Association between collagen production and mechanical stretching in dermal extracellular matrix: In vivo effect of cross-linked hyaluronic acid filler. A randomised, placebo-controlled study

Virginie Turlier a,*, Alexandre Delalleau a, Christiane Casas a, Amandine Rouquier a, Pascale Bianchi a, Sandrine Alvarez a, Gwendal Josse a, Alain Briant b, Serge Dahan b, Christine Saint-Martory a, Jennifer Theunie a, Amel Bensafi-Benaouda a, Arnaud Degouy a, Anne-Marie Schmitt a, Daniel Redoulès a

aCentre de Recherche sur la Peau Pierre Fabre, Hôtel Dieu, Toulouse, France
bDermatologists, Toulouse, France

1. Introduction

The biomechanical performance of the skin depends essentially on the integrity of the dermal extracellular matrix. This matrix is arranged in a dense network of interlinked collagen (types I and III) and elastin fibres containing water-retaining proteoglycan and glycan macro-aggregates. With age, the collagen network undergoes re-organisation (density, orientation), resulting in a reduction in collagen and proteoglycan content [1], accompanied by an increase in denatured and degraded collagen [2]. These changes lead to a loss of matrix volume and, consequently, to a deterioration in mechanical properties [3], which may induce changes in cell metabolism. Indeed, a link between the mechanical stress exerted on different types of tissues and neocollagen production has been suggested. Well-known examples are provided by studies on the adaptive response of articular tissue which show that certain types of strain clearly stimulate the anabolic response [4]. Likewise, experimental data have confirmed the anabolic effect of mechanical strains on the extracellular matrix of fibroblasts cultured in a 3-dimensional network (collagen lattices) [5]. Furthermore, owing to the high degree of biocompatibility of hyaluronic acid, the anabolic responses of tissues after injection appear very different from those seen with other types of implants, which usually lead to the formation of a fibrotic shell [6].

Hyaluronic acid (HA) fillers are reportedly effective in dermal collagen anabolism, but data demonstrating efficacy are based...
essentially on a single article [7]. In this study in 11 volunteers, Wang et al. demonstrated a significant increase in levels of dermal mRNA coding for collagen I, four and 13 weeks after injection of HA filler, and in tissue inhibitors of metalloproteinases (TIMP)-1, -2 and -3, four weeks after injection. The activating effect on collagen 1 synthesis was confirmed by a histological approach. One of the basic concepts advanced concerns the close relationship between stretching and biochemical changes in skin physiology. This stress appears to activate intracellular signalling pathways that modulate the synthesis/degradation balance of the extracellular matrix [2].

The objective of this comparative placebo-controlled study was to ascertain the association between metabolism and dermal mechanics modulated by a hyaluronic acid-based filler effect. The changes in the biochemical markers of fibroblast response were analysed over time and, in conjunction with this, we related the volume of an injected filler, measured by 3D high-frequency ultrasound, to the mechanical stresses induced. This analysis highlights the strong links between the homeostasis of collagen synthesis and degradation pathways and the mechanical stress exerted by the injection of cross-linked HA.

2. Materials and methods

2.1. Study design and patient selection

This single-centre, comparative, randomised, placebo-controlled, in vivo, blind analysis study was conducted at the Pierre Fabre Skin Research Centre – CRP, Toulouse (France), in accordance with the ethical principles of the Declaration of Helsinki and Good Clinical Practice guidelines. The protocol was approved by the Sud-Ouest et Outre Mer III Committee for the Protection of Persons (Ethics Committee) and the French Health Products Safety Agency (AFSSaPS). Each volunteer signed a written informed consent.

Sixty healthy female volunteers aged 35–75 years of skin phenotype III with a body mass index in the normal range and healthy arm skin were included.

Non-menopausal women must have been using effective contraception for at least 2 cycles before inclusion and have had a negative pregnancy test result at inclusion and at the end of the study. Menopausal women were not permitted to have modified, introduced or stopped hormonal therapy in the 3 months before inclusion or during the study. Subjects meeting the following criteria were excluded: pregnant or breast-feeding women; history of allergy to cosmetics, hyaluronic acid or any other ingredient of the test product, latex, or lidocaine- or prilocaine-mediated plaster; wound healing disorder; signs of excessive sun damage. Sun exposure within the month prior to inclusion or during the study, corticosteroids, retinoids, immunosuppressive or cardiovascular agents, or anticoagulants within the previous 3 months, and diuretics within the 2 months preceding inclusion, were other reasons for non-inclusion, as were the use of topical corticosteroids or alpha-hydroxy acids at the test sites in the 4 months before inclusion, and the introduction or modification of hormonal treatments that might affect the study results in the 3 months preceding inclusion or during the study.

