# Association between Diffuse Idiopathic Skeletal Hyperostosis and Intestinal Microbiota Dysbiosis: a case-control study

**Final Degree Project**

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## 1. Abstract

**Background**: Diffuse Idiopathic Skeletal Hyperostosis (DISH) is a chronic disease with unknown pathogenesis and no specific treatment, which is known to cause axial stiffness and increase the risk of severe spinal fractures with neurological involvement after minor trauma. Although DISH has traditionally been considered a metabolic disease, more recent studies have observed that an inflammatory background may be involved in its development. Dysbiosis has been linked to the development of multiple inflammatory diseases such as inflammatory bowel disease or axial spondylarthritis. As such, were DISH to also be linked to dysbiosis, novel preventive strategies could be developed.

**Objectives**: the main aim of this study is to analyse the association between gut microbiota dysbiosis and DISH. The secondary objectives include determining which microorganisms could be responsible for the expected gut microbiota dysbiosis in DISH patients, whether said dysbiosis is independent of the presence or absence of metabolic diseases, and if dysbiosis is present in all DISH radiographic phenotypes.

**Study design**: this study is designed as a multicentric case-control study. It will be led by the "Hospital Universitari Doctor Josep Trueta" of Girona with the collaboration of 9 other Catalan hospitals, over a period of 2 years and 7 months

**Participants and methods**: a sample of 185 cases and 370 controls matched by age and sex will be recruited over a period of 11 months using non-probabilistic consecutive sampling for cases and probabilistic random sampling for controls. Microbiota composition will be determined in faecal samples using 16S rRNA gene sequencing, and principal component analysis will then be performed in order to design a dysbiosis test algorithm which will discriminate between presence or absence of dysbiosis in both cases and controls. Radiograph images of DISH patients will be analysed to determine their radiographic pattern. All other variables will be collected either from the medical record, anamnesis or measured at the consult. Once all samples have been collected, the data will be analysed using logistic regressions and multinominal logistic regressions to adjust for all covariables.

**Keywords**: Diffuse Idiopathic Skeletal Hyperostosis (DISH), dysbiosis, inflammation

## 2. Abbreviations

**ADP**: Adiponectin

**ALL**: Anterior Longitudinal Ligament

**ASD**: Ankylosing Spinal Disorders

**ASIA**: American Spinal Injury Association

**axSpA**: Radiographic Axial Spondylarthritis

**BMI**: Body Mass Index

**BMP2**: Bone Morphogenic Protein 2

**CRP**: C Reactive Protein

**CT**: Computerised Tomography

**DISH**: Diffuse Idiopathic Skeletal Hyperostosis

**DKK-1**: Dickkopf-1

**DM**: Diabetes Mellitus

**DNA**: Deoxyribonucleic Acid

**EDISH**: Early Diffuse Idiopathic Skeletal Hyperostosis

**GH**: Growth Hormone

**GI**: Gastrointestinal

**IBD**: Inflammatory Bowel Disease

**IBS**: Irritable Bowel Syndrome

**IGF-1**: Insulin-like Growth Factor 1

**IL-6**: Interleukin 6

**MDCT**: Multidetector Computed Tomography

**MRI**: Magnetic Resonance Imaging

**MS**: Metabolic Syndrome

**MSUS**: Musculoskeletal Ultrasound

**NCEP**: National Cholesterol Education Program

**NFκB**: Nuclear Factor κB

**NGS**: Next Generation Sequencing

**NSAID**: Non-Steroidal Anti-inflammatory Drug

**OPLL**: Ossification of the Posterior Longitudinal Ligament

**PCA**: Principal Component Analysis

**PCR**: Polymerase Chain Reaction

**PGI2**: Prostaglandin I2

**PLL**: Posterior Longitudinal Ligament

**SAT**: Subcutaneous Adipose Tissue

**SCI**: Spinal Cord Injury

**SCFA**: Short Chain Fatty Acid

**SIJ**: Sacroiliac Joint

**SLE**: Systemic Lupus Erythematosus

**SpA**: Spondylarthritis

**TGF-β3**: Transforming Growth Factor β3

**Th17**: Helper T-lymphocyte 17

**TNF-α**: Tumoral Necrosis Factor α

**US**: Ultrasound

**VAT**: Visceral Adipose Tissue

**WHO**: World Health Organization

# 3. Introduction

## 3.1 Diffuse Idiopathic Skeletal Hyperostosis

### 3.1.1 General information

Diffuse Idiopathic Skeletal Hyperostosis (DISH, also known as Forestier's disease) is a systemic, non-inflammatory condition characterised by progressive calcification and ossification of entheses, ligaments and joint capsules (1). DISH was first described by Jacques Forestier and Jaume Rotés-Querol in 1950 under the name "*Senile Ankylosing Hyperostosis of the Spine*" (2), but it is now known that this disease is neither limited to elderly patients nor the spine, even if axial involvement is its main manifestation (1,3).

This disease's main characteristic is the formation of flowing calcification and ossification along the anterior longitudinal ligament (ALL) of vertebral bodies (4), causing back pain and stiffness amongst other symptoms (2,5); this mainly affects the thoracic spine, but cervical and lumbar ossification has also been described (6). OPLL is also possible in DISH patients (7). Extraspinal involvement in the form of calcification and ossification of joints and entheses is frequent (3).

### 3.1.2 DISH Epidemiology

DISH prevalence is higher in men than in women, and has been found to increase with age (5,8–10). Certain conditions such as obesity and type 2 Diabetes Mellitus have also been associated with the development of DISH, which could explain a higher prevalence in Western countries (9). Exact prevalence is unknown and varies widely with age groups (*Table 1*) (11).

*Table 1: DISH epidemiology and variation with age. Adapted from* (11)

| Author | DISH prevalence | Age group |
|---|---|---|
| Holton *et al.* (12) | 42% | 298 males aged over 65 |
| Cassim *et al.* (13) | >10% | Patients aged over 70 |
| Weinfeld *et al.* (10) | 25% males | Patients aged over 50 |
| | 15% females | |
| | 35% males | Patients aged over 70 |
| | 26% females | |

### 3.1.3 DISH Aetiology and pathogenesis

There is currently no known specific pathogenic mechanism of DISH (5,14). As previously stated, multiple studies have found an increment in DISH prevalence in association with an increase in age, which is also observed per decade of age (9,10,15).

Male sex has also been described as a risk factor for developing DISH. The prevalence gap between males and females also appears to widen with age, observing that a greater percentage of DISH diagnoses belong to male patients in elderly age groups when compared to younger age groups, where the difference of DISH prevalence between males and females narrows considerably (5).

Obesity has been linked to DISH since it was described by J. Forestier and J. Rotés-Querol (2). The presence of an elevated body mass index (BMI) is a frequent finding in patients with DISH (16,17). A study evaluating the prevalence of DISH amongst young severely-obese patients found it to be 18%, and suggested considering early-onset DISH as a musculoskeletal complication related to obesity (18). Studies have found an increase in visceral adipose tissue (VAT) surface area in DISH patients when compared to non-DISH controls, as well as an increase in VAT:SAT surface area ratio (19). Some studies have also found an increased incidence of hypertension (HT) in DISH patients (12).

Diabetes mellitus has traditionally been associated to DISH, although some studies question this relation, finding no statistically significant differences in DISH prevalence between patients with and without DM (20). Other studies, however, have found diabetes mellitus to be statistically more common in patients with DISH (16,21), especially in DISH with OPLL (22). A 2001 study by Akune *et al.* suggested that insulin may contribute to the process of ossification; since insulin receptor substrate-1 is also expressed in the spinal ligament, an excess could stimulate osteoprogenitor cells and induce ossification of enthesis (23).

Given the association of obesity, hypertension, and diabetes mellitus with DISH, it is not surprising that the metabolic syndrome (MS) is also related. According to a study published by Mader *et al.* in 2009, DISH patients were much more likely to suffer from HT and increased waist circumference; this increments the likelihood of suffering from metabolic syndrome. This same study also found an augmented prevalence of

hyperglycaemia in DISH patients, however it only contributed to the MS diagnosis when the NCEP-MS diagnostic criteria were used, and not WHO-MS criteria (24).

Smoking as a risk factor for DISH is debated (5). While some studies found a higher incidence of smoking in DISH patients when compared to non-DISH patients (25), others found no statistically significant differences (12) and others even found smoking to be less common in DISH patients than in the general population (26).

Genetic predisposition may also play a role in the pathogenesis of DISH. In dog models, DISH has been found to be much more prevalent amongst Boxers (40%) in comparison to other dog breeds (4%) (27,28). A 2006 study by Horikoshi *et. al* reported an association between ossification of the PLL and a reduced expression or function of *TGF-β3* (29). Other genes implicated in osteogenesis, such as *RUNX2*, *IL11*, *GDF5*, *NOG*, *CCDC91* and *UQCC1*, also appear to play a role in the development of DISH (30).

Vascular factors are probably implicated in DISH pathogenesis (5). Hypervascularity likely plays a role in the ossification process, observing an increment of nutrient foramina in ossification sites of vertebrae affected by DISH, although it is difficult to assess whether it is a cause or a consequence of said ossification (31).

As previously stated, DISH is a systemic disease linked to the metabolic syndrome (24), although other metabolic associations have been described. Growth hormone (GH) and IGF-1 have been associated with normal bone growth and development (32). A 1994 study observed that DISH patients have increased levels of serum GH when compared to non-DISH patients (33), but presented normal IGF-1 levels. DISH patients also present increased levels of synovial fluid GH when compared to serum GH (34).

Vitamin A and retinoids have been associated to the appearance of lesions similar to both DISH and seronegative spondyloarthropathies, being hyperostosis the main adverse reaction in prolonged treatments and high dosages (35,36). In spite of this findings, it is not clear whether vitamin A plays any significant role in DISH pathogenesis (6).

Signalling pathways have also been investigated for their role in bone formation and DISH. The Wnt-β-catenin pathway is involved in the differentiation of mesenchymal cells into osteoblasts and thus favouring bone formation; it may be linked with DISH due to

alterations in DKK-1 levels, a Wnt-inhibiting protein (6,37). Results are heterogeneous, as a study found patients with advanced DISH presented lower DKK-1 levels (38), whereas another study found no statistically significant differences between DISH patients and healthy controls (39).

NFκB may also affect DISH development (6), as it stimulates mesenchymal cells to undergo osteoblastic differentiation and has been found in higher concentrations in tissue from DISH patients (40). Other signalling pathways such as BMP2 signalling, PGI2 and endothelin 1 may play a role in DISH pathogenesis, but further studies are needed (6).

### 3.1.4 DISH as an inflammatory disease

As recently specified, the link between DISH and metabolic abnormalities is robust (24), and thus, has traditionally been considered a metabolic disease without an inflammatory base (14). However, it has been recently proposed that inflammation could play a role in DISH pathogenesis and bone formation (14,41).

Entheseal ossification, a frequent finding in DISH patients (1,3), is also a frequent finding in inflammatory diseases such as spondylarthritis (SpA), and a 2018 systematic review by *Kuperus et. al* reported co-occurrence of DISH and radiographic axial spondylitis (axSpA) in 39 cases (42). A 2021 review by Mader *et. al* suggested that local inflammation could act as an initiating factor for entheseal ossification in DISH (14). This is further supported by ultrasound findings suggestive of local inflammation, such as an increased Power Doppler activity and even erosions in DISH patients (43).

Pelvic entheseal abnormalities have been reported in DISH patients (14,44), which are also a common finding in axSpA (45). Fusion of the sacroiliac joint (SIJ), frequently seen in axSpA (46) has also been described in DISH patients (47).

As previously stated, DISH patients present an increased VAT surface area and VAT:SAT surface area ratio (19). This excessive body fat has a proinflammatory effect (given the function of adipose tissue as an endocrine organ), producing adipokines such as ADP and leptin, and other cytokines (such as IL-6 or TNF-α) (48–50). Leptin is a cytokine with proinflammatory effects, which increases TNF-α and IL-6 production (48,51).

In contrast, ADP can have either a pro-inflammatory or an anti-inflammatory effect (52), and is found in lower concentrations in patients with incremented body fat and insulin resistance (52–54). In a 2018 study by Mader *et al*, higher serum ADP levels in DISH patients were found to have a pro-ossification effect, resulting in more pronounced bony bridges (54). Ligament ossification in DISH and axSpA could be a product of low ADP levels and thus increased pro-inflammatory effects (14).

We have already mentioned the increase of nutrient foramina in ossification sites (31). Chronic inflammation acts as a promoter of angiogenesis in multiple diseases (such as MS) (55), which *Mader et al.* hypothesise could link chronic diseases and inflammation with DISH (14).

A 2023 study by Pariente *et al.* found EDISH (early DISH) patients had higher plasma C reactive protein levels in comparison to non-DISH patients, as well as an increment of other proinflammatory markers. This could be due to both MS or other proinflammatory condition, or due to EDISH itself. This same study also found trabecular bone deterioration in early stages of the disease, another marker of chronic inflammation. This findings support an inflammatory origin of DISH (41).

Given that current DISH diagnosis is based on X-ray findings, previous proinflammatory states may pass unnoticed (14). Thus, the hypothesis of an inflammatory origin to DISH should be further explored, as it could lead to new therapeutic options (14,41,56).

