50mM and 200mM). (**A**) extracts obtained at 50mM. (**B**) extracts obtained at 200mM. (**C**) comparison of the expression level between nail consistencies (Hard and Soft). A left, 50mM immunoblot comparing hard- and soft-consistency nails. A right, bar graph comparing the expression level obtained at 50mM for the two antibodies tested by nail consistency. B left, 200mM immunoblot comparing hard- and soft-consistency nails. B right, bar graph comparing the expression level obtained at 200mM for the two antibodies tested by nail consistency. C left, bar graph comparing the expression level obtained for the CTK17 antibody by the two extraction conditions and the two nail consistencies tested. C right, bar graph comparing the expression level obtained for the AE13 antibody by the two extraction conditions and the two nail consistencies tested. 50mM, concentration of 2-mercaptoethanol in the extraction buffer; 200mM, concentration of 2-mercaptoethanol in the extraction buffer; Hard, hard-consistency nails; Soft, soft-consistency nails; kDa, kilodalton; a.u., arbitrary units; *, statistically significant difference ($p<0.05$); ns, not significant; GAPDH, loading control at 50mM; Ponceau S, loading control at 200mM.

independent of gender and nail consistency (p-values≥0.942). Analysis of the results of the mean quantification of the expression level of the extracts obtained at 50mM and 200mM did not show enough evidence to reject that they were the same (p-value=0.516). For each concentration analyzed, no significant differences were observed by gender (p-values≥0.802) or nail consistency (p-values≥0.437) (Fig. 1).

For the CTK17 antibody, the comparison between the mean quantification of the expression level at 50mM and at 200mM did not vary by gender (p-value=0.341), although it varied by nail consistency (p-value=0.001). The data observed on comparing the mean quantification of the expression level of the extracts obtained at 50mM and at 200mM in hard consistency nails did not show enough evidence to reject that they were the same (p-value=0.958). However, significant differences were found on comparing the mean quantification of the expression level in the extracts obtained at 50mM and at 200mM in soft consistency nails (p-value=0.001). For each concentration, no differences were observed in relation to gender (p-values≥0.187), although differences were observed by nail consistency (p-values≤0.007) (Fig. 1).

Quantification of the expression level obtained for the AE13 antibody therefore did not vary either by gender or nail consistency (p-values≥0.942). However, the results obtained with the CTK17 antibody showed significant differences between soft consistency nails and hard consistency nails under each extraction condition tested (at 50mM and at 200mM) (p-values≤0.007) and between soft consistency nails for extracts obtained at 50mM and at 200mM (p-value=0.001).

DISCUSSION

The nail plate is a highly keratinized tissue, given that 90% of the total proteins in the nail plate are keratins or keratin-associated proteins (Rice et al., 2010) and 80-90% of these are hair keratins (Heid et al., 1988; Lynch et al., 1986). However, the extractions performed under less reducing conditions (50mM) showed a higher total protein concentration than extractions at a higher concentration of reducing agent (200mM), in which hair keratins are more soluble than epithelial keratins. Therefore, under more gentle extraction conditions, not only are the epithelial keratins soluble (Kitahara et al., 1991), but other types of proteins (membrane or intracellular) also