2.2. Study products and injection technique

The study products were administered contralaterally in the posterior aspect of the arms by two trained investigators at the injection visit (T0). The sides were randomly assigned in blocks of 4 (using in-house software) by the department of Pre-industrial Clinical Pharmacy, independently of our Skin Research Centre. We used HA filler indicated for the treatment of moderate to deep wrinkles, such as nasolabial folds (NLFs).

The control was isotonic sodium chloride injection (0.9%) – Aguettant®.

A topical local anaesthetic cream (5% lidocaine and prilocaine) was applied to the injection sites 1 h before injection. The cream was then removed and an injection of 0.5 ml of HA gel or control was administered using a linear threading technique over a distance of 2 cm (2 × 0.25 ml).

2.3. Study period

The study was conducted over a 6-month period. Subjects were divided into 3 groups of 20 (groups 1, 2 and 3), corresponding to the time of the final biopsy: 1, 3 and 6 months post-injection, respectively. Allocation to the different groups was based on subject availability and undertaken by the investigator during the pre-inclusion visit. The biopsy specimens (4 mm diameter) were taken at T0 (pre-injection baseline value) and at the end of the study (Tfinal: 1, 3 or 6 months post-injection) from each injected area.

Ultrasound measurements were taken at each time point up to Tfinal. The biopsies harvested for protein analysis were frozen immediately at –80°C and those for use for gene expression analysis were kept at 4°C in RNalater (Qiagen) overnight and then stored at –20°C until analysis.

2.4. Efficacy evaluation

The primary end-point was pro-collagen I expression 1, 3, 6 months after injection, assayed by Elisa.

Secondary efficacy analysis included changes over time in TIMP1, MMP3 and MMP9 expression assayed by Elisa and gene expression for collagen I (A1, A2), collagen III (A1), MMP3 and TIMP1 assayed by qPCR, volume and dispersion of the injected hyaluronic acid calculated from ultrasound imaging, and deformation of the different skin layers evaluated by finite element modelling.

2.5. Protein expression of procollagen I, MMPs and TIMPs

To analyse the expression of procollagen I, MMP and TIMP, the biopsy, dry-stored at –80°C, was placed in PBS/0.1% Triton buffer solution and homogenised in the Tissue Lyser (Qiagen). The resulting extract was centrifuged and the supernatant recovered. Procollagen I was assayed using the ELISA Procollagen Type I C-Peptide EIA kit (MK101, Takara) according to the supplier’s recommendations. TIMP-1 and MMP-3 and -9 were assayed in the same original extract using the Fluorokine MAP Human TIMP panel kit (LKT003, R&D) and the Fluorokine MAP MMP base kit (LMP000, R&D), respectively. The results obtained for each of the quantifications were standardised to the total protein concentration determined in the biopsy extract by OPA assay (#26025, Thermo).

2.6. Gene expression

Total RNA was isolated using the iPrep PureLink kit (Invitrogen, Carlsbad, USA) according to the manufacturer’s directions, including DNase I digestion. After reverse transcription (10 μL of total RNA) with the High Capacity cDNA kit (Applied Biosystems, Foster City, USA), amplification reactions were performed on a 7900 Real-time PCR System. The reaction volume per well consisted of 2.5 μL of cDNA diluted to one tenth, 5 μL of TaqMan Universal Master Mix (Life Technologies), 0.5 μL of 20 X Taqman probes (Gene expression assay, Life Technologies) and 2.5 μL of RNase- and Dnase-free water. RPLP0 was used as endogenous control. Results are expressed as ΔΔCT = 2^(-ΔΔCT) RPLP0-Ct target gene).
Primers and TaqMan probes used were designed by Applied Biosystems (Life Technologies): COL1A1-Hs00164004_m1, COL1A2-Hs01028956_m1, COL3A1-Hs00943809_m1, MMP3-Hs00968308_m1 and TIMP1-Hs99999139_m1. Although they do not amplify genomic DNA (the assay probe spans an exon junction) a reverse transcription control without enzyme was still performed.