#### 3.1.5 Clinical features

DISH is asymptomatic in many individuals and is often limited to a radiological incidental finding when imaging studies for other conditions are performed (5,11). Nevertheless, several symptoms may appear which can guide us towards the diagnosis.

##### Cervical spine involvement

Ossification of the ALL is a possible cause of both dysphagia and airway obstruction, due to the increased cervical bone volume displacing the oesophagus and trachea (5,57). A 2011 review by Verlaan *et al.* found 204 described cases of this phenomenon, and suggests that, even though the slow development of cervical bony bridges may be well

tolerated, trigger events such as aspiration, regurgitation, minor cervical trauma… could acutely cause local oedema which would not be easily compensated (57).

Stiffness and back pain

In their original article, J. Forestier and J. Rotés-Querol described spine stiffness as the main symptom of DISH, mainly involving the dorsal region but frequently affecting the lumbar spine as well (2). Actual reviews also support this finding (5,6).

Back pain has also been reported by some studies (58). Nevertheless, other studies found less back pain in DISH patients that in non-DISH controls, and hypothesise that this may be a consequence of vertebral fusion, resulting in increased spinal stability (12). Further research is necessary in order to evaluate whether back pain is more prevalent in EDISH stages than in advanced cases (5).

DISH is also associated with physical impairment, observing decreased grip strength and ability to bend, as well as alterations in the chair stand test. These abnormalities are used as fall predictors, implying that DISH may cause an increase in fall risk (26).

Vertebral fractures

Patients with ankylosing spinal disorders (ASD), such as those suffering from DISH or axSpA, have an increased risk of vertebral fractures as well as a higher risk of injuring their spinal cord (59–61). Most fractures affect the cervical spine, and fracture frequency decreases as lower vertebral segments are evaluated (*Table 2*) (59,62).

*Table 2: spinal fracture distribution in patients with ASD.* Adapted from (59)

| Region | Level | Fractures | axSpA | DISH | Final SCI ASIA (A-D) |
|---|---|---|---|---|---|
| Cervical (55% total fractures) | C2-T1 | 55% | 22,95% | 31,97% | 60% |
| Thoracic (32% total fractures) | T1-L1 | 30% | 6,56% | 25,41% | 31% |
| Lumbar (13% total fractures) | L1-S1 | 14% | 6,56% | 6,56% | 19% |

Ankylosis prevents the spine from absorbing the energy resulting from a fall and, at the same time, causes the spine to act like a solid rod. This implies that in ASD fractures the spine usually breaks transversely, as a long bone would, due to the bending it is subjected to (63). Most DISH patients suffer fractures through the vertebral body (61), due to the fact that ossification is thickest at the sides of the intervertebral disc, leaving the central portion of the vertebral body as the weakest section (64) (*Figure 1*). This fractures may occur even after low-energy trauma (65).

Fractures in ankylosed spines are extremely unstable, due to the ossification of ligaments and other supportive spinal tissues, causing them to fracture alongside the spine (66).

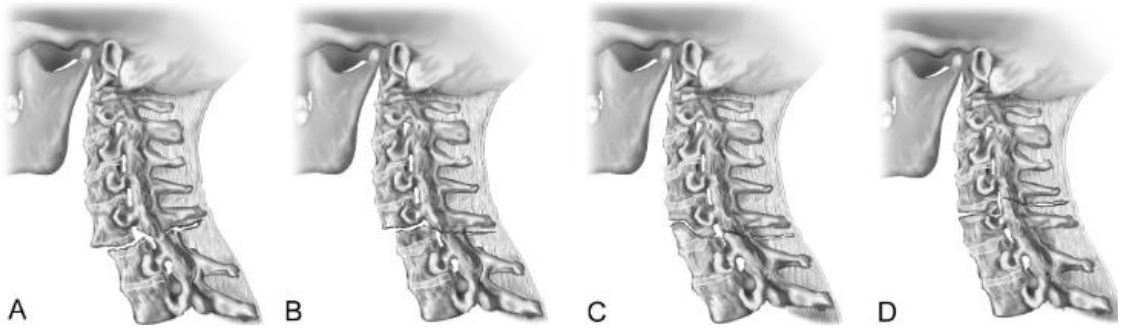



*Figure 1: types of fracture in the ankylosed spine. Extracted from* (59)

- *A: Type I, disc injury*
- *B: Type II, body injury*
- *C: Type III, anterior body or posterior disc injury*
- *D: Type IV, anterior disc or posterior body injury*

Since patients with ankylosing spine may experience back pain, fractures after minor trauma may pass unnoticed; at the same time, bone spinal alterations imply evaluating the spine is more difficult than in non-ankylosing patients. These factors imply that diagnostic delays are frequent (62). In a 2019 study by Okada *et al.* cervical fractures were found to present less diagnostic delays, as they produced more noticeable neurological symptoms at the time of injury (67).

Spinal fracture instability implies a higher risk of spinal cord injury (65), which corelates with an increased incidence of complications and mortality (62). Patients with ASD and traumatic spinal fractures have been found to present higher rates of neurologic deficit, complications and mortality than patients without ASD (59,68) (*Table 3*). Spinal epidural haematomas can develop due to the rupturing of the spinal epidural venous plexus, which may cause subacute spinal cord injury (62).

*Table 3. Medical complications associated with spine fractures in patients with ASD. Adapted from* (59).

| Adverse effect | Percentage value |
|---|---|
| Pulmonary problems | 35% |
| Urinary tract infections | 24% |
| Percutaneous external gastrostomy tube | 10% |
| Tracheostomy | 10% |
| Deep venous thrombosis | 8% |

The use of MDCT or MRI is recommended in order to properly observe signs of injured osteoligamentous compartments after minor trauma (59,69). Were spinal cord injury to be clinically suspected, an MRI should always be performed (69).

The unstable nature of fractures in ankylosed spines implies that most will require surgical fixation, and sometimes even decompression (59). A 2023 study by *Chen et al.* found that, while patients with DISH suffer less severe fractures and need surgery less often than patients with axSpA, both outcomes and one-year mortality do not differ significantly between them (70).

Extraspinal involvement

As previously mentioned, extraspinal ossification is common in DISH patients (1). These peripheral alterations are characterized by:

- Ossification of joints seldomly affected by osteoarthritis
- More frequent hypertrophic alterations in comparison to osteoarthritis
- Enthesopathies at multiple regions, usually near peripheral joints
- Other regions with entheseal ossification (3)

Entheseal ossification is frequent, especially in the elbows, shoulders, knees and heels (58,71). DISH patients usually experience peripheral pain, especially in the upper extremities, and higher rates of medial epicondylitis (58). Said peripheral involvement is usually symmetrical and similar to what can be observed in osteoarthritis, but with atypical locations also being affected (6).

### 3.1.6 Image findings

Currently, the diagnosis of DISH relies on radiographic findings (72), which are essential in order to evaluate the extent of ossification in both the peripheral joints and the spine (73). DISH can be accurately diagnosed via radiographic findings (5); CT scan is nowadays used as well, although modifications to the diagnostic criteria are suggested in order to adapt to the use of three-dimensional techniques (74).

Axial bone formation in the form of flowing ossification of paravertebral soft tissue and ALL is observed (4). These osteophytes in DISH are more often horizontal (>45º), in contrast to the more frequently vertical (<45º) osteophytes of axSpA (75).

A 2022 article by Nguyen *et al.* evaluated two types of osteophytes in DISH according to their angle morphology at the vertebral edge, setting 45º as the cut-off value (*Figure 2*):

- Jaggy type osteophytes presented with either their superior or inferior vertebral edge at a >45º angle and are associated with lower serum CRP levels. The authors consider this type of ossification to be related to degenerative processes.
- Flat type osteophytes presented with both vertebral edges at a <45º angle and are akin to ossification found in axSpA; they are more frequent in the upper thoracic spine. Since patients with predominant flat-type osteophytes also had higher serum CRP levels, it is likely that this type of bone formation is associated to an inflammatory process (56).

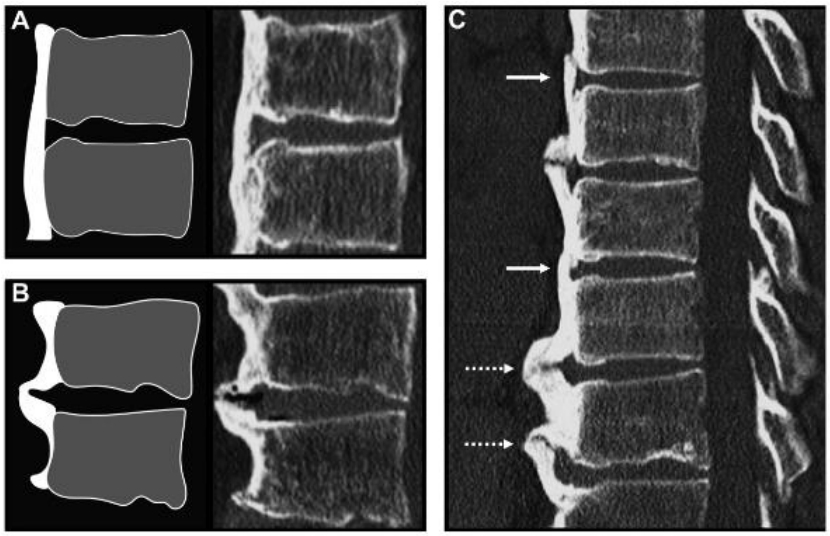



*Figure 2: morphological characteristics of ectopic ossification in DISH, extracted from* (56)*:*

*A) Flat type osteophytes*

*B) Jaggy type osteophytes*

*C) Combination of both osteophytes in a DISH patient, flat types marked by a continuous arrow and jaggy types marked by a dotted arrow*

Cervical spine

In their original study, Resnick and Niwayama found that 78% of DISH patients presented cervical abnormalities (4).

Hyperostosis begins at the inferior portion of the anterior margin of the vertebral body and develops into enthesophytes; these expand until forming bony bridges described as “candle flame” or “parrot beak” images (76). Cervical bone formation is usually anterior in relation to the vertebral body rather than lateral, and roughly symmetrical (77). Concomitant presentation of DISH with OPLL has been observed at 57.14% (56).

Thoracic spine

Thoracic involvement is the main radiographic finding in DISH patients (6,76). Resnick and Niwayama found 97% of their patients presented altered thoracic radiographs, and DISH diagnosis was mainly achieved due to these abnormalities.

Flowing ossification anterolateral to the vertebral bodies and spanning over the disk space is observed. There is an increase in bone deposition at the disk space (4). Disk degenerative changes have also been reported in DISH patients up to certain extent (78), even though the Resnick criteria consider extensive disk degeneration to exclude DISH diagnosis (4).

The main localisation of ossifications was the lower thoracic spine, but continuous bone spanning the entirety of the thoracic spine is also possible (4). The distribution of said bone bridges is asymmetrical and more frequent in the right thoracic spine, opposite the aorta, as aortic pulsation prevents the ossification of the ALL (79).

Lumbar spine

The lumbar region is the portion of the spine least affected by DISH. However, when lower or middle thoracic ossification is present, coexisting lumbar abnormalities have been observed in 30.7% and 33% of cases respectively (15). If present, radiographical alterations are akin to the ossification observed in the cervical spine (76).

Sacroiliac joints

Although the Resnick criteria exclude SIJ abnormalities for the diagnosis of DISH (4), in other studies Resnick *et al.* described the presence of para-articular osteophytes near the inferior margin of the SIJ (1). Other SIJ abnormalities seen in DISH are asymmetrical ossification of the superior ligaments and vacuum phenomena (80,81).

DISH patients may even present SIJ fusion and formation of both anterior and posterior bone bridges, alongside fusion of the entheses. This may pose a diagnostic dilemma, as it implies the differential diagnosis between DISH and axSpA can be complicated (82).

Pelvic and extra-axial findings

Extraspinal ossification in DISH is common, frequently observing formation of calcaneal posterior and inferior spurs; ossification of the patella, especially its superior margin; shoulder bone irregularities; olecranon spurs; and other zones presenting hyperostotic abnormalities such as the hands and feet (1). DISH patients also present severe pelvic juxtaarticular spurs, mainly in the acetabular margin (83).

A 2015 study by Mader *et al.* evaluated MSUS as a diagnostic technique and found that, upon entheseal US evaluation, DISH patients presented increased entheseal thickness, enthesophytes, erosions, and Power-Doppler blood flow. These alterations could help in the identification of this disease and contribute to its early diagnosis (43).

### 3.1.7 Diagnostic criteria

There are multiple classification criteria (6) (*Table 4*). However, there is still no consensus on the definition of DISH (84). The most widely used criteria nowadays are still those described by Resnick and Niwayama, although they only evaluate late stages of DISH and exclude extra-axial findings from the diagnostic process (5).

