

EXPRESSION PATTERN AND CLINICOPATHOLOGICAL RELEVANCE OF NEUROTENSIN AND ITS RECEPTORS IN COLORECTAL CANCER

Thesis submitted by

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In loving memory of my grandmother.

STATEMENT OF CONTRIBUTION AND DECLARATION OF ORIGINALITY

I declare that the following report is my own work, and that the work of others is not included except where appropriately referenced. Other contributors to the work presented include: Mr. Shengyang Qiu, Dr. James Alexander and Mr. Christos Kontovounisios.

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PUBLICATIONS DERIVED FROM THIS STUDY

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ABSTRACT

Introduction

Colorectal cancer (CRC) is the third commonest cancer diagnosed in the world with more than 1.9 million new cases and 935,000 deaths. Neurotensin and its receptors have been implicated in carcinogenesis. There is evidence in the literature to support the presence of tissue NT, NTSR1 and NTSR3 in colorectal cancers with a potential diagnostic and prognostic role in the disease.

Aims

1. To assess NT, NTSR1 and NTSR3 expression in polyps and colorectal cancer in a prospectively collected cohort of patients and to establish whether there is correlation between NT and its receptors to stage of disease and histopathological factors.
2. Assess plasma NT and correlation to tissue expression of NT, NTSR1 and NTSR3
3. Assess the diagnostic accuracy of plasma NT in distinguishing polyps and colorectal cancer from normal or benign colonic disease.

Methods

To assess aims 1 and 2, patients were prospectively recruited from 2 centres. In total, 106 patients were recruited in the study and both, blood samples and tissue were collected for analysis for plasma NT, tissue NT, NTSR1 and NTSR3 expression. Patients included in the study were either referred for a 2ww colonoscopy or had a new diagnosis of CRC.

Immunohistochemical staining of NT, NTSR1 and NTSR3 was performed on all patients and the intensity of expression was measured using the histascore system (H-score). H-score was then correlated between stage of disease, clinical and histological features.

To assess aim 3, two further prospective cohorts were included in the study to evaluate the diagnostic value of NT in detecting polyps and cancers in patients with high -risk symptoms.

Results

In the first study 106 patients were recruited but 2 excluded due to non-colorectal adenocarcinoma diagnosis. From the remaining 104, 78 had colorectal adenocarcinoma, 5 had normal colonoscopies, 17 had polyps and 4 had recurrent disease.

There was increasing expression of NT from polyps to stage IV disease but no difference in the NTSR 1 and 3 receptors. Several prognostic factors (mucin presence and LVI) were associated with higher NT expression in tissue ($p=0.01$, $p<0.01$ respectively, Kruskal Wallis test).

There was significantly reduced NTSR1 expression in cancers post chemoradiotherapy ($p<0.01$, Kruskal Wallis) and borderline difference between rectal and colon cancers ($p=0.05$, Kruskal Wallis test). There was no correlation between plasma NT and tissue NT and its receptors.

For assessing the diagnostic ability of NT, a total of 364 patients were included (135 normal, 108 cancers, 75 polyps, 4 recurrences, 27 diverticular disease and 15 colitis). ROC curve analysis and optimal cut-off values of >637.9 pg/mL gave 80.6% sensitivity and 76% specificity in distinguishing normal colons from cancers. With a cut-off of >460.8 , plasma NT showed sensitivity of 69.3% in detecting polyps from normal and specificity of 62%.

Conclusion

NT expression in colorectal cancer is associated with worsening stage of disease and presence of mucin and possibly lymphatic invasion.

Furthermore, plasma NT is a promising diagnostic marker for detecting polyps and colorectal cancer.

Future work is required to investigate the pathway through which neurotensin drives carcinogenesis which can lead to identification of therapeutic targets.

LIST OF ABBREVIATIONS

2WW	Two-week wait
95% CI	95% confidence interval
ADP	Adiponectin
AJCC	American Joint Committee on Cancer
APC	Adenomatous Polyposi Coli
BAT	Brown Adipose Tissue
BMI	Body Mass Index
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CA19-9	Carbohydrate antigen 19-9
CDX2	Caudal-type homeobox 2
CEA	Carcinoembryonic antigen
CK	Cytokeratin
CPG	cytosine-phosphorus-guanine
CRC	Colorectal cancer
CRT	Chemoradiotherapy
CRUK	Cancer Research UK
CT	Computed tomography
CV	Coefficient of variation
DAB	3,3'- Diaminobenzidine tetrahydrochloride
DAG	Diacylglycerol
DFS	Disease Free Survival
DNA	Deoxyribonucleic acid

EDTA	Ethylenediaminetetraacetic acid
EGF(R)	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked immunosorbent assay
EMVI	Extramural vascular invasion
ERK	Extracellular signal-regulated kinase
ER	Estrogen receptor
FDA	U.S. Food and Drug Administration
FIT	Faecal immunochemical test
FOBT	Faecal occult blood test
GI	Gastrointestinal
GPR	G-protein coupled receptor
H-score	Histo score
HT29	Colon cancer cell line
HRP	Horseradish peroxidase
ICHTB	Imperial College Healthcare tissue bank
IGF-1	Insulin-like growth factor 1
IP3	Inositol triphosphate
IQR	Interquartile range
IRAS	Integrated Research Application System
kDa	Kilo Dalton
Kg	kilogram
KRAS	Kirsten rat sarcoma viral oncogene homolog
L	Litre
LVI	Lymphovascular invasion

