**Calprotectin in patients with periANAL fiStulas: the CANALS study**

1. **INTRODUCTION AND RATIONALE**

Perianal fistulas are tracts that connect the intestinal lumen, usually the anal canal or rectum, with the perianal skin.(1) Cryptoglandular (CG) perianal fistulas occur in patients without Crohn’s disease caused by an infected perianal crypt gland. The incidence of CG perianal fistulas in Europe was reported to be around 2 per 10,000 per year.(2) Crohn’s disease (CD) is a chronic inflammatory condition affecting the gastrointestinal (GI) tract. The development of a perianal fistula is a common complication occurring in up to one third of Crohn’s disease patients, depending on luminal disease location.(3-6) In CD, perianal fistulas develop due to transmural inflammation rather than gland infection.(7, 8) Perianal Crohn’s disease constitutes a separate entity which warrants a comprehensive management strategy, often with combined medico-surgical treatment modalities.(9) pCD is most frequently accompanied by typical gastrointestinal symptoms such as diarrhea and abdominal discomfort. However, perianal fistulas can also precede other clinical manifestations of CD.(6, 10, 11) Conversely, patients with CG perianal fistulas may exhibit (functional) GI complaints suspicious of CD. Differentiating pCD and CG fistulas on clinical grounds can therefore be difficult and in a considerable number of patients endoscopy is required to rule out Crohn’s disease. Given the burden of endoscopy, it would be useful to have a biomarker that predicts the presence of CD with acceptable accuracy. Calprotectin consists of the two damage-associated molecular pattern (DAMP) proteins S100A8/S100A9.(12) It is an abundant calcium-binding cytosolic protein complex and its concentration in stool reflects neutrophil trafficking through the inflamed bowel wall.(13, 14) In CD the diagnostic accuracy of faecal calprotectin (FC) to detect mucosal inflammation is high.(15-20) However, no data is available on the diagnostic accuracy of FC for mucosal inflammation in pCD patients with an active perianal fistula. Active fistula tracts drain acute (neutrophils) and chronic (lymphocytes) inflammatory cells.(21)

We recently published a retrospective observation study in which we showed that:

1. Faecal calprotectin can discriminate between cryptoglandular and Crohn’s perianala fistulas, even in the absence of endoscopic inflammation and
2. In Crohn’s disease patients with an actively draining perianal fistula, specificity of faecal calprotectin to predict intestinal ulcers is low and faecal calprotectin values should be interpreted with caution.

The underlying hypothesis for the aforementioned results comes down to the loss of calprotectin via the fistula tract into the faeces. Due to distinct underlying etiopathogenesis, active cryptoglandular fistulas might not produce calprotectin in a similar amount as active Crohn’s disease perianal fistulas. Furthermore, due to the loss of calprotectin via the fistula fluid into the stools of Crohn’s disease patients with an active perianal fistula, it seems an elevated FC concentration does not accurately predict the presence of intestinal inflammation and should be interpreted with caution.

1. **OBJECTIVES**
   1. **Primary objectives**
      1. Determine the diagnostic accuracy of faecal calprotectin for differentiating active Crohn’s disease perianal fistulas and cryptoglandular perianal fistulas
   2. **Secondary objective**
      1. Determine the diagnostic accuracy of faecal calprotectin for the presence of active intestinal inflammation in patients with an active perianal fistula
      2. Determine local calprotectin production in an active fistula tract determined by fistula fluid calprotectin concentration
      3. Determine the association between the anatomical nature of the fistula tract (simple vs complex) and faecal calprotectin and fistula fluid calprotectin concentrations
      4. Determine the association between the epithelialization status of the fistula tract and faecal calprotectin and fistula fluid calprotectin concentrations
      5. Determine the correlation between the Perianal Disease Activity Index (PDAI) and faecal calprotectin concentration
      6. Determine the correlation between calprotectin concentration in faeces and fistula fluid
      7. Determine the correlation between calprotectin concentration in fistula fluid and fistula scraping
      8. Determine the correlation between calprotectin concentration in faeces and fistula scraping
2. **STUDY DESIGN**

Prospective cross-sectional study

1. **STUDY POPULATION**

Biomaterial for analysis will be collected from 54 patients with an active perianal fistula (27 Crohn’s disease perianal fistulas & 27 cryptoglandular perianal fistulas) who require surgical examination or intervention under anesthesia at the outpatient surgery center of the Amsterdam UMC, location AMC.