*Table 4. Suggested main diagnostic features of DISH, extracted from* (6)

| Definition | Number of vertebrae connected by bony bridges | Peripheral enthesopathies | SIJ involvement |
|---|---|---|---|
| Resnick and Niwayama (4) | 4 in the thoracic spine | Not required | Not involved |
| Arlet and Mazieres (85) | 3 in the lower spine | Not required | Ossification in the vicinity of SIJ allowed |
| Utsinger (71)<br>Definite DISH<br>Probable DISH<br>Possible DISH | <br>4 in the thoracolumbar spine<br>2 in the thoracolumbar spine<br>2 in the thoracolumbar spine<br>None | <br>Not required<br>Bilateral enthesopathies<br>Not required<br>Presence of symmetrical enthesopathies, preferably in >2 anatomical sites | Involvement of SIJ is not an exclusion criterion |
| Rogers and Waldron (86) | 3 in the thoracic spine | Peripheral calcification or ossification of ligaments and/or entheses | No reference |

Resnick and Niwayama criteria

Defined by Donald Resnick and Gen Niwayama in 1976, these criteria are exclusively based on the spinal radiographic findings in advanced DISH patients. DISH diagnosis is achieved when all the following characteristics are met:

- Flowing ossification of 4 or more contiguous vertebral bodies along their anterolateral aspect
- Preserved vertebral disk height in affected regions, without extensive signs of disk degeneration such as vacuuming of sclerosis of the vertebral body margin
- Preserved sacroiliac joints and absence of joint ankylosis in bone apophyses (4)

The main limitation of these criteria is that, since peripheral manifestations are ignored, a loss of diagnostic sensitivity is observed even if specificity is high (71).

Utsinger criteria

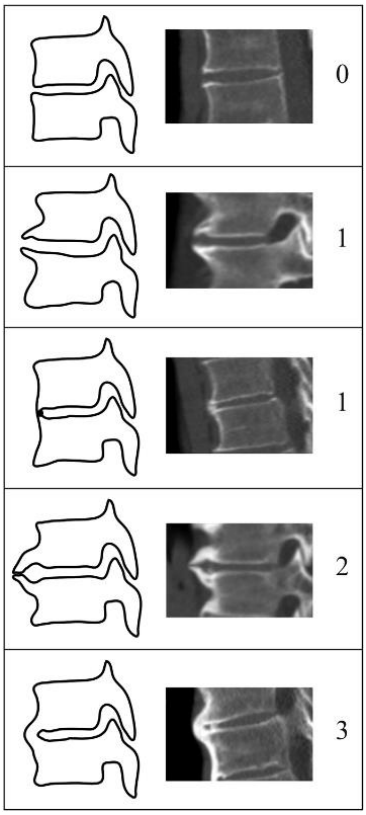
Created by Peter D. Utsinger in 1985, these diagnostic criteria aim for higher specificity while maintaining high sensitivity:

- Diagnostic criteria:
    1. Flowing ossification of the anterolateral aspect of 4 or more contiguous vertebral bodies
    2. Flowing ossification of the anterolateral aspect of 2 or more contiguous vertebral bodies
    3. Extra-axial symmetrical enthesopathies with well-delimited cortical margins affecting the posterior heel, superior patella or olecranon
- Exclusion criteria:
    - Disk height abnormalities in affected areas
    - Ankylosis of joint apophyses
- DISH categories:
    - Definite DISH: the patient meets criteria 1
    - Probable DISH: the patient meets criteria 2 and 3
    - Possible DISH: the patient may meet:
        - Criteria 2 and 3

- Criteria 2
- Criteria 3, especially when concomitance of calcaneal and patella or olecranon spurs is observed (71)

New criteria for EDISH

A 2019 study by Kuperus *et al.* developed a set of criteria for the CT diagnosis of EDISH to distinguish patients with definite DISH, no DISH and EDISH. A score was associated to each vertebral segment based on the amount of bone bridge formation between vertebral bodies (*Figure 3*) (87).




- *Score 0: normal vertebral bodies*
- *Score 1: any of the following:*
  - *Anterior osteophytes connected or not to the vertebral body but separated between them by 2 millimetres or more.*
  - *Small bone bridge between 2 contiguous vertebral bodies, lacking abundant bone formation.*
- *Score 2: any of the following:*
  - *Anterior osteophytes with a separation of less than 2 millimetres.*
  - *Bone connection in 2 or less CT sections.*
- *Score 3: complete bone bridge in more than 2 CT sections.*

*Figure 3: scoring system for the definition of EDISH. Extracted from* (87).

EDISH could be diagnosed in the following situations:

- Presence of 3 near-complete bone bridges.
- If all the following are met:
  - Presence of a complete bone bridge.
  - Presence of an adjacent almost-complete bone bridge.
  - Presence of an adjacent segment with at least new bone formation.

These criteria had a 96% sensibility and an 83% specificity for EDISH diagnosis (87).

### 3.1.8 DISH phenotypes

In a 2021 cross-sectional study by Clavaguera *et al.*, the authors described three different DISH phenotypes by clinical symptoms and radiologic findings:

- Axial Pattern: spinal manifestations are dominant, with few peripheral alterations; mostly found in older-age men, these patients exhibit a higher incidence of cardiovascular events. This group met all Resnick criteria but were less symptomatic.
- Mixed Pattern: pattern characterised by both frequent extraspinal and axial ossification and clinical symptoms; it is also more frequent in elderly patients, but with smaller male predominance than the axial pattern. These patients were usually diagnosed combining clinical and radiological findings. Cervical spine ossification is more common.
- Peripheral Pattern: these patients mainly exhibit clinical enthesopathies with limited axial manifestations, and frequently present hip ossification (65%). It is more frequently found in women and younger patients. A minimum of three enthesopathies are necessary to diagnose this pattern, but, by definition, it does not fulfil the Resnick criteria.

The presence of these phenotypes, especially the peripheral pattern, manifests the limitations of the Resnick criteria and the necessity to include extraspinal manifestations of DISH in the diagnosis, in order to fully encompass this disease's spectrum (88).

### 3.1.9 Treatment

The knowledge gap on DISH pathogenesis implies that no specific treatment for this disease is currently known and instead symptomatic treatment is performed (5,6). In general lines, DISH treatment should focus on (89):

- General measures: physical activity may be useful to decrease pain and improve mobility (6,89), and alongside weight reduction and diets poorer in saturated fats and carbohydrates could also disrupt DISH progression (89).

- Symptomatic treatment: protective bandages or insoles for plantar spurs can also help in improving symptoms of peripheral pain (89). Oral NSAIDs, analgesics or tramadol may be useful in DISH patients (6). Most treatments for osteoarthritis appear to relief symptoms in DISH patients as well (89); as such, topic NSAIDs may be as effective as oral drugs, but with less gastrointestinal adverse effects (90). Local infiltration of anaesthetics and corticosteroids is also possible for severely symptomatic patients (89).
- Correction of metabolic abnormalities: ACE inhibitors, calcium channel blockers and α-blockers in order to treat arterial HT are preferred as they neither disrupt lipoprotein metabolism nor worsen insulin resistance (91). Hyperglycaemia and hyperinsulinemia can be controlled by glucose lowering oral drugs (89).
- Surgical interventions: osteophytectomy is a possible surgical treatment in cases of cervical DISH causing dysphagia or airway compression and should always be considered when these conditions are refractory to medical management (92). As previously mentioned, surgical management of spinal fractures in DISH patients is usually necessary (59).

## 3.2 Dysbiosis and its relationship with inflammatory diseases

### 3.2.1 Introduction to gut microbiota

Human gut microbiota is a diverse community of gastrointestinal microorganisms (93), comprised by multiple bacteria as well as archaea, viruses, fungi and other eukaryotic microorganisms (94–96). This microbial diversity is not fixed, but rather it is fluid and changes with time in healthy adults (97).

Gut microbiota play an essential role in human physiology, interacting with us on a metabolic, protective, structural and neurological level (*Figure 4*) (98). A diverse gut microbiome is beneficial, as it implies more functional niches are occupied and thus helps in preventing colonisation by pathogenic microorganisms (93).

However, gut microbiota has also been related to other conditions, such as obesity subsets developing specific metabolic risk profiles (99), or Alzheimer's disease through the microbiota-gut-brain axis (100)

Indeed, drastic changes to the composition, distribution and metabolic activities of said microbiota, known as dysbiosis, are linked to multiple diseases such as IBD, allergic disorders (101), or type 2 diabetes mellitus (102).

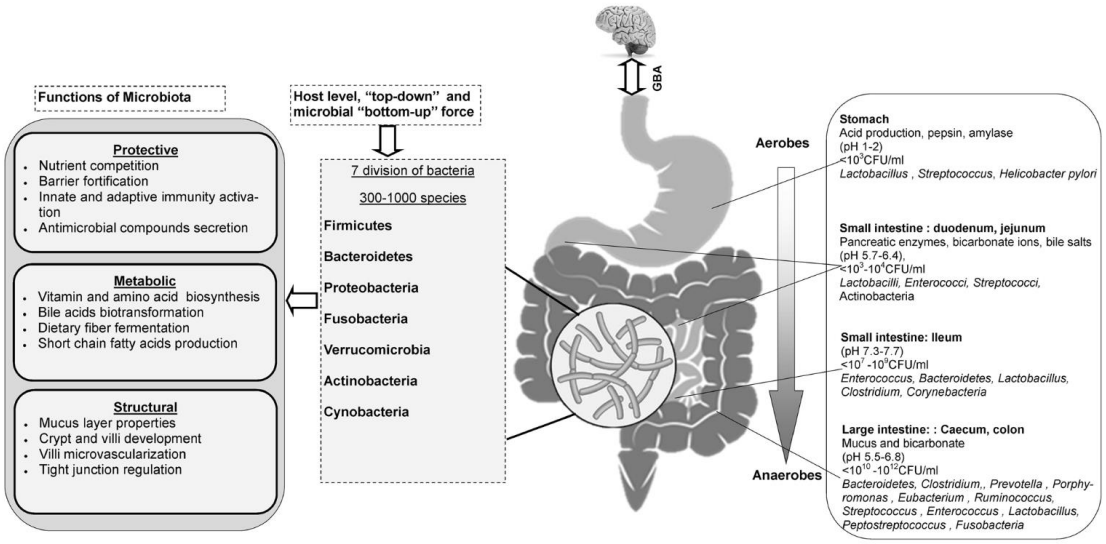



*Figure 4: normal gastrointestinal microbiome in the human gut and main functions. Extracted form* (98).

### 3.2.2 Microbiota and immune modulation

Gut microbiota is known to participate in the regulation of host immunity and GI homeostasis (103). For example, short chain fatty acids (SCFA), which are end products of gut microbiota metabolism (104), have been found to supress NF-κβ activation as well as inhibit the secretion of certain proinflammatory cytokines such as TNF-α (105).

A 2016 study by Schirmer *et al.* aimed to study the gut microbiome with production of inflammatory cytokines and found that metabolism of microbial tryptophan was an important mediator of cytokine production, mainly inhibiting TNF-α production via its metabolite tryptophol. Other microbial metabolites such as palmitoleic acid inhibited TNF-α, IL-1β or IL-6 response. This results also apply to the involvement of specific microorganisms, such as *S. aureus* which was associated with the Th17 response while other microorganisms did not influence it (106).

Thus, it is not surprising to discover that microbiota abnormalities in relation to the immune system may play a role in the development of inflammatory diseases, and that solving this disbalances could help in treating chronic inflammation (103). Studies have shown that inflammatory diseases such as IBD present important GI dysbiosis with depleted populations of *Bacteroidetes* and *Lachnospiraceae* (107).

Although further research is needed, therapeutic microbiome interventions have been shown to influence the immune system and further advancements are expected to occur (93).

### 3.2.3 Microbiota and rheumatic diseases

Similarly, gut dysbiosis has also been found in rheumatic diseases, such as rheumatoid arthritis, where a 2015 study by Zhang *et al.* even found partial reversal of microbiota disbalances after treatment (108). Other rheumatic diseases like SLE (systemic lupus erythematosus) (109) or primary Sjogren syndrome also present dysbiosis (110).

A 2022 meta-analysis by Wang *et al.* found that, when evaluating rheumatic diseases and dysbiosis:

- α-diversity was moderately decreased in rheumatic diseases in general, but only specifically in rheumatoid arthritis, gout, SLE and fibromyalgia
- β-diversity was consistently altered in axSpA and IgG4-related diseases
- A lack of anti-inflammatory and an increase of pro-inflammatory bacteria (such as *Streptococcus*) was found in rheumatoid arthritis, SLE and Sjögren's syndrome
- Disease-specific alterations still require further studying (111).

In axSpA patients, a decrease in microbial α-diversity has been observed when compared to healthy controls, as well as a lesser presence of butyrate-producing bacteria; in fact, in these patients, bone formation decreased when butyrate was administered, thus remarking the benefits of SCFA production by gut bacteria and the potential therapeutic implications of correcting dysbiosis (112). A 2024 meta-analysis by Su *et al*. found that the populations of *Bifidobacterium* decreased in axSpA patients, while the abundance of *Bacteroidetes* increased (113).

## 4. Justification

Diffuse Idiopathic Skeletal Hyperostosis (DISH) is a chronic disease with unknown prevalence, but its association with obesity, diabetes and old age implies it will likely become more common in the near future and in western countries. Although it is not infrequent for this disease to be asymptomatic, many other patients can present spinal rigidity, peripheral pain, physical impairment and severe symptoms such as unstable spinal fractures and subsequent spinal cord injury. DISH has no specific treatment, and only symptomatic measures can be applied.