LN	Lymph node
MAPK	Mitogen activated protein kinase
mIR	MicroRNA
MLH	MutL Homolog 1
MSH	MutS Homolog
Mm	Millimitres
MMR	Mismatch repair
MRI	Magnetic resonance imaging
mSEPT9	Methylated Septin9
MSI	Microsatellite instability
NFkB	Nuclear factor kappa B
Ng	Nanogram
NHS	National Health System
NICE	National Institute for Health and Care Excellence
NK	Neurokinins
Nm	Nanometre
NT	Neurotensin
NTSR1	Neurotensin Receptor 1
NTSR2	Neurotensin Receptor 2
NTSR3	Neurotensin Receptor 3
OD	Optical density
OS	Overall Survival
Pg	Picogram
PI3K	Phosphatidylinositol-3 – kinase

PIP2	Phosphatidylinositol 4,5-biphosphate
PKC	Protein Kinase C
PNI	Perineural invasion
RAS	Rat sarcoma viral oncogene homolog
REC	Research Ethics Committee
ROC	Receiver operating curve
SATB2	special AT-rich sequence-binding protein 2
SD	Standard deviation
SP	Substance P
TGF β	Tissue growth factor beta
Tis	Carcinoma in situ
TNM	Tumour, Node, Metastasis staging system
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
WAT	White Adipose Tissue
μg	Microgram
μm	Micrometre

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(Taken from Mr Shengyang Qiu's PhD thesis.

Figure 4.2. A. ROC curve analysis for normal vs cancer **B.** ROC curve analysis for normal Vs polyps **C.** ROC curve analysis early vs late disease **D.** ROC curve analysis for normal vs all abnormal colonic pathology

1.1 COLORECTAL CANCER

1.1.1 EPIDEMIOLOGY OF COLORECTAL CANCER

Colorectal cancer (CRC) is the third commonest cancer diagnosed in the world with more than 1.9 million new cases and 935,000 deaths, putting it in second place in terms of mortality (2).

There is a significant variation of colon and rectal cancer incidence between different world regions with transitioned countries having a 4-fold increased incident rate compared to transitioning countries(2). This could be attributed to changes in diet, increased rates of obesity and more sedentary lifestyle as well as higher intake of red or processed meat(2). Certain high-income countries have shown a decline in colorectal cancer incidence in adults ≥ 50 years old which reflects population lifestyle changes and uptake of screening(3). This is accompanied by a concomitant higher incidence of early onset CRC diagnosis which may be attributed to lifestyle changes but certainly needs further investigation to determine the reason for this change (2, 4).

The 5-year survival rates for early and localised disease reaches 90 percent but those with metastatic disease have a survival of less than 20 percent(4, 5). The median life expectancy without treatment of metastatic CRC is 6 to 12 months. The addition of multiple systemic therapies, which depends on the molecular tumour profile and the patient's general fitness, can extend survival to up to 3 years(4). In a small percentage, (10 to 20 percent of patients with metastatic CRC) cure can be achieved(4).

1.1.2 AETIOLOGY AND PATHOGENESIS OF COLORECTAL CANCER

CRC is caused by an interaction between genetic and environmental factors. They can be classified into sporadic, familial and hereditary types(6). Approximately 75 percent of cases are sporadic and have no apparent hereditary cause(1). Twenty-five percent of patients with familial CRC have a family history of the disease but do not have a known associated gene(7). Only 5 percent of patients, with a family history of CRC, are due to inherited mutations of major CRC genes(6).

Most CRCs arise from precancerous polyps first suggested by Gilbertson in the 1960s (8). By the end of 1980s, Fearon and Vogelstein described the polyp-to-carcinoma progression as a series of genetic mutations driving the carcinogenesis(8). One such mutation which occurs early in the adenoma-carcinoma sequence affects the adenomatous polyposis coli (APC) tumour suppressor gene located on chromosome 5q21(9). Other genes affected are K-ras, p53, 18q loss, SMAD2 and SMAD4. Although 40 percent of the Western population will develop adenomas, only 3 percent will go on to develop cancer and currently there are no reliable indicators to predict which ones will(9).

In addition to the adenoma-to-carcinoma pathway, CRC can develop via the serrated pathway(10). The term '*serrated polyps*' (SPs) includes hyperplastic polyps (HP), sessile serrated lesions (SSLs) and traditional serrated adenomas (TSAs)(10). Most serrated polyps are hyperplastic polyps, which are usually small ($\leq 5\text{mm}$), mostly located in the distal colon and not considered as a precursor to CRC(10). SSLs comprise 25 percent of the SPs and are usually large and present mostly in the proximal colon(10). Finally, TSAs are the least common SPs and are usually larger than SSLs, are usually polypoid or pedunculated lesions and are mainly found in the distal colon(10). Both TSAs and SSLs are precursors to CRC(10).

Twenty-five percent of sporadic CRCs, arise from serrated lesions. A strong risk factor for developing serrated polyps is tobacco use, whilst higher alcohol intake and obesity have a weaker association with SSLs(10). Aspirin and non-steroidal anti-inflammatory drug use seem to have a protective effect(10, 11).