2.7. Ultrasound monitoring

High-frequency ultrasound at 20 MHz [8–10] enables the structures of the dermis to be imaged by analysing the radio-frequency signal emitted in the skin by a transducer. The signals received undergo morphological processing to map the echogenicity of the area examined.

The apparatus used for this study was a Dermcup 2020 (ATYS, France) coupled to a three-dimensional scanner to visualise the volume of the region concerned. The acquisition performed, consisting of 300 2D images, allowed a volume of 16 mm × 16 mm × 6.24 mm to be imaged at a resolution of 550 × 300 × 268 voxels.

2.8. Calculation of volume and dispersion parameters

Manual contouring of the cavities containing the injected hyaluronic acid was performed using a specifically developed software platform. The injected volumes were depicted in three dimensions using VTK® graphics libraries and by concatenating the contours generated using a simple linear interpolation of the existing contours. The volume (V) and surface area (A) of the resultant object [11] were calculated and appeared essential parameters for its caracterisation. Dispersion of the object was calculated by determining the ratio between the surface area (A) of the object and the theoretical area of a sphere of corresponding volume (V). The sphere represents the theoretical minimum dispersion of the injected volume. The dispersion (D) could be expressed as follows:

\[ D = \frac{A}{(36\pi V^2)^{1/3}} \]  

(1)

This calculation allowed sensitive discrimination between different distributions of hyaluronic acid in the skin.

2.9. Finite element modelling

Finite element modelling [12] of this process was performed to analyse the effects of an injection on the different skin layers. In this case, only the dermis and hypodermis, with respective thicknesses of 1.22 mm (mean of each subject) and 2 mm, were considered [13,14]. The system was reduced to two dimensions (plane strains). The geometry concerned corresponded to the exploratory field of the probe (16 mm). Meshing was obtained by second order triangular elements. To encourage lateral dispersion (generally observed on ultrasound), the injection was simulated by imposing excess hydrostatic pressure \( P = 0.1 \) kPa within an elliptical cavity with axes of 0.4 mm (diameter of injection needle) and 1 mm (chosen arbitrarily). Neo-Hookean hyperelastic behaviour [15] was used to model the dermis and hypodermis, with respective elastic constants of \( X^D = 30 \) kPa and \( X^h = 10^{-2} \) kPa [10,11]. A coefficient of compressibility of \( v = 0.45 \) was assumed, irrespective of the materials. The relationship between this structure and the underlying tissues was modelled through restrained boundary conditions of the nodes corresponding to the inferior aspect of the hypodermis [13].

Because of the difference in viscosity of the two products, the different sensation of volunteers during injection of the two products, the appearance of the areas after injection (domed in the case of HA) and the obvious differences between ultrasound images, the study cannot be blinded. However, to maintain some
integrity, biochemical analysis of biopsies were performed blind (label tubes hidden) by persons involved in this task only.

2.10. Statistical analysis

Statistical analysis was performed by a Biometry Unit independent of the Pierre Fabre Skin Research Centre. Sixty subjects, divided 20 to a group, were planned to be enrolled to ensure a minimum sample size of 16 per group. The purpose of the analysis was to compare treatments within each treatment group and to analyse changes over time. The type I (α) risk was set at 0.05 for all of the study. Analysis were performed on the per protocol population (PP: randomised subjects receiving 1 dose of study medication and not exhibiting any major protocol deviation).

Statistical analysis was performed with SAS 8.2 software as follows: the primary endpoint was evaluated by analysis of variance (paired t-test) for repeated measures on the changes from baseline (T0), taking into consideration time, product, site, sequence and interactions as fixed effects and subject as a random effect factor. This analysis provided the results of intra- and inter-group comparisons. The same analysis was used for secondary endpoints.

3. Results

3.1. Subjects

Sixty subjects were included from February to October 2009. One withdrew prematurely and six others exhibited a major protocol deviation (Fig. 1). Fifty-three subjects were analysed, with the following numbers in each group: 1 month: 17, 3 months: 17, and 6 months: 19. The mean age was 53.5 ± 13.8 years (median: 44.0 years, minimum: 35; maximum: 73 years). Mean ages (±SD) for groups 1, 2 and 3 were 55.35 (±13.12), 53.93 (±15.01) and 54.15 (±14.22) years, respectively. There were no significant intergroup differences in subject characteristics and biochemical marker content at baseline.