The pathogenesis of DISH is unknown. Hyperinsulinemia, GH and IGF-1 and other metabolic factors have been investigated as possible causes for the development of said disease. However, a new hypothesis of DISH as an inflammatory disease has recently emerged as a contender for explaining the development of this condition, with studies showing that certain cases of DISH have distinct clinical and radiological characteristics and are simultaneously associated to higher C reactive protein (CRP) levels.

Dysbiosis is the "disturbance to gut microbiota homeostasis due to an imbalance in the flora itself, changes in their functional composition and metabolic activities, or changes in their local distribution" (101), and has been linked to the development of multiple inflammatory diseases such as IBD, rheumatoid arthritis or axSpA, due to the interactions between microbiota and the immune system. In fact, symptoms of rheumatic diseases such as axSpA show certain improvement after administering molecules that would be produced by the normal microbiota. Thus, the correction of dysbiosis could help not only in treating but also preventing certain inflammatory conditions.

DISH, as a possible inflammatory disease, could exhibit microbiota imbalances. The main reason for the development of this protocol is the knowledge gap of DISH pathogenesis. Were dysbiosis to be linked to DISH, this study could serve as a rallying point for understanding whether microbiota imbalances also play an active role in DISH pathogenesis and even explore new options for prevention altogether.

Thus, the aim of this study is to analyse the association between gut microbiota dysbiosis and DISH diagnosis.

## 5. Hypotheses

### 5.1 Primary hypothesis

Patients with Diffuse Idiopathic Skeletal Hyperostosis (DISH) will have higher odds of presenting intestinal microbiota dysbiosis in stool microbiome analysis than non-DISH controls.

### 5.2 Secondary hypotheses

The secondary hypotheses are:

- Intestinal microbiota dysbiosis in all DISH patients will be present independent of presence of metabolic disease (obesity or diabetes mellitus).
- Gut microbiota composition in DISH patients will differ from non-DISH patients.
- Gut microbiota dysbiosis will more frequently be present in DISH patients with pattern equivalence or a flat-dominant pattern in their radiographic phenotype.

## 6. Objectives

### 6.1 Main objective

To analyse the association of intestinal microbiota dysbiosis and Diffuse Idiopathic Skeletal Hyperostosis (DISH) of adult patients in Catalonia.

### 6.2 Secondary objectives

- To analyse the association of intestinal microbiota dysbiosis and diffuse idiopathic skeletal hyperostosis (DISH) in adult patients in Catalonia by the presence of metabolic disease (obesity or diabetes mellitus).
- To compare the proportion of each phyla's microorganisms present in DISH and non-DISH subjects.
- To analyse if the proportion of intestinal microbiota dysbiosis differs among the three different DISH radiographic phenotypes (jaggy-dominant pattern, flat-dominant pattern, pattern equivalence). This last objective will not be evaluated in our control population, only in DISH patients.

## 7. Material and methods

### 7.1 Study design

This study follows a multicentre case-control design study.

### 7.2 Study population and setting

The population of the subsequent study is comprised of patients with a DISH diagnosis according to the 1976 Resnick criteria (3.1.7 Diagnostic Criteria). A total of 10 reference hospitals from Catalunya with a rheumatology unit will be invited to take part in this study (catchment area in brackets):

- Hospital Universitari Doctor Josep Trueta (800,000)
- Hospital de Santa Caterina (147,222)
- Hospital Universitari Germans Trias i Pujol (800,000)
- Hospital de la Santa Creu i Sant Pau (403,047)
- Hospital Clínic de Barcelona (540,000)
- Hospital Universitari Vall d'Hebron (430,000)
- Hospital Universitari Parc Taulí (406,913)
- Hospital Universitari de Bellvitge (201,192)
- Hospital Universitari Joan XXIII de Tarragona (600,000)
- Hospital Universitari Arnau de Vilanova de Lleida (450,000)

### 7.3 Inclusion and exclusion criteria

- **Inclusion criteria**:
    - **Cases**:
        - Diagnosis of Diffuse Idiopathic Skeletal Hyperostosis according to the Resnick Criteria (3.1.7 Diagnostic Criteria)
        - Individuals aged 40 or more years
        - Patients in full use of their mental capacity who agree to sign the informed consent

- **Controls**:
    - Healthy patients with no diagnosis of DISH or chronic rheumatic diseases.
    - Individuals aged 40 or more years
    - Patients in full use of their mental capacity who agree to sign the informed consent

- **Exclusion criteria**:
    - History of diseases that affect the digestive tube, such as inflammatory bowel disease or irritable bowel syndrome.
    - Previous surgery that could alter the proper function of the gastrointestinal tract, such as any colon resection or bariatric surgery.
    - Use of antibiotics or probiotics in the past six months.
    - History of other rheumatic diseases such as rheumatoid arthritis or SLE.
    - Diagnosis of pathologies that pose a significant challenge for the differential diagnosis of DISH, such as axSpA or very severe arthrosis.
    - Refusal or inability to sign the informed consent form or to provide stool samples.

The 6-month limit for antibiotic or probiotic consumption has been set based on the findings of the 2018 study by Palleja *et al.* where they found gut microbiota α-diversity could take as much as 6 months to recover (114).

## 7.4 Sample and sampling

### 7.4.1 Sampling method

Case selection will be performed following a non-probabilistic consecutive sampling method. All patients with a previous or recent DISH diagnosis according to the Resnick Criteria (3.1.7 Diagnostic Criteria) at any of the 10 participating hospitals and who meet all inclusion criteria and none of the exclusion criteria, will be given an information sheet (***Annex I***). This document explains the aim of the study, relevance, all required tests (microbiota detection by stool test), confidentiality guarantees, and a reminder on the possibility of dropping out of the study if the patient wishes to do so. After receiving the information sheet, they will be asked if they wish to join the study and, if they respond positively, they will subsequently be given the informed consent form (***Annex II***) in order to sign it.

Healthy controls will be recruited by probabilistic random sampling from the "Registre Central de Persones Assegurades" of Catalunya. Case and controls will be matched by age (intervals of 2.5 years) and sex by a ratio of 2 controls for 1 case (2:1 ratio). Once randomly selected they will be contacted, briefly informed about the aims of the study and asked to come to the rheumatology consult of their reference hospital in order to be given the information sheet (***Annex I***) and sign the informed consent form (***Annex II***). If they accept, the attending clinician will collect the necessary covariables (7.5.3 Covariables) and will conduct an exhaustive physical examination to rule out possible signs of DISH, exploring the standard spinal rigidity signs evaluated in patients with hyperostosis. If clinical signs of DISH are present in controls, they will not be considered eligible to participate in this study. Radiographs will not be performed, in order to prevent unnecessary exposure to radiation.

### 7.4.2 Sample size

Sample size calculus was performed using the GRANMO version 8.0 software. Accepting an alpha risk of 0.05 and a power of 0.8 in a two-sided test, 185 cases and 370 controls are necessary to recognise an Odds Ratio greater than or equal to 2. A proportion of exposed subjects in the control group has been estimated to be 0.16. A drop-out rate of 10% has been anticipated.

We have considered the prevalence of dysbiosis of 16% as per the results of the 2015 study by Casén *et al.* which evaluated the frequency of dysbiosis in patients with IBS, IBD and healthy controls (115).

### 7.4.3 Estimated recruitment time

Since this is a multicentric study and the different hospitals participating have different catchment areas, an estimation of the number of DISH diagnosis per month has been calculated using the "Hospital Universitari Doctor Josep Trueta" as a reference.

Approximately 3 DISH cases per month who meet all Resnick criteria (3.1.7 Diagnostic Criteria) are diagnosed at the "Hospital Universitari Doctor Josep Trueta". If a similar diagnostic rate is assumed in all participating hospitals, and given their respective catchment area, the amount of expected DISH cases diagnosed per month is that described in the following table (***Table 4***). Thus, in order to recruit the 185 DISH cases, a total time of 11 months is required.

*Table 4: expected diagnosed cases of DISH who meet the Resnick criteria (3.1.7 Diagnostic criteria) per month and per hospital, estimated on their respective catchment area (7.2 Study Population and Setting).*

| Reference Hospital | Catchment area | Cases/month |
|---|---|---|
| Hospital Universitari Doctor Josep Trueta | 800,000 | 3 |
| Hospital de Santa Caterina | 147,222 | 0.55 |
| Hospital Universitari Germans Trias i Pujol | 800,000 | 3 |
| Hospital de la Santa Creu i Sant Pau | 403,047 | 1.5 |
| Hospital Clínic de Barcelona | 540,000 | 2 |
| Hospital Universitari Vall d'Hebron | 430,000 | 1.61 |
| Hospital Universitari Parc Taulí | 406,913 | 1.52 |
| Hospital Universitari de Bellvitge | 201,192 | 0.75 |
| Hospital Universitari Joan XXIII de Tarragona | 600,000 | 2.25 |
| Hospital Universitari Arnau de Vilanova de Lleida | 450,000 | 1.69 |
| **Total** | | 17,87 |

### 7.4.4 Participant withdrawal

An expected drop-out rate of approximately 0.1 (10%) has been used to calculate sample size. Any participant may decide to withdraw from the study at any time and without need for any justification; this will not affect any current or future medical treatment, or the medical attention received

- **Withdrawal before sample collection**: subjects may drop out of the study before sample collection by signing the "withdraw informed consent" form (***Annex V***). Participants who fail to show to their scheduled appointment with rheumatology will be contacted to reschedule; if contact is not possible or they do not wish to reschedule, they will also be considered as withdrawn from the study. Furthermore, participants who consume antibiotics or probiotics in the 6 months prior to stool sample collection, will also be withdrawn from the study, as this would alter their gut microbiota composition. Any collected data on the withdrawn subjects will be eliminated from the study. Additional recruitment will be performed to maintain the representativity of the evaluated sample in case of subject withdrawal before the end of the sample collection phase.
- **Withdrawal after sample collection**: any subject may drop out of the study once sample collection is completed by signing the "withdraw informed consent" form (***Annex V***). All collected information on that subject will be eliminated from the study. However, no additional subjects will be recruited in this eventuality.

### 7.5 Variables and data collection

All collected data will be stored in a REDCap database, after the ethics committee of all hospitals have approved this study. All clinicians of the participating hospitals will have access to REDCap.

REDCap does not allow the introduction of identifying personal data, thus all study participants, both cases and controls, will have a unique ID. Each recruiting hospital will create a register containing the relationship between REDCap IDs and hospital history of the recruited participants. Thus, all data included in the database will be anonymised.

#### 7.5.1 Independent variables

- **Gut microbiota dysbiosis**: dichotomous nominal qualitative variable, described as presence or absence of gut microbiota dysbiosis in stool samples. In order to achieve this, mass gene sequencing of stool samples is required:
  1. All subjects in both groups will be given their own stool sample containers by the rheumatologists at their reference hospital. A new appointment with the Rheumatology Unit will be scheduled for them to bring their stool samples back to the hospital.
  2. All participants are required to collect their own stool samples in their containers and store them inside a freezer immediately, before bringing them to the Rheumatology Unit of either of the participating hospitals, where they will be labelled with an anonymous barcode and frozen at -80ºC for their correct preservation. Stool samples can be transported at most 2 hours at room temperature; if a longer transportation time is required, they must be stored in a cooled compartment. In order to prevent study subjects from keeping their stool samples for prolonged periods of time, they will be recommended to collect said samples approximately 24 hours before their scheduled appointment.
  3. All stool samples will be sent to the Microbiology Service in the “Parc Científic i Tecnològic” of the “Universitat de Girona” to be properly analysed, following

the protocol included in the "16S Metagenomic Sequencing Library Preparation" for the Illumina MiSeq System.

4. DNA extraction will be realised following the NucleoSpin® 96 DNA Stool Kit (Macherey-Nagel GmbH&Co).
5. An Amplicon PCR will be subsequently performed targeting the V3 and V4 regions of the rRNA 16S gene, on a 96-well PCR plate and in a thermal cycler using DNA polymerase (KAPA HiFi HotStart ReadyMix) and the primers included in "Illumina 16S Metagenomic Sequencing Library Preparation":
   - Forward primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTACGGGNGGCWGCAG-3'
   - Reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACTACHVGGGTATCTAATCC-3'
6. In order to visualise the size of the PCR product, an electrophoresis on agarose gel will be performed.
7. Following the first Amplicon PCR, a PCR clean-up to eliminate free primers and dimer species is necessary, using AMPure XP beads (Beckman Coulter).
8. After 16S V3 and V4 purification, an index PCR using the Nextera XT Index Kit will be performed, followed by a second clean-up with AMPure XP beads before the quantification process.
9. Library quantification will be performed with a Qubit™ 4 Fluorometer Kit (Thermo Fisher Scientific), followed by normalization and pooling of the library, and finally denaturing.
10. Finally, NGS will be performed using the MiSeq® system in order to achieve mass sequencing of the bacteria in the stool samples. This will allow us to obtain information on both the composition of the individual's gut microbiome based on the Greengenes database (classified at different taxonomic levels: kingdom, phylum, class, order, family, genus and species), and the relative abundance of the main species. This information will be further analysed with the QIIME 2 Visualisations program, which allows for the generation of operational taxonomic units (thus clustering closely related microbes together).