Modifiable risk factors of CRC include low fibre and calcium diet, higher animal products and processed meat intake, lack of exercise, obesity, tobacco use and moderate to excess alcohol intake(5). Non-modifiable risk factors include personal or family history of colorectal polyps or cancer, hereditary conditions, history of inflammatory bowel disease and increasing age(5).

Most CRCs are adenocarcinomas (more than 90 percent) arising from epithelial cells of the colorectal mucosa and are mostly characterised by the presence of glands(12). Most of the colorectal adenocarcinomas are moderately differentiated, 10 percent are well differentiated and 20 percent poorly differentiated(12). Rare types of CRC include: neuroendocrine, squamous cell carcinomas, adenosquamous spindle cell and undifferentiated carcinomas(12).

1.2 MARKERS IN COLORECTAL CANCER

1.2.1 DIAGNOSTIC MARKERS IN COLORECTAL CANCER

The stage of disease at diagnosis is the most important factor with regards to survival. If the cancer is detected at an early stage, the reported 5-year survival is 90 percent, but this decreases to 14 percent if the disease is advanced at initial diagnosis(13).

The natural history of CRC is to develop from a benign adenoma to invasive adenocarcinoma over a 5-to-10-year period(14, 15). The early stages of disease are usually accompanied by non-specific symptoms, if any exist at all. It is therefore vital to find a safe, acceptable, sensitive, specific and cost-effective test that detects the early stages of disease(16).

The current gold standard diagnostic investigation for identifying colonic pathology is colonoscopy. Colonoscopies are estimated to reduce colorectal cancer incidence by 59 percent and mortality by 68 per cent(17). The test is however invasive, has low adherence and has potential risks such as bleeding, colonic perforation and cardiorespiratory complications(13, 18, 19). The alternative is a virtual colonoscopy or CT colonogram which although is less invasive, it still requires bowel preparation and can cause discomfort to the patient(20). Other screening investigations include flexible sigmoidoscopy, faecal occult blood test (FOBT), and faecal immunohistochemistry (FIT), which is a DNA-based faecal test(20).

FIT test has been introduced in the bowel cancer screening programme in England and Wales, since June 2019. It is offered every 2 years to men and women aged 50 to 74 since 2021. FIT testing has also been offered in England and Wales as a diagnostic adjunct in patients without per rectal bleeding and with low-risk symptoms(21). It has also been used to triage 2WW referred patients during the COVID19 pandemic, during which a negative (<10ug/gm) FIT test indicates that the symptoms experienced by the patient are unlikely to be caused by cancer and hence an investigation can be deferred(22). Its negative predictive value (NPV) for CRC is more than 95 percent if negative (<10ug/gm). If the FIT test is >100ug/gm, there is a 25 percent chance of CRC or other significant colorectal pathology(22). The NPV and positive predictive value (PPV) of 10-100ug/gm is still

unknown(22). Although FIT testing is easier to use compared to the guaiac test, an aversion to handling stool is an important reason for its low uptake (60 percent) (21).

Blood molecular markers in current use, such as carcinoembryonic antigen (CEA) and cancer antigen (CA) 19-9, are for surveillance and monitoring response to treatment only and will be discussed in further detail in section 1.2.2. Their sensitivity and specificity range from 40 to 70 percent and 73 to 90 percent, respectively(16). This makes them unsuitable for use as a diagnostic tool.

Methylated septin 9 (mSEPT9), which is a more recent molecular epigenetic biomarker and detects hypermethylation of the SEPT9 gene located on the 17q25 chromosome(23). It has shown 76 percent sensitivity and 87 percent specificity, making sensitivity (but not specificity) comparable to FIT test(24). In 2016, the FDA approved the Epi proColon Kit version 1.0 (and later updated to version 2.0), to presumably detect early CRC(25). However, most studies agree that it has a low sensitivity in detecting stage I CRC (35 percent) and even lower for advanced adenomas (14.4 percent)(26) which renders it ineffective in detecting important pre-cancerous lesions(27) and is no longer recommended as a screening tool in the US(28) .

Other blood markers under investigation include ‘liquid biopsies’ which assess presence of tumour components in blood such as circulating tumour cells, circulating tumour DNA, nucleic acids (i.e. microRNAs, long non-coding RNAs,mRNAs), proteins, cytokines and antibodies(20, 29). Their clinical application is still fraught with challenges and need further clinical validation(29, 30).

Immunohistochemistry and polymerase chain reaction tests (PCR) tests for deficiencies in mismatch repair (MMR), microsatellite instability, KRAS and BRAF mutations have diagnostic, predictive and therapeutic implications(6). IHC markers for colorectal adenocarcinoma in current use are cytokeratin (CK)20, CK7 and CDX2(12). The commonest immunophenotype is CK20 positivity and CK7 negativity, however 20 percent of the colorectal adenocarcinomas may show a CK7-positive/CK20-negative or CK7-negative/CK20-negative staining pattern(12).

CDX2 is a marker of enteric differentiation and is present in more than 90 percent of colorectal adenocarcinomas(12). CDX2 is an adverse prognostic feature (discussed in section 1.2.2).