3.2. Protein markers

The values of the biochemical markers of the dermal matrix were comparable in the 3 groups at inclusion. In group 1 (one month post-injection), a highly significant increase in procollagen levels was found in the area treated with filler compared with the control (+66%, p = 0.0016) and with baseline (+138%, p < 0.0001). Analysis showed that this increase was still present 3 months post-injection: +40% relative to the control side (p = 0.0005); +27% relative to the baseline content (p = 0.014) (Fig. 2a). Similarly, analysis of the changes from baseline at 1 month showed a significant increase in TIMP-1 content in the filler group compared with the control (+19%, p = 0.0485) and with baseline (+28%, p = 0.0086) (Fig. 2b).

No difference was observed three months post-injection, whether between the treated and control areas or from baseline. Conversely, at 6 months, the opposite course was noted for TIMP-1 content between treated and control areas, with a significant increase for the control versus treated area (p = 0.032) and versus baseline (p = 0.049) (Fig. 2b).

The mean dermal MMP-9 content (not presented here) did not differ significantly between treated and control areas in the 3 groups. Similarly, no significant difference was apparent in the MMP-3 content measured in the treated and control areas one month after the injection. Conversely, the differences observed between these same two areas 3 months post-injection were significant (p = 0.044). Furthermore, inclusion of the MMP-3/TIMP-1 ratio revealed a statistically significant decrease between the two products, expressed in the first month (p = 0.01) and still present at 3 months (p = 0.022) (results not presented here).

3.3. Transcriptional markers

Quantitative RT-PCR provided additional evidence of the effect of an injection of cross-linked hyaluronic acid on the modulation of procollagen expression and substantiated the results obtained using the Elisa technique (Fig. 3). The results showed a significant increase at 1 month in relative expression of the procollagen I isoforms A1 and A2 (p = 0.007 and p = 0.0057, respectively) and of procollagen III (p = 0.004) at the sites treated by filler compared with those treated with control. This stimulant effect then declined over time. Three months after injection of filler, a significant increase from their baseline expression was still found in the relative expression of the A1 and A2 isoforms of procollagen I (p = 0.0266 and p = 0.0086, respectively) and procollagen III (p = 0.0126). By contrast, intergroup analysis failed to reveal a significant difference from procollagen expression on the control side.

An increase in relative TIMP1 expression at 1 month was also found at filler-treated sites compared with the control (p = 0.0135)
and with baseline expression \( p < 0.0001 \) (Fig. 3). However, as MMP-3 expression was below the limit of detection for half the samples studied, these data were un evaluable.

3.4. Volume and dispersion

Only the ultrasound data for the patients from group 3 (6 month follow-up) were analysed to monitor the injections over time. Fifteen of these nineteen subjects were evaluable.

Fig. 4 shows a clear 2D illustration of an injected environment (a) (dark non-echogenic areas) versus a non-injected environment (b). The majority of the images analysed showed the filler to be localised in the hypodermis as far as its interface with the dermis (Fig. 4a). The mean individual variation was expressed as a percentage of the injected volume. Fig. 5 clearly demonstrates the changes due to the consumption and displacement of hyaluronic acid over time. Following injection, a highly significant decrease \( p < 0.0001 \) was observed over time (Figs. 5 and 6a). Forty-three percent of the initial volume injected was found at 1 month and 26% and 20% after 3 and 6 months, respectively.

Concurrently with this decrease in volume, an increase was noted in the calculated dispersion values between the first and third months (Figs. 5 and 6b). In qualitative terms, the volume appeared less massive in terms of its organisation and the density of the injected filler decreased. On the other hand, these dispersion values exhibited no significant change subsequently (Fig. 6b). This suggests that the appearance of the implant remained unchanged between 3 and 6 months, with hyaluronic acid simply being degraded according to the same distribution.