Once the samples have been processed and microbiota composition and prevalence established, we will proceed with the construction of a dysbiosis test algorithm, as developed by Casen *et al.* in their 2015 article (115):

1. Principal Component Analysis (PCA) will be used to build a normal microbiota profile from the microbiota samples of control (non-DISH) patients. Then, confidence intervals for the values of Hotelling's *T*-square and *Q* statistics will be set in order to define a limit for what we define as normobiosis in our healthy controls. Thus, we can draw a rectangle with a corner located at the origin of our principal components that encompasses inside all individuals without dysbiosis, while all values outside its border correspond to individuals with dysbiosis.
2. Euclidean distance from the origin is then used to combine the dimensions of the PCA. Then, log-normal distribution will be performed by assigning estimated portions of said distribution to a scale of 0 to 5, obtaining a numeric value for the degree of gut microbiota alterations, henceforth known as Dysbiosis Index. All study subjects, both cases and controls, will then be assigned a Dysbiosis Index value in relation to their distribution.
3. As identified by the confidence interval, a Dysbiosis Index of 0, 1 or 2 will be described as normal microbiota composition (no dysbiosis); in contrast, a Dysbiosis Index of 3, 4 or 5 will be considered dysbiosis. Thus, the variable dysbiosis can be defined as a dichotomous qualitative nominal variable (presence/absence of dysbiosis).

- **Gut microbiota composition**: independent variable for our second secondary objective. The definition of this variable corresponds to the first 10 steps of the variable "gut microbiota dysbiosis" and differs from it in the fact that no posterior algorithm is built. Thus, the data obtained will be represented in relative numeric indexes (relative proportion of each phylum) and treated as a quantitative continuous variable.

### 7.5.2 Dependent variables

- **DISH** diagnosis: dependent variable for our main objective, it will be expressed as a dichotomous nominal qualitative variable: as presence or absence of DISH.

- DISH group (cases): this group will be constructed by the rheumatology units of the participating hospitals and will include patients from the catchment areas of any said hospitals with a DISH diagnosis. This diagnosis will be achieved based on simple radiograph findings provided by the radiology unit (flowing calcification of vertebral bodies) and the fulfilling of the Resnick criteria (4):
    - Flowing ossification of 4 or more contiguous vertebral bodies along their anterolateral aspect
    - Preserved vertebral disk height in affected regions, without extensive signs of disk degeneration such as vacuuming of sclerosis of the vertebral body margin
    - Preserved sacroiliac joints and absence of joint ankylosis in bone apophyses
- Non-DISH group (controls): subjects from the catchment areas of the participating hospitals and registered in the "Registre Central de persones Assegurades" who do not fulfil the Resnick criteria will be contacted and recruited into this control group. They will be matched by age (2.5-year intervals) and sex.

- **Radiological osteophyte pattern**: dependent variable for our third secondary objective. Spinal osteophytes will be evaluated by the rheumatologist based on his/her findings on a simple radiograph, distinguishing between: flat type osteophytes, with both the superior and inferior vertebral edges at a <45º angle; and jaggy type osteophytes, with either vertebral edge at a >45º angle. The type of osteophyte will be treated as a qualitative nominal variable, thus classifying patients into three categories:
    - Jaggy-dominant pattern: ≥60% of jaggy type osteophytes.
    - Pattern equivalence: both jaggy and flat type osteophytes are present and represent <60% and >40% of total axial bone formations.
    - Flat-dominant pattern: ≤40% of jaggy type osteophytes.

### 7.5.3 Covariables

Multiple covariables will be evaluated to prevent them from acting as confounding factors, due to their relationship with the development of DISH and/or changes in the gut microbiota composition. The information on certain variables (age, sex, BMI, ethnicity, smoking status, diabetes mellitus, time since DISH symptoms and diet) will be extracted via the clinical history and the anamnesis in the first visit to the Rheumatology Unit.

- **Age**: will be expressed in years since birth and thus expressed as a quantitative discrete variable.
- **Sex**: will be defined as a qualitative dichotomous variable, female or male[1], based on what sex was assigned at birth.
- **Ethnicity**: it is defined by the National Cancer Institute's dictionary as the "social and cultural characteristics, backgrounds or experiences shared by a group of people", which include "language, religion, beliefs, values and behaviours that are often handed down from one generation to the next". Ethnicity will be presented as a qualitative nominal variable and classified into the following groups: Caucasian, Hispanic American, Afro-descendant, Maghrebi, Asian or Others.
- **Diabetes mellitus**: diabetes mellitus will be treated as a qualitative dichotomous variable: DM diagnosis, or no DM diagnosis. Said diagnosis must have been performed by a medical professional and recorded in the clinical history.
- **Body Mass Index** (**BMI**): BMI is defined as the subject's mass in kilograms divided by the square of his/her height in meters ($kg/m^2$). Weighing the subject shall be performed on an electronic scale with 100 g precision; all clothing items must be removed, shoes included, except for underwear. The subject's height will be measured using a stadiometer and he/she must take all footwear items out, socks included. Once weight and height have been measured, BMI will be calculated and the results presented as a qualitative ordinal variable with the following categories:
    - Underweight (BMI <18.5 $kg/m^2$)
    - Normal weight (BMI 18.5 – 24.99 $kg/m^2$)

1. The concepts of female (XX) and male (XY) refer exclusively to biological sex

    - Overweight (BMI 25.0 – 29.99 kg/$m^2$)
    - Obese (BMI ≥30 kg/$m^2$)
- **Metabolic disease**: for the purposes of this study, we will consider the presence of metabolic disease if the study subject presents either diabetes mellitus or obesity, as previously defined in the "diabetes mellitus" and "BMI" covariables. Metabolic disease will be considered as a qualitative dichotomous variable, classifying subjects as: presence of metabolic disease or absence of metabolic disease.
- **Smoking status**: smoking status will be treated as a qualitative nominal variable; only smoked tobacco will be accounted for. Study subjects will be classified into three categories:
    - Non-smokers: subjects who have smoked less than 100 cigarettes in their entire lifetime or patients who have never smoked.
    - Ex-smokers: subjects who previously fulfilled the definition for smokers but have stopped smoking at least a year before being interviewed.
    - Smokers: any subject who has smoked at least 100 cigarettes in their entire lifetime until the interview.
- **Diet**: diet information will be collected by the investigator in the anamnesis during the first visit at the Rheumatology Unit. Diet information will be expressed as a qualitative nominal variable differentiating 3 separate categories:
    - Omnivore diet: the subject has eaten some kind of meat in the last 6 months.
    - Vegetarian diet: the subject has eaten no meat products in the last 6 months but does eat other animal-derived foods such as eggs or milk.
    - Vegan diet: the subject has eaten no animal-derived products for the last 6 months.
- **Time since DISH symptoms**: time since DISH symptoms first appeared will be expressed in years and will be treated as a quantitative discrete variable.

### 7.5.4 Data collection

Data collection will start simultaneously with subject recruitment, will approximately last for 11 months and will be performed by the rheumatologists from the 10 hospitals participating in this study.

Case and control recruitment will follow the indications specified in the "Sampling Method" section (7.4.1 Sampling Method). Once the informed consent has been signed, the attending rheumatologist will complete the anamnesis as required for all covariables.

Upon collection of all covariables, participants will be provided with a container for stool sample collection and a new appointment with rheumatology will be scheduled. Participants will be instructed on how to collect their faeces samples and told to bring them to the Rheumatology unit on the day of their scheduled appointment. As previously mentioned in the "Participant Withdrawal" section (7.4.4 Participant Withdrawal), if any study participant consumes antibiotics or probiotics in the 6 months prior to stool sample collection, they will be withdrawn from the study.

Once collected, samples will be frozen and then transferred to the Microbiology service of the "Parc Científic i Tecnològic" of the "Universitat de Girona" for analysis, as described in the "Independent variables" section (7.5.1 Independent variables).

## 8. Statistical analysis

The statistician will perform the statistical analysis using the software "R Project for Statistical Computing" (R Core Team 2019). Statistical significance will be considered only when a p-value of <0.05 is obtained; a Confidence Interval (CI) of 95% is set for all analyses.

### 8.1 Descriptive analysis

Sample description for all qualitative variables (dysbiosis presence or absence, DISH diagnosis or non-DISH patients, sex, ethnicity, diabetes mellitus diagnosis, BMI, smoking status and diet) will be done using frequencies and percentages.

For the remaining quantitative variables (age and time since DISH symptoms), sample description will be done by using means and standard deviations if the evaluated data follows a normal distribution. If no normal distribution is followed, medians and interquartile ranges (Q3 – Q1) will be used instead.

### 8.2 Bivariate analysis

In order to contrast whether differences exist in control and case groups, we will compare the dependent variables and covariables. A Chi-squared test will be performed to analyse the difference of proportions of DISH diagnosis in relation to dysbiosis presence or its absence. If the expected number of cases in a cell is too small to perform a $\chi^2$ test, Fisher's exact test will be performed instead.

For our second secondary objective, a $\chi^2$ test will be performed evaluating the different proportions of DISH radiographic phenotypes in our DISH population, in relation to the presence or absence of dysbiosis. As previously mentioned, a Fisher's exact test will be performed if the number of cases in a cell is not high enough to use the $\chi^2$ test.

For our third secondary objective a Mann-Whitney's U test will be performed, as it is expected that the independent variable "gut microbiota composition" will not follow a normal distribution.

### 8.3 Multivariate analysis

Multivariate analysis will be performed adjusting for those covariables with statistically significant differences in the bivariate analysis. A logistic regression analysis will be performed for our main objective. In order to contrast our first secondary objective, the covariable “metabolic disease” will be included in said logistic regression even if no statistically significant differences are observed in the bivariate analysis. For our second secondary objective, a multinomial logistic regression will be used instead.

## 9. Ethical and legal aspects

All aspects of this study will be performed following the human rights and ethical principles established by the World Medical Association in the "Declaration of Helsinki: ethical principles for medical research involving human subjects" of 1964 and revised in 2024. As such, after the creation of this protocol, it will be submitted to the Drug Research Ethics Committee (CEIm) of the "Hospital Universitari Doctor Josep Trueta" and the respective Ethics Committee of all other participating hospitals. All modifications or recommendations provided by the Ethics Committee will be accounted for and included in the protocol. Before starting patient recruitment, final approval by the Ethics Committee must be obtained.

In accordance with the "*Llei 41/2002, del 14 de novembre, bàsica reguladora de l'autonomia del pacient i de derets i obligacions en matèria d'informació i documentació clínica*", all participants in this study will be informed on the aims, all procedures, risks and benefits, as well as all measures taken to ensure protection of personal information and stool samples; all this information will be collected in the Information Sheet (***Annex I***), which will be handed to the participant. Subsequently, participants will be handed and asked to voluntarily sign the Informed Consent (***Annex II***). Furthermore, they will also be informed on the possibility of withdrawing said consent at any time during the study through the Withdrawal Consent Form (***Annex V***) and of asking all the collected information to be destroyed and neither published nor used for further research.

Since stool samples from the participants are collected, and as stipulated by the "Llei 14/2007, de 3 de juliol, de recerca biomèdica" and the "Reial decret 1716/2011, de 18 de novembre, pel qual s'estableixen els requisits bàsics d'autorització i funcionament dels biobancs amb fins de recerca biomèdica i del tractament de les mostres biològiques d'origen humà, i es regula el funcionament i l'organització del Registre Nacional de Biobancs per a recerca biomèdica", patients will be informed on what a biobank is, the storage process of all collected stool samples, the possible future uses for said stored samples, and reminded of the possibility of requesting the destruction of their samples. This information will be collected in the Biobank Information Sheet (***Annex III***), which will be also handed to the participants. Furthermore, upon receiving said information, the participants will be handed a Biobank Informed Consent (***Annex IV***) which will also

be asked to voluntarily sign and reminded on the possibility of rescinding their consent at any time.

The personal data of study participants is strictly confidential and in compliance with the "Llei orgànica 3/2018, de 5 de desembre, de protecció de dades personals i garantia dels drets digitals" and the "Reglament (UE) 2016/679 del Parlament Europeu i del Consell, de 27 d'abril de 2016, relatiu a la protecció de les persones físiques respecte al tractament de dades personals i a la lliure circulació d'aquestes dades i pel qual es deroga la Directiva 95/46/CE (reglament general de protección de dades – RGPD)" will be anonymised (by an external team) and access restricted to only the researchers who take part in this study. Only the data necessary for this study will be collected. In addition, study participants are free to access all of their collected data and may request its elimination at any time during or after the study.

Furthermore, this project follows the "Principles of Biomedical Ethics" by Beauchamp and Childress, through the following actions:

- **Autonomy**: as stated, the participant's autonomy will be maintained by the providing of the Information sheet (***Annex I***), Biobank Information sheet (***Annex III***), Informed Consent (***Annex II***), Biobank Informed Consent (***Annex IV***) and the Withdrawal Consent (***Annex V***). It is imperative for all clinicians to emphasise the voluntary nature of this study, as well as the possibility of withdrawing from it at any time if they wish to do so.
- **Beneficence**: the aim of the study is to determine if a statistically significant relation between DISH and dysbiosis exists, which could act as a catalyst to allow the scientific community to better understand the pathogenesis of this disease and thus try to prevent DISH development altogether, thus benefiting in the long-term patients at risk of developing DISH.
- **Non-maleficence**: no invasive procedures are performed on any study subject and there is no necessity for interrupting the symptomatic treatment of DISH, thus preserving this principle.
- **Justice**: all participants in both registers of both groups have the same probability of being recruited into the study, independently of socioeconomic status.