Microsatellite instability testing (MSI) in CRC tissue is also a tissue biomarker for diagnosing Lynch syndrome, but also has a prognostic and predictive role (also discussed in section 1.2.2). Microsatellites are short tandem repeats of DNA sequences present in the genome(1). MSI is caused by errors in the mismatch repair (MMR) system, which corrects errors during DNA replication. MSI is present in 95 percent of all Lynch syndrome-associated cancers(12). The MSI status is evaluated in tumoural DNA by a PCR-based technique that amplifies DNA at several microsatellite sites from a CRC tissue and healthy tissue sample. IHC uses antibodies to detect an increased or abnormal expression of the MMR proteins in the cancer tissue. Absent or reduced nuclear staining of 1 or more of the MMR proteins suggests that there is a pathogenic mutation on a gene encoding these proteins and should prompt further testing(31). Sporadic CRCs also have the MSI phenotype in about 15 percent

of cases. These cases usually are due to somatic hypermethyations of the *MLH1* gene promoter, which lead to silencing expression from that allele in all somatic tissues. In 40 to 87 percent of all sporadic microsatellite unstable tumours with hypermethylation of the *MLH1* gene, there is a V600E missense mutation in the BRAF oncogene(12). This particular mutation is not present in Lynch syndrome MSI tumours in which the MSI phenotype is due to genetic alteration of the MMR genes (*MLH1* on chromosome 3p21, *MSH2* on chromosome 2p16, *MSH6* on chromosome 2p16, *PMS2* on chromosome 7p22, *MLH3* on chromosome 2p16 and *MSH3* on chromosome 5q11) and not due to epimutation(12, 32). The latest NICE guidelines recommend testing all colorectal patients for MSI instability or perform IHC for MMR proteins in order to identify patients at high risk of LS(31).

Other tissue diagnostic biomarkers include: special AT-rich sequence-binding protein 2 (SATB2) which is part of a transcription factor family involved in skeletogenesis(30). One potential application of this marker includes its use to determine the origin of adenocarcinomas of unknown primary and if this is combined with CK20 it is a highly specific marker for colon and rectal adenocarcinoma(30).

1.2.2 PROGNOSTIC AND PREDICTIVE MARKERS IN COLORECTAL CANCER

CRC patient prognosis is based on clinicopathological features and the overall stage of disease at the time of diagnosis: the overall survival rate for stage I is 90 percent, 70 percent for stage II, 58 percent for stage III and 15 percent for stage IV(1). Additional high-risk features include presence of lymphovascular and perineural invasion, poor histological differentiation, tumour budding, bowel obstruction or perforation and advanced tumour stage (T4) at the time of surgery(1, 33).

DNA testing of tumour samples have identified certain genes whose expression or absence can help tailor treatment in patients with metastatic disease(1) (table 1.1 summarises these).

Status	Prevalence	Prognostic value	Predictive value	
			Non-metastatic	Metastatic
Microsatellite instability (MSI) high	Overall: 15% (3% are associated with Lynch syndrome) Stage II/III: 15% Stage IV: 4.5%	Increased OS in stage II Poor OS in stage III	☹️ 5-FU monotherapy (stage II) 😊 FOLFOX (stage III)	😊 Immunotherapy
KRAS/NRAS mutation	45%	Bad prognostic (debatable in stage II)	No impact on treatment	☹️ Anti-EGFR therapy
BRAF mutation	7-10%	Bad prognostic especially in the metastatic setting	Currently no impact on treatment	☹️ Anti-EGFR therapy (conflicting data) 😊 Combi therapy: Anti-BRAF/Anti-EGFR
CDX2 absence/low expression	7%	Overall reduced DFS and OS in stage II/III disease	In stage II disease had improved 5-year DFS (91%) with adjuvant chemotherapy compared to those who had no chemotherapy(33)	Reduced PFS with first line chemotherapy compared to CDX2 positive tumours (3 versus 10 months)

Table 1.1: Clinical molecular markers in current use for CRC (table adapted from (1). EGFR:

epidermal growth factor receptor, DFS: disease free survival, OS: overall survival, PFS:

progression-free survival. 😞 no benefit; 😊 benefit

Prognostic tissue biomarkers in current clinical use are based on the mutational status of the genes which are important in CRC carcinogenesis: *NRAS*, *KRAS* and *BRAF*, *CDX2* or with DNA mismatch repair (MMR) system defects which lead to microsatellite instability (1). The four MMR genes are *MSH2*, *MLH1*, *MSH6* and *PMS2*. MMR defects lead to failure to correct the insertion or deletion of repeating units during DNA replication, resulting in a hypermutable phenotype (MSI-high is characterised by 2 or more loci)(1). MSI tumours are present in 15 percent of all CRC, and of these, 3 percent are associated with Lynch syndrome. The rest are due to sporadic hypermethylation of the *MLH1* gene promoter(1). MSI prevalence is stage-dependent and 15 percent of stage II and III CRC patients are dMMR and only 5 percent of stage IV CRC are dMMR(34). The presence of MSI instability predicts a good outcome in stage I and II disease but a poor outcome in stage III(35). MSI-H tumours have unstable microsatellites and are highly immunogenic hence why they respond to immunotherapy with anti-PD-1/ PDL-1 if they are at an advanced stage (6, 35). If the tumours is MSI-H and not advanced, they have a good prognosis and will not respond to 5-FU-based chemotherapy(6).