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| **Population: 27** patients with active Crohn’s disease perianal fistula |
| Inclusion criteria: |
| * Patients ≥ 16 years old * Established diagnosis, based on a combination of history, physical examination, family history, laboratory tests, endoscopy tests including histopathologic examination of mucosal biopsies, imaging studies and occasionally intraoperative findings * Active perianal fistula as defined by spontaneous drainage or drainage upon gentle finger compression * Clinical indication for surgical examination or intervention under anesthesia at discretion of the treating physician at the outpatients surgery center of the Amsterdam UMC, location AMC * Written informed consent |
| Exclusion criteria: |
| * Age < 16 years at inclusion * Perianal abscess * Perianal hidradenitis suppurativa * Clinical signs of an infectious gastroenteritis * Patients with an ileostomy or colostomy * No internal opening during surgical examination * Drugs that could induce a faecal calprotectin elevation: oral glucocorticosteroids, Non-Steroidal Anti-inflammatory Drugs (NSAIDS), aminosalicylates, proton pump inhibitors and statins. |

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| **Population: 2**7 patients with active Cryptoglandular perianal fistula |
| Inclusion criteria: |
| * Patients ≥ 16 years old * Established diagnosis of a cryptoglandular perianal fistula, based on a combination of history and physical examination and supplemented by the prior exclusion of Crohn’s disease by at least one previous endoscopy * Active perianal fistula as defined by spontaneous drainage or drainage upon gently finger compression * Clinical indication for surgical examination or intervention under anesthesia at discretion of the treating physician at the outpatients surgery center of the Amsterdam UMC, location AMC * Written informed consent |
| Exclusion criteria: |
| * Age < 16 years at inclusion * Perianal abscess * Perianal hidradenitis suppurativa * Clinical signs of an infectious gastroenteritis * No internal opening during surgical examination * Drugs that could induce a faecal calprotectin elevation: oral glucocorticosteroids, Non-Steroidal Anti-inflammatory Drugs (NSAIDS), aminosalicylates, proton pump inhibitors and statins. |

1. **TREATMENT OF SUBJECTS**

Patients will receive fistula treatment in accordance with standard clinical practice.

1. **METHODS**
   1. **Study procedures**
      1. **Specifically for the Crohn’s disease perianal fistula patients**
         1. *Demographic and clinical data collection*

Date of Crohn’s disease diagnosis, Montreal classification, biochemical markers of disease (CRP, faecal calprotectin, Hemoglobin, white blood cell count) within 3 months of surgical procedure.

* + - 1. *Disease activity assessment*

To assess the diagnostic accuracy of faecal calprotectin for the presence of luminal disease activity, a ileocolonoscopy will be performed in all Crohn’s disease patients who have a clinical indication for an endoscopy (independent of this research, ie **standard clinical care**). Active intestinal disease will be defined as a Simple Endoscopic Score for Crohn’s Disease (SES-CD) of > 6 in general or ≥4 in case of isolated ileal disease.

The remaining Crohn’s disease patients (without indication for endoscopic investigation) will receive an abdominal ultrasound by one of the IBD ultrasound specialists from our department. The ultrasound will solely focus on the gastrointestinal tract. Other abdominal organs will not be investigated. To assess Crohn’s disease activity, the following parameters will be assessed: bowel wall thickness (BWT), Doppler enhancement, bowel wall stratification, fatty wrapping, compressibility, peristalsis and haustrations.(22) Furthermore, patients will be stratified in an active or quiescent disease group. Stratification in these two groups will be based on bowel wall thickness and Doppler enhancement (22):

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| **Luminal disease activity assessment by ultrasound investigation** | | |
| **Groups** | **Bowel wall thickness** | **Doppler signal** |
| **Active luminal disease** | ≥ 3mm | Increased Doppler signal |
| **Quiescent luminal disease** | < 3mm | No Doppler signal |

As the visualization of the rectum is limited when using ultrasound, the presence of proctitis will be assessed during the investigation under anesthesia by the GI surgeon. The endoscopy or abdominal ultrasound will be performed within four weeks of the examination under anesthesia

* + 1. **For all patients**
       1. *Demographic and clinical data collection*

Gender, age, height, weight, duration of the currently active perianal fistula, medical history, surgical history, current medication, perianal disease activity index (***appendix 1***), MRI specific fistula information (anatomical characteristics of the fistula (simple vs complex // single vs multiple tracts // superficial, intersphincteric, intrasphincteric, transsphincteric, extrasphincteric, suprasphincteric, rectovaginal tracts) and finally the presence of anorectal stenosis as observed during the examination under anesthesia.

* + - 1. *Collection of biomaterial*

At time of surgical examination/intervention at the outpatient surgery center of the Amsterdam UMC, location AMC, the following additional biomaterial will be collected: faeces, fistula fluid, fistula tract scraping and mucosal biopsies of the internal opening of the fistula tract.