Fig. 3. Expressions of transcriptional markers and comparison of different time courses. (3a) Col IA1 (3b) Col IA2 (3c) Col IIIA1 3d: TIMP-1. Significance: * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \). Real-time reverse transcriptase-polymerase chain reaction (PCR) analysis of the genes encoding procollagen IA1 (3a), procollagen IA2 (3b), procollagen IIIA1 (3c) and TIMP-1 (3d). Real-time PCR assay and isolation of total RNA are described in Section 2. mRNA levels were expressed normalised by the reference gene (RPLP0). Filler injection produced an increase in procollagens at 1 and 3 months compared with control (NaCl). TIMP-1 relative expression was significantly increased 1 month after filler injection compared with control and baseline values, and after NaCl injection compared with baseline. Six months after filler injection none of the markers expression remained significantly increased compared with control.

Fig. 4. Example of 2D ultrasound images for (a) an injected and (b) a non-injected area. The dermis appears as the homogeneous, echogenic (bright) structure in the top half of the image. The injected HA appears as the non-echogenic (dark) area within the hypodermis.
4. Discussion

This study was conducted to determine whether the mechanical stretching generated by HA injection could exert beneficial effects on constituents of dermal extracellular matrix. 3D ultrasound enables the filler volumes to be visualised and quantified after injection. Owing to their identical structures, it was not possible to distinguish strictly between injected and native HA. Nevertheless, injected sites were clearly identified on the images by the observation of hypo-echogenic areas.

Our analysis reveals that cross-linked HA accumulates in the hypodermis from its interface with the dermis, confirming an observation made in the nasolabial folds [16]. The increase in pressure occasioned by the injection probably causes the filler to flow naturally towards an area that deforms more easily than the dermis, i.e. the hypodermis, which is much less rigid [13].

In addition, we had demonstrated by immunohistochemistry in a previous study that the filler tended to migrate towards the softer parts of the dermis [17]. We were not able to differentiate injected HA gel strictly from native HA. Nevertheless, large hypo-echogenic areas were seen on high-frequency ultrasound images and large zones stained with Alcian blue were identified by histology. These observations are characteristic of the presence of HA gel [18] and were visible on the injected side only.

Finite element modelling was undertaken to corroborate this hypothesis (Fig. 7). This allows digital simulation of the action of the filler on the value and localisation of tissue stresses. Fig. 8 shows clearly that, as a result of differences in the rigidity of the dermis and hypodermis, the material tends to flow towards the latter structure. This appears to be the site of tensile stresses located predominantly in the superficial dermis. The changes in these stresses occur quasi-linearly with the excess pressure applied to the system. The values obtained and the deformation appear minor, but it is not possible to model the whole injection because of the marked distortion of the meshing. The results obtained, correlated with the ultrasound measurements of volume and dispersion of the filler, establish strong links between mechanical stresses and metabolic mechanisms.

Thus, the maximum volume and the minimum dispersion noted after 1 month necessarily occasion greater mechanical stress in the superficial dermis. Similarly, while the dispersion remains constant, the decrease in volume between 3 and 6 months results in a reduction in these stresses. This effect is amplified by a long-term relaxation phenomenon described in the literature in relation to skin expanders for autologous grafts [19]. The reduction in volume at 1, 3 and 6 months may be due to degradation of the hyaluronic acid injected or simply to migration, since the exploratory field of the probe precluded us from confirming one hypothesis or the other (limitation of the technique).

This stress induces deformation of the tissue matrix that might affect deformation-sensitive components on fibroblasts (mechanoreceptors). In fact, mechanoreceptors convert the mechanical signal into an intracellular biochemical signal involving molecular transformations that cause changes in the biochemical parameters. Transduction mechanisms have not been elucidated yet, but several articles suggest a role for integrins, in particular integrins...
suggests collagens, of MMP-3/TIMP-1 measured response although control. activity TIMP-1 gen reduction mechanical Fig. 7. Meshing generated to simulate the effect of the injection on skin stresses (superficial dermis, papillary dermis and hypodermis, depicted in red, green and blue, respectively). Injection was modelled by an elliptical area in which an increasing hydrostatic pressure load was applied.

α1β1 and α2β1, in the mechanical interaction between actin in the cytoskeleton and collagen fibres in the perception of mechanical stress [20,21].

The biochemical parameters sensitive to the mechanical effect are endogenous materials present in the dermis, predominantly collagens, but also molecules that regulate the metabolism of these materials. The main catabolic actors are the enzymes involved in the degradation of collagen, MMPs, and TIMPs, the latter being modulators of increased catabolic activity.