Caudal Type Homeobox transcription factor 2 (CDX2) is expressed in the nucleus of the intestinal epithelial cells and plays a role in intestinal development and differentiation (30). CDX2 downregulation is associated with more advanced tumour with liver metastases(36, 37). Patients with stage II disease and CDX2-negative disease, were found to have

significantly shorter 5-year DFS compared to patients with stage II disease with CDX2-positive tumours(33). The stage II CDX2-negative colon cancer patients who underwent adjuvant chemotherapy had a significantly better five-year DFS compared to those who did not undergo chemotherapy(33).

Cytokeratin 7 (CK7) is present in glandular malignancies (i.e. breast, respiratory tract and biliary tract). Its presence in metastatic lymph nodes is associated with a shorter OS and is a poor prognostic factor. Its presence in the primary tumour does not seem to affect survival(38).

CK20 loss is also associated with a lower DSF and OS and is more commonly found in older patients, right-sided tumours and more advanced disease(30).

Cytokeratin 18 (CK18) is involved in cell proliferation, apoptosis and cell signalling(30). It is upregulated in many types of human cancers (e.g. hepatocellular and cervical carcinomas). In CRC, high CK18 expression is positively correlated with advanced clinical stage, lymph node positivity and poor differentiation(30).

CK15 is involved in maintaining cytoplasmic stability and high expression in CRC has been associated with a poorer prognosis and lower overall survival compared to patients with low CK15 expression(30).

Finally, telomerase is an enzyme complex which helps to avoid senescence in cells(30). It is present in germ line cells and 80 to 90 percent of human cancer cells(39). Telomerase

contains a telomere-specific reverse transcriptase (hTERT) which increases the risk of recurrence and is associated with a worse median overall survival and lower disease-free survival(40).

1.2.3 THERAPEUTIC MARKERS IN COLORECTAL CANCER

Targeted molecular therapy was originally proposed in the 1900s and was introduced in cancer therapy in 1988 and has been evolving ever since(41). The role of molecular therapy is to work on cancer cells and their microenvironment in order to disrupt cell proliferation, migration and differentiation(41).

The first FDA-approved targeted treatment agent was cetuximab and then bevacizumab in 2004(41). Cetuximab and panitumumab are anti-EGFR agents and bevacizumab, regorafenib and ramucirumab are anti-VEGF/VEGFR agents. Both anti-EGFR and anti-angiogenic therapies in metastatic CRC have shown important benefits but the preferred first-line choice is an ongoing debate(41). Cetuximab seems to be a better choice in RAS-wild-type cases as shown in a meta-analysis that included the FIRE-3, CALGB and PEAK trials(42). Left-sided tumours respond more to cetuximab whilst right-sided tumours tend to respond more to bevacizumab, although if the BRAF-mutated cases were removed, panitumumab still showed some benefit in right-sided tumours(43).

Apart from directly blocking pathways involved in cancer proliferation and migration, methods to enhance immunorecognition has been a topic of great interest. Cancer cells exhibit an immune escape by secreting immunosuppressive factors, such as TGF- β and IL-6(41, 44, 45). Immune checkpoint blockade agents aim to enhance the immune response

and avoid the cancer's ability to escape T cell detection(41). An example of this is PD-1 inhibitor, pembrolizumab which is an FDA-approved immunotherapy agent used in advanced renal cancer, melanoma and NSCLC(41). In metastatic CRC, the first PD-1 blocker to be FDA-approved was pembrolizumab. The KEYNOTE-016 study found that this agent had a 40 percent response rate and 20 week PFS of 78 percent, in dMMR CRC patients(46). The KEYNOTE-164 study subsequently showed that pembrolizumab had an objective response rate of 52 percent, a 59 percent PFS and OS of 72 percent in patients with MSI-H metastatic cancers(47). Nivolumab has also been FDA-approved since the Checkmate-142 trial showing an overall response rate of 31.1 percent(41).

Unfortunately, all targeted molecular therapies eventually are let down due to the development of tumour resistance. Several strategies to overcome this have been investigated including combining it with radiotherapy, switching to a different agent or use bispecific antibody therapy(41).

Other therapeutic markers targeting IGF-1R, Wnt, Notch, TGF- β have been explored without any significant progress yet(41).

1.3 THE PROGNOSTIC AND THERAPEUTIC ROLE OF HORMONES IN COLORECTAL CANCER

Hormone therapy in breast, ovarian, endometrial and prostate cancer has revolutionised treatment. The GIT expresses more than 30 hormone genes, making it the largest endocrine organ in the body(48). Yet, there is currently no clinical use of hormones in colorectal cancer(48). In this section, a summary of research investigating the role of hormones in CRC as prognostic and/or therapeutic markers will be discussed.

1.3.1 ADIPOSE TISSUE HORMONES

Obesity is a risk factor to developing CRC and adipose tissue hormones may exert a tumorigenic effect(48). There are two types of adipose tissue: the white adipose tissue (WAT) and the brown adipose tissue (BAT). Hormones and cytokines are secreted from WAT.

Adiponectin (APN) is a 30kDa complement C1q-related protein and is mainly secreted from WAT. It has 3 receptors: AdipoR1, AdipoR2 and T-cadherin(49). APN is involved in many signalling pathways which suppress inflammation, insulin resistance and endothelial cell migration(48, 50). Low levels of circulating APN have been linked to obesity-related CRC (50, 51). Adiponectin supplementation in vitro and animal studies has shown promise in suppressing carcinogenesis(50, 52, 53). The prognostic role of APN and its receptors shows conflicting evidence in literature and needs further investigation(48).