The authors declare no conflicts of interest and no economic interests associated to the development of this study. Furthermore, all obtained data will be transparently published, whether favourable or unfavourable.

## 10. Work plan

### 10.1 Research team members

The research team for this study will be composed by different members:

- **Principal investigator** (PI): the professional in charge of the study. He/she is responsible the development of the study protocol, information sheets, informed consent and the recruitment of other clinicians willing to voluntarily participate in the study.
- **Rheumatologists** (RH): all clinicians who take part in this study are rheumatology specialists. They oversee recruiting both cases and controls, as well as interviewing the participants, performing the physical examination and informing them on the aims, procedures and legal aspects of the study.
- **Microbiologists** (MB): specialists in microbiology will be necessary to extract the DNA from the faeces samples, amplify the 16S rRNA gene and prepare the library for the NGS.
- **Bioinformatician** (BI): he/she will construct and analyse the database obtained from the crude 16S rRNA sequences from the NGS. The bioinformatician will also be responsible for generating the operative taxonomic units and performing the principal component analysis to build the dysbiosis test algorithm.
- **Statistician** (**ST**): the statistician will be responsible for performing the statistical analysis, as well as aiding in managing the obtained data

### 10.2 Stages of the study

This study is expected to be performed over a total time of 2 years and 7 months, and will be divided into six different stages according to the procedures performed in each phase:

- **Stage 1: protocol and study design** (estimated duration of 6 months)
    1. **Investigation and protocol elaboration**: the principal investigator will conduct the bibliographic research to elaborate the protocol, establish objectives and hypothesis, define variables and explain which statistical analysis will be performed. Additionally, the principal investigator will also prepare the informed consent and information sheet.

2. **Professional recruitment**: the principal investigator will establish contact with the other rheumatologists that will partake in this study. These clinicians may also contribute with suggestions or ideas to the project.
3. **Ethics Committee evaluation**: the project will then be presented to the "Comitè Ètic d'Investigació amb medicaments" (CEIm) or the "Comitè Ètic d'Investigació Clínica" (CEIC) of each of the participating hospitals, organisms which will be responsible for declaring whether the project is ethically acceptable or if further modifications are required.
4. **Final project elaboration**: any modifications in accordance with Ethics Committee requirements will be applied and the final version of the protocol will again be presented to this organism for approval.
5. **REDCap database creation**: by the statistician and principal investigator; all other rheumatologists participating in this study will be given access.

- **Stage 2: team members training** (estimated time of 2 months)
    1. **Training sessions**: a small number of training sessions will be held with all the participating rheumatologists, with the aim of promoting standardisation of how the protocol is to be followed, how information is to be collected, which tests will be performed in the physical exploration, and how data sheets should be filled. Furthermore, the principal investigator will contact the microbiologists in order to prepare all necessary laboratory materials.
- **Stage 3: data collection and processing** (estimated time of 16 months)
    1. **Participant recruitment**: cases from the 10 participating hospitals will be recruited following a non-probabilistic consecutive sampling method. Meanwhile, control recruitment will be performed from the "Registre Central de Persones Assegurades" of Catalunya via probabilistic aleatoric sampling. Subjects that meet all inclusion criteria and none of the exclusion criteria will be given the information sheet (***Annex I***) and asked to sign the informed consent (***Annex II***), and further required information complemented as explained in the "Data Collection" section (7.5.4 Data Collection). All information on subjects will then be stored in the REDCap database.

2. **Sample collection**: stool samples will be collected following instructions detailed in the "Data Collection" section. Upon collection, all faeces' samples will be coded, frozen and sent to the Microbiology Service of the "Parc Científic i Tecnològic" of the "Universitat de Girona".
3. **Sample processing**: it will be performed by the microbiologists who will extract the DNA, perform the amplicon PCR and prepare the library for mass sequencing, following the previously stated NGS protocol.

- **Stage 4: statistical analysis and interpretation** (estimated time of 3 months)
    1. **Diversity analysis**: the bioinformatician will analyse the crude results obtained from the NGS and adapt them in order to obtain the diversity indexes of the different bacteria populations.
    2. **Dysbiosis test algorithm**: using Principal Component Analysis, the bioinformatician and statistician will elaborate the dysbiosis test algorithm to evaluate the presence or absence of dysbiosis in both cases and controls.
    3. **Analysis and interpretation**: the statistician and principal investigator will analyse all obtained data using the previously mentioned tests to extract conclusions. Then, a meeting with the other professionals who took part in the study will be held to arrive at a conclusion.
- **Stage 5**: **final report elaboration** (estimated time of 2 months)
    1. **Final report and proofreading**: the final report will be elaborated by the leading investigator and then proofread by an English translator.
- **Stage 6**: **publishing and dissemination** (estimated time of 2 months and onward)
    1. **Publishing**: results will be published in the form of a scientific article and then submitted to a specialised scientific journal. Once published, the preprint will be published to a public repertoire.
    2. **Dissemination**: all relevant findings will be, if possible, shared with the rest of the scientific community through conferences and congresses.

## 10.3 Chronogram

*Table 5: chronogram (PI = principal investigator, RH = rheumatologists, CEIC = "Comitè Ètic d'Investigació Clínica", CEIm = "Comitè Ètic d'Investigació en medicaments", ST = statistician, MB = microbiologist, BI = bioinformatician)*

| Stage | Staff | Year and month | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2025 | | | | | | | | | | 2026 | | | | | | | | | | | | 2027 | | | | | | | | |
| | | Mar | April | May | June | July | Aug | Sept | Oct | Nov | Dec | Jan | Feb | Mar | Apr | May | Jun | Jul | Aug | Sept | Oct | Nov | Dec | Jan | Feb | Mar | Apr | May | Jun | Jul | Aug | Sept |
| **Stage 1: protocol and study design** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Investigation and protocol elaboration | PI | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Professional recruitment | PI, RH | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Ethics commitee evaluation | CEIC CEIm | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Final project elaboration | PI, RH | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Computer database creation | PI, ST | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **Stage 2: team member training** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Training sessions | PI, RH, MB | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **Stage 3: data collection and processing** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Participant recruitment | RH | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Sample collection | RH | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Sample processing | MB | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **Stage 4: statistical analysis and interpretation** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Diversity analysis | BI | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Dysbiosis test algorithm | BI, ST | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Analysis and interpretation | ST, PI, RH | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **Stage 5: final report elaboration** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Final report and proofreading | PI | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **Stage 6: publishing and dissemination** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Publishing | PI | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Dissemination | PI | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

## 11. Budget

- **External staff expenses**:
    - A statistician will be hired to design the dysbiosis test algorithm and assist in the analysis and interpretation of the collected data. He/she will be engaged for two months, estimating approximately 120 worked hours with a salary of 40 €/h.
    - A bioinformatician will be hired to perform the diversity analysis and assist the statistician in the development of the dysbiosis test algorithm. A total engagement period of two months and 120 worked hours are estimated, with a salary of 40 €/h.
    - Microbiologists will also be hired to conduct the DNA extraction, the amplicon PCR and the 16S rDNA sequencing. They will be hired for a total of 640 work hours for 5 months, with a compensation of 35 €/h. Further classification of the amount of time allocated to each activity can be found in the following table (***Table 6***).
    - Attention should be directed to the fact that neither the rheumatologists nor the principal investigator will receive any financial compensation from the study. Any economic gain will be solely derived from their assigned research hours as part of their employment contract
- **Transportation expenses**: sample transportation from each of the participating hospitals to the "Parc Científic i Tecnològic" of the "Universitat de Girona" will be accomplished by hiring the services of FedEx®. In accordance with their tariffs for national transportation, the expected price is:
    - Transports inside the province of Girona: 44.62€
    - Transports from the provinces of Barcelona, Lleida or Tarragona: 54.69€

  No patient transportation will be performed; thus, it will incur no expenses.
- **Material expenses**: multiple expenses are associated with the material required for the evaluation of gut microbiota:
    - 555 stool sample containers will be required, at an expected price of 0.06€ per container

- 2 NucleoSpin® 96 DNA Stool Kits (Macherey-Nagel GmbH&Co), for a cost of 1,377 €/kit. Every kit contains 4 96-well plaques for a total of 384 wells per kit, thus 2 kits are required for all 555 samples.
- 3 Ion 16S™ Metagenomics Kits (Thermo Fisher Scientific), for a cost of 1,280 €/kit. These kits contain the primers required to perform the Amplicon PCR and allow 250 reactions per kit, thus a total of 3 kits are required to cover for all 555 samples.
- 6 polymerase KAPA HiFi HotStart Ready Mix (Thermo Fisher Scientific), for a total of 213 €/mix. Every polymerase mix allows 100 reactions, thus a total of 6 mixes are necessary.
- 3 E-Gel™ 48 agarose gels, 1% agarose and SYBR™ safe DNA gel stain (Thermo Fisher Scientific), for a total of 274 €/kit. Every kit contains 8 48-well gels, for a total of 384 wells in total, thus 3 gel kits are required.
- 4 AMPure XP beads kits (Beckman Coulter), for 874.49€/5 mL-kit. These kits are essential for PCR-clean up. A single 5 mL kit can be used to clean 384 wells; thus, 2 kits are required to clean all samples. However, since 2 PCR-clean ups are necessary, a total of 4 kits will be required.
- 2 Nextera® XT Index Kit (Illumina) for NGS, for 1,222€/unit and a total of 384 samples/unit. A total of 2 kits are necessary to account for all 555 samples.
- 2 Qubit® assay kits. 1 500-reactions/kit for 397€, and 1 100-reactions/kit for 133€ will be required to account for all 555 samples.
- 1 Fluorometer Qubit® 4 with Wi-Fi, for a total of 3,810€
- Laboratory supplies such as pipette tips, gloves or other equipment are expected to cost around 1800€
- Additionally, printing costs will also be accounted for. Estimating a total of 0.1€ per page, the following documents and total pages will be printed:
    - 555 Information Sheets, 4 pages per document (total 2220 pages)
    - 555 Informed Consent Forms, 2 pages per document (total 1110 pages)
    - 555 Biobank Information Sheets, 4 pages per document (total 2220 pages)

        - 555 Biobank Informed Consent Forms, 3 pages per document (total 1665 pages)
        - 555 Withdrawal Informed Consent forms, 1 page per document (total 555 pages)
        - Thus, a total of 7,770 copies will be printed for a total of 777€
    - No hospital admissions are necessary and will not incur any expenses
- **Publication and divulgation expenses**:
    - The article will be proofread and corrected by an English translator, for an expected compensation of 500€
    - As explained the section "Stages of the study" (10.2 Stages of the study), this study will be published and submitted to a specialised journal. An expected 2000€ cost is expected.
    - Finally, attendance to a congress to divulge the results of the study is also accounted for. Approximately 2 attendees and 1500€ per attendee are estimated. However, this could vary depending on the number of congresses attended to and of attendees per congress.

*Table 6: budget*

| Expenses | Units and cost per unit | Total cost |
|---|---|---|
| **External staff expenses** | | **32,000€** |
| Statistician | 40€/h x 120 h | 4,800 € |
| Bioinformatician | 40€/h x 120h | 4,800 € |
| Microbiologist (DNA extraction) | 35€/h x 160 h | 5,600€ |
| Microbiologist (Amplicon PCR) | 35€/h x 160 h | 5,600€ |
| Microbiologist (Sequencing) | 35€/h x 320 h | 11,200€ |
| **Transportation** | | **526.76€** |
| Sample transportation (FedEx Custom Critical) | 44.62€ x 2 transports (Girona)<br>54.69€ x 6 transports (Barcelona)<br>54.69 x 1 transport (Tarragona)<br>54.69 x 1 transport (Lleida) | 526.76€ |

| **Material expenses** | | **21,586.26€** |
|---|---|---|
| Stool sample containers | 0.06€/container x 555 containers | 33.3€ |
| NucleoSpin® 96 DNA Stool Kit (Macherey-Nagel GmbH&Co) | 1,377 €/kit (4 96-well plaques) x 2 kits | 2,754€ |
| Primers: Ion 16S™ Metagenomics Kit (Thermo Fisher Scientific) | 1,280 €/kit (250 reactions/kit) x 3 kits | 3,840€ |
| Polymerase: KAPA HiFi HotStart Ready Mix (Thermo Fisher Scientific) | 213 €/mix (100 reactions) x 6 mixes | 1,278€ |
| E-Gel™ 48 agarose gels, 1% agarose and SYBR™ safe DNA gel stain (Thermo Fisher Scientific) | 274€/gel kit (8 gels/kit, 48 wells/gel) x 3 kits | 822€ |
| AMPure XP beads (Beckman Coulter) | 874.49€/5 mL (up to 384-wells cleaned) x 4 kits | 3,497.96€ |
| Nextera® XT Index Kit (Illumina) | 1,222€/unit (384 samples/unit) x 2 units | 2,444€ |
| Qubit® assay Kit | 397€/500 reaction kit, 133€/100 reaction kit x 1 500 reaction kit + 1 100 reaction kit | 530€ |
| Fluorometer Qubit® 4 with Wi-Fi | 3,810€/fluorometer x 1 fluorometer | 3,810 |
| Laboratory supplies (pipette tips, test tubes, buffers, gloves…) | ------ | 1,800€ |
| Printing costs | 0,1€/page x 7,770 copies | 777€ |
| **Publication and divulgation expenses** | | **5,500€** |
| English proofreading | 500€/article x 1 article | 500€ |
| Publication fees | 2000€/article x 1 article | 2,000€ |
| Congress attendance | 1500€/attendant x 2 attendants | 3,000€ |
| **Total cost** | | **59,613.02€** |

## 12. Limitations and strengths

### 12.1 Study limitations

The main limitations of this study are the following:

Case-control study

- Although this study follows a case-control model, exposure to our independent variable (gut microbiota dysbiosis) is only evaluated in a specific time in space. This means that no temporal relation can be established between the presence of gut microbiota dysbiosis and DISH. Furthermore, case-control studies cannot establish causal relations. However, the aim of this study is to describe a possible association between gut microbiota dysbiosis and DISH, not to establish causality.