* + - * 1. *Faeces*

During the surgical procedure one scoop of faecal material will be collected from the rectum and stored in a transparent container. Faecal calprotectin will be measured by a quantitative enzyme linked immunosorbent assay (EliA, Calprotectin 2 test, Phadia AB, Freiburg, Germany). The upper detection limit of this test is 6000mcg/g. Faecal calprotectin will be measured according to the manufacturer’s instructions and samples will be processed within 3 days after collection.

* + - * 1. *Fistula fluid*

During the surgical procedure the fistula tract will be flushed with an amount of 10mL of 0.9% saline solution. The drainage of the fistula tract (comprising both fistula production and saline) will be collected in a transparent container. Faecal calprotectin will be measured using the same ELISA and processing methods as specified above.

* + - * 1. *Fistula tract scraping*

During the surgical procedure, biological tissue/debris will be scraped from within the fistula tract using a curette surgical instrument. Scrapings will be collected in a separate transparent and labeled container. Again faecal calprotectin will be measured using the same ELISA and processing methods as specified above.

* + - 1. *Data collection during fistula examination under anesthesia*

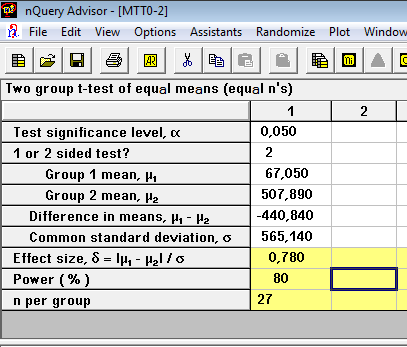
The gastrointestinal surgeon who performs the fistula examination will collect/report the following additional data:

* Complex vs simple fistula as defined by at least > 1/3 of the external sphincter being involved
* Single vs multiple tracts
* Single vs multiple internal openings
* Presence of epithelialization of the tract (or conversely, presence of mucoid or purulent secretion)
* Signs of proctitis
* Presence of anorectal stenosis
* Presence of a perianal abscess (exclusion criterion)

1. **STATISTICAL ANALYSIS**
   1. **Sample size calculation**

Due the lack of data on the discriminative value of faecal calprotectin for the differentiation between Crohn’s disease and cryptoglandular perianal fistula, no exact sample size can be calculated. However in our pilot retrospective study some preliminary data was obtained. In the Crohn’s disease patients with an active perianal fistula without endoscopic luminal inflammation (N=18), mean faecal calprotectin concentration (standard deviation) was 507.89 mcg/g (558.37mcg/g). In the cryptoglandular perianal fistula population (N=19), mean faecal calprotectin concentration (standard deviation) was 67.05 mcg/g (87.208 mcg/g).

So for our primary objective (association between FC and underlying cause of the perianal fistula), using a two-sided T test with a type I error rate of 5%, a group 1 mean of 67.05, a group 2 mean of 507.890 and a common standard deviation of 565.14, it is estimated that 56 patients (27 patients in each arm) are needed to provide 80% power in a two-sided test model.



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APPENDIX 1

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| **Perianal disease activity index** (23) | | |
| **Item** | | **points** |
| **Discharge** | |  |
|  | *No discharge* | 0 |
|  | *Minimal mucous discharge* | 1 |
|  | *Moderate mucous or purulent discharge* | 2 |
|  | *Substantial discharge* | 3 |
|  | *Gross fecal soiling* | 4 |
| **Pain/restriction of activities** | |  |
|  | *No activity restriction* | 0 |
|  | *Mild discomfort, no restriction* | 1 |
|  | *Moderate discomfort, some limitation* | 2 |
|  | *Marked discomfort, marked limitation* | 3 |
|  | *Severe pain, severe limitation* | 4 |
| **Restriction of sexual activity** | |  |
|  | *No restriction of sexual activity* | 0 |
|  | *Slight restriction of sexual activity* | 1 |
|  | *Moderate limitation of sexual activity* | 2 |
|  | *Marked limitation of sexual activity* | 3 |
|  | *Unable to engage in sexual activity* | 4 |
| **Type of perianal disease** | |  |
|  | *No perianal disease* | 0 |
|  | *Anal fissure or mucosal tear* | 1 |
|  | *<3 perianal fistulas* | 2 |
|  | *>3 perianal fistulas* | 3 |
|  | *Anal sphincter ulceration or fistulas with significant undermining skin* | 4 |
| **Degree of induration** | |  |
|  | *No induration* | 0 |
|  | *Minimal induration* | 1 |
|  | *Moderate induration* | 2 |
|  | *Substantial induration* | 3 |
|  | *Gross fluctuance/abscess* | 4 |