Leptin is an adipokine and is involved in regulating hyperphagy, glucose homeostasis, growth, immune response and angiogenesis(48, 54). Only two out of the six leptin isoform receptors are involved in intracellular signalling and OB-Rb is the longest isoform(54). It is normally produced by the hypothalamus and is involved in appetite regulation(54). Leptin's role in CRC carcinogenesis has been studied both in vitro and animal models(55-57), there only a few studies looking at its prognostic role and have conflicting evidence(48).

1.3.2 REPRODUCTIVE HORMONES

Oestrogen's role in CRC was first observed in 1969 where an increased risk of breast and colon cancer was observed in a group of nuns who had a longer exposure to oestrogen(58). There are three forms of oestrogen receptors: ER α , ER β and G-protein coupled oestrogen

receptor 30 (GPR30)(59). The main ER subtype in normal colonic epithelium is ER β (60). Its expression is progressively lost with advancing malignancy, whilst ER α expression increases(61). Estrogen and its receptors' role in prognosis show conflicting evidence and need further investigation(48).

Testosterone's role in colon carcinogenesis remains unclear as some studies actually show a protective effect(62, 63) whilst others show an increased risk of CRC(64). Androgen receptor A and B (AR-A and AR-B) are expressed on normal colonic epithelium. CRC epithelium only expressed AR-A and binds to dihydrotestosterone suggesting a role in carcinogenesis (DHT)(64). Animal studies have shown tumour regression with activation of membrane androgen receptors which shows promise as a novel therapeutic target(65). AR expression has shown a potential as a prognostic marker(66) but the literature on this is sparse.

1.3.3 PANCREAS AND LIVER HORMONES

Chronic hyperinsulinaemia which is associated with type 2 Diabetes Mellitus (T2DM) as well as insulin treatment are both associated with a higher risk of CRC(48, 67). Insulin resistance is linked to higher circulating insulin-like growth factor binding protein -1 and -2 (IGFBP-1/-2) which leads to higher IGF-1 which in turn promotes cell proliferation(68). Studies have found higher IGF-1 in CRC patients compared to normal(69, 70) and animal models with an IGF-1R knockdown inhibits CRC growth(71). Dalotuzumab (MK-0646) antibody and Figitumumab (IgG2 monoclonal antibody) to IGF1-R have not progressed beyond phase II trials as they did not show improvement in survival outcomes(72, 73).

1.3.4 CENTRAL NERVOUS SYSTEM

Growth hormone (GH) is a peptide hormone, secreted by the pituitary gland and directly acts on tissue or via IGF-1's action on the liver, to promote cell proliferation and differentiation(74). In vitro and in vivo xenografted mouse models, have shown that a neutralizing GHR antibody and somatostatin agonists causes primary and metastatic liver tumour shrinkage(74). Unfortunately, anticancer therapies targeting the GH and IGF system have so far had discouraging results(75).

1.3.5 GASTROINTESTINAL TRACT

Gastrin releasing peptide (GRP) which is released from the vagus nerve, stimulates the release of gastrin from the G cells in the stomach. When gastrin binds to CCKBR receptors on enterochromaffin (ECF) and parietal cells, gastric acid is secreted(48). Gastrin's role in CRC shows conflicting evidence but this could be due to the fact that the amidated form of the protein is measured and not the non-amidated form which is predominantly expressed in CRC(76).

Progastrin (PG) which binds to the annexin II (ANXII) receptor promotes mitogenesis and tumour growth. Overexpression of PG in patients with hyperplastic polyps had a 5-year neoplasm-free survival rate of 38 percent compared to 100 percent in patients with no or low PG expression(77).

GRP and its receptor GRPR are not normally expressed on normal colonic epithelial cells but are overexpressed in various cancers including colon, pancreas and lung cancer(48). Its overexpression in CRC makes it an attractive diagnostic and therapeutic target(78). A natural

GRPR-targeting peptide, bombesin (BBN) have been used in radiolabelled form of imaging with promising results as a non-invasive early CRC detection tool(78).

Guanylyl and uroguanylyl are peptide hormones secreted by the small bowel and colon. They are involved in epithelial homeostasis via the guanylyl cyclase enzyme (GCC2C)(79). GCC2C is found in 95 percent of primary and metastatic CRCs whilst uroguanylin and guanylin disappears in early CRC tumorigenesis(79). Phase I trial using a replication-deficient human type 5 recombinant adenovirus (Ad5) vaccine encoding guanylyl cyclase C fused to the Pan DR Epitope (PADRE) in 10 patients demonstrated that GUCY2C is a safe immunogenic target(80). Antibody-drug conjugates (ADCs) in Phase I trials have showed antitumour activity and chimeric antigen receptors (CARs) in murine models have shown increased survival, regression of metastases, and without toxicity(81, 82). A phase II trial results for the Ad5.F35-hGCC-PADRE vaccine are still awaited.

Neurokinins (NK) and substance P (SP) are peptide hormones whose overexpression has been associated with lymph node metastasis, TNM stage and worse survival(83, 84). Use of NK1R antagonists inhibited cancer stem cells in animal studies which are thought to cause resistance to chemotherapy and radiotherapy and could have a role in CRC recurrence(85).