DISH studies

- DISH studies are only performed using the Resnick criteria, which although restrictive are the only validated criteria and the ones accepted in research. Thus, patients that do not fulfil said criteria but do fulfil the Utsinger criteria for DISH diagnosis cannot be included. As previously mentioned, some patients present with increased peripheral entheseal ossification without the degree of axial involvement required for the Resnick criteria. This group of patients are not represented in this study and their microbiota is not accounted for.

Sample size

- There is no known prevalence of dysbiosis in the general population, thus sample size has been calculated using the data from a single study. However, if the real prevalence of dysbiosis in our population were lower, then it would be necessary to increase sample size or risk committing a type 2 error. Thus, if the dysbiosis prevalence obtained in our study is lower than expected, we will recalculate sample size.

Microbiota determination

- There is currently no universally accepted consensus on what constitutes a healthy microbiome, and the gut microbiota composition can vary due to factors such as geographic location or diet. Furthermore, the gut microbiome is not static and exhibits intraindividual variability across time, and cross-sectional case-control studies such as this one do not describe these variations. Instead, other models which evaluate microbiota changes through time, could better evaluate the presence of dysbiosis and adjust for the effects of other factors such as diet. In this study, we will consider as normal gut microbiota the results obtained in our control group; however, other studies should be performed in other areas.

Stool sample

- Stool sample use also has some inherent problems. Mainly, the fact that faeces do not contain a representative sample of the complete human gut microbiome, as some bacteria have stronger adhesive mechanisms to the intestinal wall and thus are present in lesser proportions in stool. Furthermore, bacteria from the upper digestive tract and their DNA may be damaged as they pass through the intestines and the colon, hindering their recognition during stool analysis (116). Other sample collection methods such as intestinal biopsies are more representative, but their invasive nature implies their use is not as frequent.
- The patients are asked to collect the samples themselves; thus, it is possible that mistakes are made in the collection process that could alter the results. To help to prevent this, the collection process will be explained to them both verbally and in the information sheet (***Annex I***).

16s rRNA sequencing

- 16S rRNA gene sequencing has certain implicit limitations that increase the probability of a misclassification bias occurring:
    - The procedure described is only useful for identifying bacterial DNA but does not detect genetic material for other constitutive species of human gut microbiome, such as viruses or fungi. Thus, no information on possible alterations of said species would be obtained.

- Not all types of gut bacteria can be detected using sequencing-based techniques but can be detected using culture-based traditional methods. Similarly, other gut bacteria can only be detected via sequencing and not in cultures. Since this study only describes gut microbiota via sequencing, some of the diversity of the bacterial community may be lost in the analysis.
- Not every 16S rRNA gene is amplified equally, due to primer affinity (117), thus we may obtain a false relative abundance for certain species.
- Furthermore, even if algorithms have been developed to prevent them, some chimera generation can still happen during PCR, resulting in the erroneous creation of new species or misclassification of existing ones. Similarly, sequencing errors may occur, even if rare (117).

### 12.2 Study strengths

The main strengths of this study are the following:

Case-control study

- The case-control design allows this study to be relatively short. Since DISH is a chronic disease that takes years to develop, other designs such as a cohort study would require decades in order to obtain any result.
- Matching allows both groups to be as homogeneous as possible, thus controlling for both age and sex which are potential confounders to this study.
- Since this is a multicentric study, study subject recruitment will certainly be faster than what it would be were only one centre involved. It also enhances this study's external validity.

DISH studies

- This study's objective aims to describe a poorly known disease. By investigating this knowledge gap and improving the understanding of DISH physiopathology, new DISH studies could be conceived to develop novel prevention strategies and thus prevent the consequences of this disease altogether.

Data and sample collection

- The simplicity of the instructions that study participants have to follow, has many inherent benefits, mainly:
    - Since stool samples are relatively easy to collect and only one sample is required to be collected by the study subjects, the probability of errors occurring during sample collection is lower.
    - Only two visits with rheumatology are scheduled, which implies study subjects will not be required to invest extensive amounts of time in this study. This reduces the probabilities of subject withdrawal.
    - No follow-up is required, thus diminishing the drop-out probability
- No invasive tests are performed during this study, implying that study subjects incur no risks derived from their participation. This also implies that acceptance in participating in the study will likely be higher than expected if invasive methods were used.
- The fact that highly trained rheumatologists are involved in subject recruitment implies that many of the questions asked and the disease evaluated are already a part of their daily clinical practice. Thus, promoting accuracy in diagnosing and admitting both cases and controls.

## 13. Healthcare impact

As previously mentioned, DISH is a chronic disease with no definite pathogenesis and no effective treatment, other than symptomatic measures, and it is very likely that DISH prevalence will increase in the future. Were this study to prove a relation exists between DISH and the presence of gut microbiota dysbiosis, it would act as a starting point for other studies to investigate whether these imbalances in the gut microbiome play an active role in the development of DISH.

Dysbiosis has been found in other rheumatic diseases and is known to play a role in inflammation and immune regulation. Furthermore, microbiome-based interventions as a tool for treating certain diseases such as IBD, are already under investigation (118) and as medicine advances said interventions could prove a new tool against many diseases. If dysbiosis were to play an important role in DISH pathogenesis, treating the gut microbiota could be a novel avenue for the treatment of DISH, or even prevent the onset of this disease.

## 14. Feasibility

We consider this study to be feasible due to different factors. Firstly, all clinicians involved in the study would be medical professionals who specialise in rheumatology. Thus, it is expected both that the collection of variables will be performed by experienced personnel. Furthermore, all patients with a DISH diagnosis are already followed at either of the hospitals included in this study, thus no modification of their follow-up is expected.

Secondly, this protocol will be submitted to the ethics committee of the "Hospital Universitari Doctor Josep Trueta" and all other participating hospitals, which will enforce any modifications needed to ensure no ethical principles are being violated.

In addition, this study is estimated to require a total of 2 years and 7 months. Given the sample size and time required to recruit all cases and collect and process all samples, we consider this to be a reasonable amount of time for performing this study, while at the same time being short enough to prevent new studies evaluating the same objectives from emerging.

Thus, we consider this study to be feasible regarding the expertise of professionals involved, the mandatory compliance with ethical standards and the estimated timeline to perform it.

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## 16. Annexes

### 16.1 Annex I: Information Sheet

## Full d'Informació al/la participant

**Títol de l'estudi**: Association between Diffuse Idiopathic Skeletal Hyperostosis and Intestinal Microbiota Dysbiosis: a case-control study

**Centres de referència**: ……

**Investigador/a principal**:.....

Benvolgut/da,

Ens dirigim a vostè per tal de convidar-lo/a a participar a l'estudi liderat pel servei de reumatologia de l'Hospital Universitari Doctor Josep Trueta, que investiga l'associació entre la Hiperostosi Esquelètica Difusa Idiopàtica i la disbiosi de la microbiota intestinal.

En aquest document podrà trobar tota la informació necessària per comprendre el funcionament de l'estudi i els objectius d'aquest, així com per poder decidir de forma voluntària i mitjançant el consentiment informat si accepta o no participar-hi sota les condicions exposades posteriorment. Tot i així, si té qualsevol tipus de dubte sobre el document, no dubti a preguntar-ho al professional que li ha entregat.

La participació en dit estudi és completament voluntària i no està associada a cap tipus de remuneració econòmica. Igualment, la qualitat i el tracte assistencial per part dels metges no es veuran afectats de cap manera si rebutja participar-hi. En cas que accepti participar, té el dret de revocar en qualsevol moment el consentiment, sense necessitat d'explicacions i sense que això afecti al tracte mèdic rebut.

La seva col·laboració seria essencial per l'ampliació del coneixement científic de l'àmbit de l'estudi, pel que es sol·licita que es llegeixi atentament la informació exposada a continuació i així ajudar a la decisió del lector.

**Generalitats de l'estudi**

Aquest és un estudi dissenyat i liderat pel servei de reumatologia de l'Hospital Universitari Doctor Josep Trueta (HUDJT) i de Sata Caterina de Salt, amb la col·laboració dels hospitals Hospital de la Santa Creu i Sant Pau, Hospital Universitari de Vall d'Hebron, Hospital Universitari de Bellvitge, Hospital Universitari Germans Tries i Pujol, Hospital Universitari Parc Taulí, Hospital Clínic de Barcelona, Hospital Arnai de Vilanova de Lleida i Hospital Joan XXIII de Tarragona. Dit projecte ha estat valorat i aprovat pel Comitè d'Ètica de cada un dels hospitals participants.

**Objectiu de l'estudi**

L'objectiu de dit estudi és valorar si existeix una relació entre la Hiperostosi Esquelètica Difusa Idiopàtica (DISH) i la presència de disbiosi de la microbiota intestinal.

La disbiosi intestinal és l'alteració de la composició o funció dels microorganismes que conformen la microbiota intestinal. S'ha observat que alguns d'aquests microorganismes participen de la regulació del sistema immunitari de l'hoste, tal que les seves alteracions poden alterar dit sistema, fins i tot relacionant-se amb malalties inflamatòries. La DISH és una malaltia crònica i sense tractament que causa rigidesa i major risc de fractures de la columna vertebral. Si bé no es coneix el mecanisme pel que apareix aquesta malaltia, múltiples estudis han observat que certs casos de DISH tenen una base inflamatòria. L'objectiu d'aquest projecte és valorar si, com a malaltia inflamatòria, la DISH presenta també disbiosi intestinal mitjançant la valoració de mostres de femta. Si es confirmés, aquest estudi podria ser un punt de partida per altres projectes i així comprendre millor com es desenvolupa dita malaltia per així desenvolupar mesures per la seva prevenció.

**Característiques de la participació en l'estudi**

La participació en dit estudi és completament voluntària, de forma que no s'oferirà cap remuneració econòmica als participants. Qualsevol participant por decidir d'abandonar l'estudi en qualsevol moment i sense necessitat de justificar-ho, sense que això suposi cap repercussió en l'atenció sanitària rebuda posteriorment.

Per poder participar a l'estudi és necessari ser un subjecte de més de 40 anys, no patir d'altres malalties inflamatòries intestinals ni malalties reumàtiques, no haver consumit ni antibiòtics ni probiòtics en els darrers 6 mesos, i estar en ple ús de les seves capacitats mentals per comprendre aquest document i signar el consentiment informat.

**Quin és el meu paper a l'estudi?**

Si el subjecte decideix participar a l'estudi, se li entregaran els documents informatius necessaris, se li realitzarà a la mateixa consulta una recol·lecció d'aquelles dades necessàries per la interpretació dels resultats de l'estudi i se li entregarà un recipient per recol·lectar mostres de femta que s'haurà d'emportar a casa. Tot seguit, es programarà una segona visita amb reumatologia.

El participant haurà de recol·lectar ell mateix les mostres de femta a casa, preferiblement en les 24 hores abans de la nova visita amb reumatologia i congelar-les. Després, serà el participant qui portarà les mostres de femta a dita visita, on el servei de reumatologia s'encarregarà de mantenir-les congelades i posteriorment transportar-les pel seu processament. L'objectiu de dit processament és l'obtenció de la composició de la microbiota intestinal del participant per determinar possibles alteracions en aquesta. Si fos necessària l'administració d'un antibiòtic o probiòtic en els 6 mesos previs a la recol·lecció de mostres, el participant serà exclòs de l'estudi.

**Confidencialitat, protecció de dades i tractament de mostres**

L'anàlisi i recol·lecció de dades serà realitzada de forma anònima i amb caràcter estrictament confidencial, així com ho manifesta la Llei Orgànica de Protecció de Dades Personals i Garantia dels Drets Digitals (3/2018) i el Reglament (UE) (2016/679) del Parlament Europeu i del Consell.