The results presented here show that the synthesis of neocollagen I and of procollagen I and III mRNA is significantly increased 1 month after injection of the filler compared with baseline and control. At the same time, a significant increase is observed in dermal TIMP-1 levels, confirmed by RT-PCR, and a significant decrease in the MMP-3/TIMP-1 ratio, raising the possibility of reduced proteolytic activity in the dermis exposed to mechanical stress. However, a slight increase in procollagen I levels is observed on the control side 1 month after injection compared with baseline. As with the filler, this suggests a metabolic change induced by a modification of the mechanical stress. However, it has been reported that the metabolic response to mechanical stress decreases with age and that this lack of response may be linked to the decrease in actin proteosynthesis, as has been established in isolated fibroblasts obtained from donors of varying ages [22].

The ultrasound measurements at 3 months show a loss of filler volume and an increase in its dispersion, testifying to a significant reduction in the stress exerted in the extracellular matrix measured 3 months after injection. At the same time, a marked reduction is noted in the metabolic response of the dermis, although the effect of the filler still persists at 3 months relative to the baseline value and the control side. The increase induced in procollagen I content by the filler is attenuated by comparison with the variations observed in the first month, but the increased expression of the different procollagens remains significant relative to the baseline values. In the case of TIMP1, a significant change from baseline and from the control is no longer observed at 3 months. On the other hand, with the MMP-3/TIMP-1 ratio, a significant decrease is found at 3 months between filler-injected sites, baseline and control.

At six months there is no longer any significant difference from baseline except for TIMP-1. Similarly, ultrasound observations of the fate of the filler corroborate the existence of an increased dispersion of the volume and validate the resultant loss of compression. This indicates a substantial reduction in the mechanical stress exerted in the dermis. Thus, the changes in anabolic markers are accompanied by those in catabolic markers, the two processes being intimately linked.

To conclude, these results confirm those of Fisher and Voorhees [2] and further elucidate the effect of a mechanical action induced by hyaluronic acid filling on dermal metabolism. Physiologically, the mechanical stresses can activate certain signal transduction pathways, causing the production of second messengers and other cell mediators. These events induce a change in the transcriptional activity of the fibroblast under stress and consequently of protein mRNA levels in the extracellular matrix [5]. Another phenomenon associated with the progressive degradation of hyaluronic acid might contribute to a change in fibroblast metabolism. Thus, certain polysaccharide fragments can interact directly with fibroblasts via membrane receptors and in so doing engender beneficial anabolic effects [5].

Fig. 8. Finite element analysis (plane strain) of the effect of injection on (a) the vertical displacement field in deformed geometry (magnification 1×) and (b) tensile stress (σ_11 in MPa) in deformed geometry (magnification 10×).
Although the mechanism of action remains unclear, by integrating both mechanical and biological aspects our studies highlight the role that may be played by the mechanical stress generated by the filler on the biochemical response of dermal cells.

In fact, the age-related reduction in mechanical stretching due to decreased HA content and fragmented collagen fibres in the skin is associated with a slowdown in biochemical response [1], so that the mechanical action of the filler would counteract this ageing phenomenon.

Role of the sponsor

The sponsor had no role in the design and conduct of the study, in the collection, analysis, and interpretation of data, or in the preparation of the manuscript, review, or approval of the manuscript.

Financial disclosure

Drs. A. Briant, S. Dahan are private practitioners and served as consultants and investigators for this study, for which they received honoraria. All other authors (V. Turlier, A. Delalleau, C. Casas, A. Rouquier, P. Bianchi, S. Alvarez, G. Josse, C. Saint-Martory, J. Theunis, A. Bensaï-Benaouda, A. Degouy, A.M. Schmitt, D. Redouïs) are employed by Pierre Fabre Laboratoires and received salaries, but they do not have any relevant financial interests in the findings from this manuscript.

Funding

This study was supported by Pierre Fabre Dermocosmétique (PFDC).

Acknowledgements

We are grateful to Dr. Marielle Romet for providing editorial assistance on behalf of Laboratoires Pierre Fabre Dermo-Cosmé tique, and to Laboratoires Pierre Fabre Dermo-Cosmétique for their financial support. We are grateful to Dr. Didier Cousiotou for help in designing the study.

References