Finally, neurotensin is a hormone expressed both in the central nervous system and the small intestine. It is this hormone's link to CRC and adenomas which we will focus on this thesis and section 1.4 looks into this hormone and its receptors into more detail.

1.4 NEUROTENSIN

1.4.1 NEUROTENSIN AND NTSR RECEPTORS

Neurotensin was first detected by Carraway and Leeman in bovine hypothalamus in 1973. It is a 13-amino acid regulatory peptide present in both the central nervous system and the gastrointestinal tract (N cells of the jejunum and ileum)(86).

NT is produced from pre-pro-neurotensin and its physiological functions include increasing intestinal motility, pancreatic and biliary secretions and promoting growth of GI cells, pancreas, adrenal gland and liver(87). In the CNS, it is thought to inhibit dopamine production and has analgesic properties acting through opioid pathways(87).

NT's role in cancer seems to stem from Ras dysregulation and changes to methylation patterns of cytosine-phosphorus-guanine (CpG) sites in the NT promoter region(87).

NT has 3 main receptors: Neurotensin receptor 1 (NTSR1), NTSR2 and NTSR3 (or sortilin 1). NTSR1 and NTSR2 are both transmembrane G-protein coupled receptors and the NTSR3 is a single transmembrane domain sorting receptor(87). NTSR1 is the most studied in its involvement in cancer(87). It composed of 424 amino acids and has a high affinity for NT. The NT/NTSR1 complex leads to phospholipase C (PLC) activation which in turn produces inositol triphosphate (IP3) and diacylglycerol (DAG)(88). Protein Kinase C (PKC) activation and intracellular calcium mobilisation then leads to cell proliferation, migration and invasion(89).

NT's role in cancer seems to stem from Ras dysregulation and changes to methylation patterns of cytosine-phosphorus-guanine (CpG) sites in the NT promoter region(87).

NTSR2 is a low affinity, 410 amino acid receptor which has been reported in prostate cancer, glioma and chronic lymphocytic leukaemia (CLL)(90). There is still little information on its role in CRC and its mechanism of action(87).

NTSR3 (sortilin 1) receptor is a single transmembrane domain receptor and is not specific to NT(87). NTSR3's role in cancer is largely mediated by soluble NTSR3 (sNTSR3) which increases calcium concentration and induces focal adhesion kinase (FAK)/Src-dependent activation of inositol 1,4,5-triphosphate (IP3) kinase pathway(87). Sortilin has also been implicated in angiogenesis via the tyrosine kinase and epidermal growth factor complex(87).

1.4.2 NEUROTENSIN AND NTSR RECEPTORS IN NON-GASTROINTESTINAL CANCERS

Lung cancer

NT has been postulated to be one of the regulatory peptides in both the SCLC and NSCLC in in-vitro studies(87). The postulated mechanism is that NT and NTSR1 interaction causes EGFR, HER2 and HER3 over-expression and activation of lung tumour cells(87). In one study, blocking the NTSR1 receptor with SR48692 in human NSCLC cells, resulted in a potentiated effect of gefitinib, a tyrosine kinase inhibitor already in clinical use, in inhibiting the growth of NCI-H1299 and A549 cells(87).

NTSR1 present in the cytoplasm is correlated with poor prognosis in lung adenocarcinoma, but its presence on the cell surface, as it is in lung squamous cell carcinoma, has no effect to prognosis(91). NTSR1 overexpression has also been correlated to worse sensitivity to platinum-based chemotherapy in patients with NSCLC and a more aggressive tumour(92).

The same group of researchers studied the effect of NTSR2 in non-squamous NSLC and they did not find a correlation to OS and NTSR2 expression(92).

NTSR3 or sortilin 1 normally cycles between the cell membrane and the trans-Golgi network(87). It binds both stimulated and unstimulated EGFR, leading to its internalisation(87). This eventually leads to EGFR expulsion via exosomes or receptor degradation and hence signal termination(87). Al-Akhrass et al found that NTSR3 is a favourable prognostic marker in lung adenocarcinoma patients(93). His team also demonstrated that sortilin expression decreased significantly with pathologic grade of NSCLCs (grades I-III) and was associated with poor prognosis(93).

Currently, only EGFR-mutated tumours are eligible to receive EGFR tyrosine kinase inhibitors, which represent only 10 percent of all lung adenocarcinomas(87). Preclinical trials of erlotinib showed good response in xenografted models harbouring NT/NTSR1 but no effect if NT/NTSR1 were absent(87). A phase II clinical trial of Afatinib (a selective irreversible erbb family blocker) used as a third- or further-line treatment in patients with stage IV bronchial adenocarcinoma which harbor wild-type EGFR and express the Neurotensin-neurotensin receptor complex (THEN), has been withdrawn due to 'medical reasons' which are not further explained(87).

Pancreatic cancer

Pancreatic cancer is an extremely aggressive cancer with poor prognosis, and this is due to the late stage of diagnosis(87). NT has been found to increase pancreatic secretions among other physiological functions. NT and its receptors play a role in the pathological growth of

cancer cells, including human pancreatic cancer cells, as shown in in-vitro studies(87). Addition of NT to pancreatic cell lines stimulates DNA synthesis via protein kinase (ERK-1 and ERK-2) activation(87). The combination of NT and epidermal growth factor (EGF) in PANC-1 and Mia PACA-2 cells result in a prolonged duration of ERK pathway activation leading to DNA synthesis(87). Both NT and EGF induce extracellular acidification and intracellular alkalinization through the Na/H exchanger1 (NHE1) leading to interleukin-8 (IL-8) synthesis and promoting glycolysis leading to increased tumor cell invasion(87, 94). Furthermore, NT/NTSR3 complex modifies the expression of α V and β 5 integrin subunits in PDACs cultured on vitronectin. This results in a reduced level of migration of collectively migrating PDACs cultured on vitronectin. This results in a reduced level of migration of collectively migrating PDACs via the PI3 kinase pathway, whilst increases the migration potential of individually migrating PDACs via the EGFR/ERK pathway(87).