Un cop finalitzat el projecte, és possible que es conservi un excedent de la mostra de femta de cada participant. Al full de consentiment informat se li proposaran les següents opcions sobre el destí d'aquestes mostres:

- Destrucció de tota mostra excedent
- Ús de l'excedent en projectes futurs en la mateixa línia de recerca
- Emmagatzematge de l'excedent al Biobanc de l'hospital corresponent

**Riscs i beneficis**

Donat que no es realitzen proves invasives, no es contempla cap risc per les persones que participen en dit estudi. La participació en aquest tampoc suposa la modificació de cap tractament. Si fos necessària l'administració d'un antibiòtic o altre fàrmac que pugui alterar la microbiota intestinal, sempre prendrà preferència l'administració d'aquests fàrmacs abans que la participació en l'estudi.

Tot i que és possible que el subjecte no n'obtingui cap benefici directe, la participació en l'estudi suposaria una gran contribució a la comprensió de la DISH i els mecanismes associats al seu desenvolupament, el que podria ajudar a la comunitat científica en el seu conjunt a obtenir estratègies per prevenir-la.

**Informació sobre els resultats de la investigació**

Si el participant ho desitja i ho sol·licita expressament, se li informarà sobre els resultats obtinguts a l'estudi. El participant està també en el seu dret de no ser informat sobre els resultats de la investigació si així ho desitja.

**Dubtes i contacte**

Davant qualsevol dubte o pregunta associada a aquest estudi, pot contactar amb l'equip investigador fent ús del telèfon ..... o mitjançant el correu electrònic .....

Moltes gràcies per la seva paciència i col·laboració.

### 16.2 Annex II: Informed Consent Form

## Consentiment informat

**Títol de l'estudi**: Association between Diffuse Idiopathic Skeletal Hyperostosis and Intestinal Microbiota Dysbiosis: a case-control study

**Centres de referència**: ……..

**Investigador/a principal**:.....

Jo, ..... amb DNI ....., declaro que:

- Se m'ha entregat una còpia del full d'informació al/la participant sobre aquest estudi, així com una còpia d'aquest consentiment informat.
- He pogut llegir atentament i he entès la informació compresa al full d'informació al/la pacient que he rebut.
- Se m'ha explicat de forma coherent i suficient la informació necessària en relació als objectius i finalitat de l'estudi i el meu paper en aquest.
- He pogut formular els dubtes que m'hagin sorgit sobre l'estudi i he rebut de forma satisfactòria una explicació per part del metge de referència.
- Entenc que les mostres obtingudes seran completament confidencials i que tota mostra excedent pot ser usada per futures investigacions en la mateixa línia de recerca o destruïdes segons les meves preferències.
- Entenc que la meva participació en l'estudi és completament voluntària i que conseqüentment no rebré cap compensació econòmica.
- Entenc que puc revocar el consentiment informat en qualsevol moment sense necessitat de justificar la meva decisió i sense que això repercuteixi en cap moment i de qualsevol manera en l'atenció sanitària rebuda.

En base a la informació present:

- Expresso la meva conformitat per participar voluntàriament en l'estudi usant mostres de femta:

  ☐ Sí ☐ No

- En cas que fos necessari obtenir més mostres biològiques o informació addicional rellevant per l'estudi, autoritzo a ser contactat/da pels investigadors:

  ☐ Sí ☐ No

- Si romangués un excedent de la meva mostra havent finalitzat aquest estudi, i havent estat informat/da sobre les possibles opcions, manifesto que:

  A) Sol·licito la destrucció de la mostra excedent ☐

  B) Permeto l'ús de l'excedent en investigacions futures en la mateixa línia de recerca ☐

  C) Permeto l'emmagatzematge de l'excedent de mostres al Biobanc de l'hospital corresponent ☐

Així doncs, ACCEPTO explícitament participar en aquest estudi, així com la recol·lecció i ús de les meves dades tal i com s'explica en el Full d'Informació al/la Participant.

A ....., dia ..... de ..... de 20.....

**Signatura de l'/la investigador/a** **Signatura del/la participant**

DNI: ..... DNI: .....

### 16.3 Annex III: Biobank Information Sheet


## Full d'informació al/la pacient per l'obtenció i ús de mostres biològiques i/o dades clíniques per investigació biomèdica i conservació en el biobanc

A la majoria d'hospitals, a més de l'activitat assistencial, es realitzen també tasques investigació biomèdica per progressar en el coneixement, diagnòstic, tractament i prevenció de malalties. Aquelles mostres i dades recollides a la pràctica clínica pels pacients i les obtingudes de donants sans són essencials per la investigació, pel que sol·licitem que es llegeixi detingudament aquest document i el consentiment informat adjuntat.

### Què és un biobanc?

Els biobancs són unes institucions regulades segons el disposat a la "Llei 14/2007, de 3 de juliol, d'Investigació Biomèdica" i el "Reial Decret 1716/2011, de 18 de novembre, de Regulació dels Biobancs". Són entitats que afavoreixen la recerca biomèdica mitjançant l'obtenció, emmagatzematge, gestió i distribució de mostres biològiques, amb l'objectiu de millorar els coneixements, detecció, prevenció, pronòstic i/o tractament de les malalties. Tota mostra o dada emmagatzemada al biobanc, sempre i que compleixi amb els requeriments d'un comitè científic i un d'ètica de les institucions responsables, pot ser cedida pel seu ús en estudis d'investigació, tant a nivell nacional com internacional. La cessió d'aquestes mostres, sempre que sigui possible, es farà sense informació personal associada, però en certs casos pot ser necessari l'accés a la història clínica o als resultats d'altres proves per completar la investigació.

**Quines mostres es recullen?**

Les mostres biològiques normalment provenen d'excedents de proves o intervencions quirúrgiques prèviament realitzades o que es realitzaran properament. Tot i així, en certs casos on es requereixin mostres addicionals, la institució sanitària pertinent es pot posar en contacte amb vostè per sol·licitar la seva col·laboració, manifestant sempre els motius i sol·licitant novament el consentiment informat. La recollida d'aquestes mostres es pot realitzar a qualsevol centre de salut integrat per l'IDIBGI: Hospital Universitari Doctor Josep Trueta (Institut Català de la Salut), Institut d'Assistència Sanitària, Institut Català d'Oncologia, Institut de Diagnòstic per la Imatge i els centres d'Atenció Primària de l'Institut Català de la Salut de Girona.

En relació a l'estudi prèviament esmentat (Association between Diffuse Idiopathic Skeletal Hyperostosis and Intestinal Microbiota Dysbiosis: a case-control study), les mostres de femta són obtingudes de forma no invasiva, recollides pel propi pacient i sols incorporades al biobanc com a excedent un cop finalitzat l'estudi si el participant així ho manifesta. Aquest excedent, si el participant ho desitja, serà emmagatzemat al biobanc fins que s'esgoti. Tot i així, com a propietari de les mostres, vostè pot disposar de les mostres en tot moment sempre i quan aquestes encara estiguin disponibles.

**Accés a les dades**

L'accés a les seves mostres, dades i historial clínic quedarà restringit exclusivament al personal autoritzat del Biobanc i només en situacions que sigui necessari. Tota la informació recollida serà codificada. Com s'ha esmentat abans, com a propietari de les mostres vostè pot accedir a dites mostres en tot moment sempre i quan encara estiguin disponibles.

**Protecció de dades**

Totes les dades recollides s'han de tractar sempre mantenint la seva confidencialitat, tal i com disposa el Reglament (UE) 2016/679, del Parlament Europeu i del Consell de 27 d'abril del 2016, reglament general de protecció de dades, i de la Llei Orgànica 3/2018,

de 5 de desembre, de protecció de dades personals i garantia dels drets digitals. L'IDIBGI actuarà com a responsable de les dades.

Com a responsable de les dades, pot exercir el seu dret d'accés, rectificació, supressió, objecció, portabilitat i limitació escrivint al correu electrònic a Biobanc@IDIBGI.org, trucant al 972 940 282 o accedint personalment al biobanc (Hospital Universitari Doctor Josep Trueta, Avinguda de França, 17007, Girona).

**Voluntarietat de la donació**

La donació de la mostra és voluntària i altruista, pel que no suposa cap tipus de retribució econòmica pel donant. Degut a que la participació és en l'àmbit de la recerca, no es preveu que vostè obtingui directament cap benefici per la seva salut, però sí que pot ajudar a beneficiar la salut de la societat en un futur.

**Voluntarietat de la participació en l'estudi i consentiment**

La participació és també voluntària i es confirma mitjançant el consentiment informat, al qual pot negar-s'hi o revocar-lo en qualsevol moment sense necessitat de justificar-ho i sense que això tingui cap altra repercussió en l'assistència sanitària rebuda pel subjecte. La cancel·lació del consentiment s'ha de sol·licitar per escrit i personalment a la Direcció del Biobanc de l'IDIBGI, a l'adreça mencionada anteriorment.

També es pot sol·licitar l'anonimització de les mostres, de forma que es desvincularà qualsevol mostra o dada clínica de les seves dades personals i, conseqüentment, de la seva identitat.

**Obtenció sobre informació de resultats i investigació**

Si vostè ho sol·licita expressament, el Biobanc li proporcionarà la informació sobre les investigacions en que s'han utilitzat les seves mostres i els resultats globals d'aquestes, excepte en cas de cancel·lació o anonimització.

Degut a que els mètodes que s'utilitzen en investigació biomèdica són diferents dels que s'usen en pràctica clínica, els resultats no han de ser considerats amb valor clínic. Tot i això, en cas que els resultats de les investigacions aportessin dades rellevants a nivell clínic o genètic pel propietari de les dades, aquests li seran comunicats per part del biobanc. En cas que la informació sigui rellevant per la seva família, vostè serà responsable de decidir si vol que se'ls comuniqui aquesta informació.

El subjecte també està en el seu dret de no ser informat sobre els resultats obtinguts en investigació. Tot i així, complint amb el marc legal, si la informació obtinguda pot evitar un prejudici per la salut dels familiars biològics, davant la seva negativa de compartir la informació es convocarà un comitè d'experts que haurà de decidir si cal informar als afectats o representants legals.

Davant qualsevol dubte, pregunti al personal sanitari que li ha comunicat aquesta informació, o al seu metge que el posarà en contacte amb l'autoritat corresponent. Moltes gràcies per la seva atenció.

### 16.4 Annex IV: Biobank Informed Consent Form


## Consentiment Informat per l'obtenció i ús de mostres biològiques i/o dades clíniques per investigació biomèdica i conservació en el biobanc

Jo, ....., amb DNI .....
declaro que:

- He rebut una còpia del Full d'Informació al/la Pacient per l'obtenció i utilització de mostres biològiques i/o dades clíniques per a investigació biomèdica i la seva conservació en el Biobanc, així com d'aquest consentiment informat associat
- He pogut llegir atentament i he entès la informació compresa al Full d'Informació al/la Pacient que he rebut.
- Se m'ha explicat de forma coherent i suficient la informació compresa en dit Full d'Informació al/la Pacient
- He pogut formular els dubtes que m'hagin sorgit sobre el Biobanc i he rebut de forma satisfactòria una explicació per part del professional sanitari de referència.
- Entenc que les mostres obtingudes seran completament confidencials i que s'emmagatzemaran per ser utilitzades en futures investigacions.
- Entenc que la meva donació és completament voluntària i que conseqüentment no rebré cap compensació econòmica.
- Entenc que puc revocar el consentiment informat en qualsevol moment sense necessitat de justificar la meva decisió i sense que això repercuteixi en cap moment i de qualsevol manera en l'atenció sanitària rebuda.

En base a la informació present:

- Dono el meu consentiment per tal que s'emmagatzemi el meu material biològic i/o les dades associades al Biobanc de l'IDIBGI:

  ☐ Sí ☐ No

- Dono el meu consentiment a que s'utilitzin les meves mostres biològiques i la informació clínica en investigacions:
  - Nacionals: ☐ Sí ☐ No
  - Internacionals: ☐ Sí ☐ No
- Dono el meu consentiment per usar l'excedent de mostres biològiques i/o les dades associades per la investigació biomèdica:

  ☐ Sí ☐ No

- Dono el meu consentiment per obtenir i utilitzar material biològic addicional i/o dades associades per la investigació biomèdica:

  ☐ Sí ☐ No

- Dono el meu consentiment per utilitzar mostres biològiques emmagatzemades prèviament a l'hospital amb la finalitat de la investigació biomèdica:

  ☐ Sí ☐ No

- Dono el meu consentiment a ser contactat si es requerís més informació o més mostres addicionals:

  ☐ Sí ☐ No

  Telèfon/correu de contacte (sols en cas afirmatiu):

  ……

- Dono el meu consentiment a que se'm comuniqui la informació obtinguda en projectes d'investigació usant les meves mostres i que sigui clínicament rellevant i pugui afectar a la meva salut o de la meva família biològica:

  ☐ Sí ☐ No

Així mateix, expresso la meva voluntat que es respectin les següents restriccions sobre l'ús de les meves mostres biològiques i/o dades:

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A ....., dia ..... de ..... del 20.....

**Signatura del/la professional** **Signatura del/la participant**

DNI: ..... DNI: .....

### 16.5 Annex V: Withdrawal Consent Form

## Revocació del consentiment

Jo, ....., amb DNI ..... declaro que vull revocar el consentiment informat per la participació a l'estudi: "Association between Diffuse Idiopathic Skeletal Hyperostosis and Intestinal Microbiota Dysbiosis: a case-control study"

A ....., dia ..... de ..... del 20.....

**Signatura del/la participant**

DNI: .....