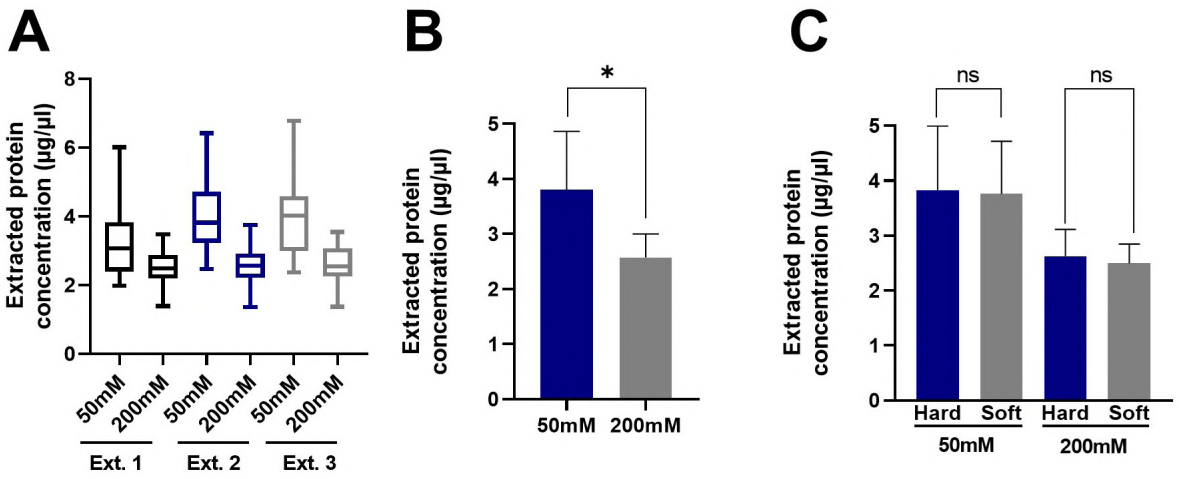

Fig. 2.- Principal results obtained from the extraction experiments. (**A**) Box and whisker plot of the three independent repetitions of the extraction procedure. (**B**) Bar graph comparing the mean extracted protein concentration under the two extraction conditions tested (50mM and 200mM). (**C**) Bar graph comparing the mean extracted protein concentration under the two extraction conditions tested by nail consistency (Hard and Soft). 50mM, concentration of 2-mercaptoethanol in the extraction buffer; 200mM, concentration of 2-mercaptoethanol in the extraction buffer; Ext. 1, independent extraction number 1; Ext. 2, independent extraction number 2; Ext. 3, independent extraction number 3; *, statistically significant difference (p<0.05); ns, not significant; Hard, hard consistency nails; Soft, soft consistency nails.

solubilize, producing a higher extraction yield than under more reducing conditions (Rice et al., 2010). This is the case of the protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which participates in glycolysis and was detected only in extracts performed at 50mM, where it was used as loading control. Moreover, the results obtained in the three independent repetitions of protein extraction showed greater variability in extractions performed at 50mM than at 200mM. These results support the hypothesis that a higher diversity of proteins is extracted under less reducing conditions, whereas under more reducing conditions the type of proteins solubilized is more homogeneous and variability is lower. However, the nail plate is not characterized as a very active tissue from a metabolic point of view, because it is made up of cells with pyknotic nuclei or largely denucleated cells (Runne et al., 1981). It is likely that, together with the minority non-keratin proteins present in the nail plate, some hair keratins are also extracted under less reducing conditions (Rice et al., 2010), even though they are almost insoluble in these conditions (Kitahara et al., 1991).

The AE13 antibody was not useful for differentiating between hard- and soft-consistency nails, because its expression level did not vary either by nail consistency or extraction conditions. Previous studies with this antibody reported exclusive staining in extracts obtained under more reducing conditions (Kitahara et al., 1991). However, we cannot state that only hair keratins will be extracted under these conditions, which would explain the expression level of the extracts obtained at 50mM. A minor tendency was observed in the slightly higher expression level in soft-consistency nails, which was higher under the more reducing extraction conditions. Although the results were not statistically significant, the extracts from soft consistency nails may contain a higher relative concentration of target hair keratins of the AE13 antibody than the extracts from hard consistency nails.

The results showed that hard-consistency nails express more K17 than soft consistency nails. K17 has been detected in the nail matrix associated with precursor cells, in the nail bed (McGowan et al., 2000) and in the nail plate (Heid et al., 1988). It has also been associated with epithelia subjected to high physical stress, with high cell turnover rates (Swensson et al., 1998). Nails are constantly subjected to bending forces by activities of daily living (Forslind et al., 1980). It seems logical that hard-consistency nails would be less adapted to these forces than soft-consistency nails, which are probably more flexible and adaptable. These less adaptable nail plates would be subjected to greater stress, which could cause an increase in K17 synthesis and, therefore, a greater presence of this keratin in the nail plate of hard-consistency nails. Moreover, this difference in expression by nail consistency is independent of the extraction conditions, although in soft consistency nails the expression level is higher under less reducing extraction conditions, supporting an earlier description of keratin solubility and reducing power (Kitahara et al., 1991).

Nail consistency varies by age, gender, and sports activity. Adult males and young people who do sport have a higher frequency of hard-consistency nails than adult females and sedentary young people, whose most frequent nail consistencies are medium and soft (Pérez Pico et al., 2017; Pérez Pico et al., 2019). In developing a predictive model of nail consistency, gender was not shown to be a good predictive variable (Mingorance et al., 2021), and neither were the results obtained with the two antibodies tested, because no relation was established between nail consistency and gender. Therefore, although the frequency of each nail consistency varies in the population, hard- and soft-consistency nails show no differences by gender.

CONCLUSIONS

This study provides experimental evidence to show that hard-consistency nails and soft-consistency nails have different expression levels of K17, a type I epithelial keratin, and that expression is greater in hard-consistency nails. The role of K17 in healthy nail plates strengthens the hypothesis that nail consistency varies and establishes differences at molecular level. With greater knowledge of the characteristics of the nail plate, health professionals will be better equipped to prevent, diagnose, and treat numerous nail and

systemic diseases. We consider nail consistency to be one of the key characteristics that health professionals should assess during clinical examination. However, more studies are needed, both to define the determining factors of nail consistency and to associate this characteristic with the most common nail and systemic diseases.

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