Pancreatic ductal adenocarcinoma overexpresses NTSR1 and NT, whilst normal pancreatic tissue does not(87). The intensity of NTSR1 increases with the stage of pancreatic cancer and metastases express NTSR1 at the same level as the primary tumour(87). Preliminary studies showed that NTSR1 is a promising target for therapeutic and diagnostic purposes(87). An NTSR1 antagonist, ^{77}Lu -3BP-227, used as an intravenous agent to assess uptake and then one of the patients proceeded to have 3 further intraperitoneal doses(95). This resulted in partial remission in one patient with improved quality of life and 11-month survival despite a very poor prognosis. There is currently an ongoing phase I and II trial, in evaluating ^{77}Lu -3BP-227's safety and efficacy in various cancers including pancreatic cancer(95).

Breast cancer

NT and NTSR1 have been implicated in breast cancer progression and high levels of pro NT in the blood is linked to a higher risk of breast cancer. The NT/NTSR1 complex enhances tumour growth and metastasis(87).

High NTSR1 in patients with IDCs is correlated with larger tumour size, Scarff-Bloom-Richardson (SBR) histoprognostic grade 3 and number of positive lymph nodes(87). These patients also have worse survival compared to those with low NTSR1 expression (10-year survival rate of 66.2 percent versus 96.5 percent, $p = 0.01$)(87).

The NT/NTSR1 complex enhances tumour aggressiveness by increasing human epidermal growth factor receptor (HER) expression and activation via the release of specific EGFR, HER2 and HER3 ligands(87). Use of lapatinib, an EGFR/HER2 tyrosine kinase inhibitor, and metformin reduced the growth of cells overexpressing NT/NTSR1 in an experimental mouse model, suggesting NT/NTSR1 as a potential therapeutic target(87).

Prostate cancer

Androgen treatment provides a temporary therapeutic response in prostate cancer, as eventually, a more aggressive and androgen-independent tumour tends to develop after initial treatment(87).

The NT receptor pathway has been particularly implicated as an alternative growth pathway, especially in the absence of androgens(87). NT, binding to NTSR1 causes prostatic cancer cell mitosis through Src-, MMP- and PKC-dependent transactivation of EGFR, which ultimately stimulate the MAP-kinase pathway in a PI₃K-dependent manner(87). NT and its

receptors have been implicated in the neuroendocrine differentiation of prostate cancers(87). Moreover, using the NTSR1 antagonist, SR48692, inhibits Neurotensin-mediated prostatic growth(96, 97).

So far, NTSR expression has not been correlated with T stage of disease, Gleason score but there is evidence that there is more frequent NTSR1 overexpression in metastatic lymph nodes compared to primary tumours(98). The authors of this study concluded that this may open a new perspective in imaging and radionuclide therapy in prostate cancer.

Head and neck tumours

A study by Shimizu and colleagues in 2008 identified high mRNA expression levels of NT and NTSR1 in head and neck squamous cell carcinomas (HNSCC) were associated with worse survival rates(99). In HNSCC cells, NT promoted invasion and migrations, whilst NTSR1 knockdown had the opposite effect(99).

Pleural mesothelioma

Malignant pleural mesothelioma (MPM) studies show that inhibition of NT reduces cell migration and collagen invasion of mesothelioma cells. Thirty percent and 77 percent of normal pleural express NT and NTSR1 respectively. In MPM, this percentage rises to 71.1 and 90.4 percent for NT and NTSR1(100). High expression of NT is associated with a poor prognosis(100).

Glioma

Gliomas comprise 80 percent of all malignant brain tumours and despite medical advances in surgery, radiotherapy and chemotherapy, their high recurrence rate accounts for the high mortality for these patients(101).

NT is known to be present in the CNS and is highly expressed in the hypothalamus, median eminence, pituitary stalk, substantia nigra, locus coeruleus, raphe nuclei and brainstem structures(87). Both NT and NTSR1 expression is correlated with increasing glioma tumour grade. In-vivo experiments using xenografted mice models with NTSR1 blockade significantly prolonged survival(101). Glioblastoma multiforme, which is the most aggressive form of glioma, has the highest expression of NT and NTSR1(87). Although the exact mechanism of NT in GBM is not well understood, several in-vitro studies showed that NT acts on glioblastoma stem cells (GSC) via an IL-8/CXCL8 mechanism(102). NT also seems to have an important role in protecting glioblastoma from intrinsic apoptosis via c-Myc/LIN28/Let-71-3p/Bcl-w axis(103). NT increases tumour sphere formation and regulates stem-like traits via an EGFR-dependent increase of IL-8 secretion(87, 102).

Liver cancer

NT has been reported to promote cytokine release, particularly IL-8 via the MAPK and NF- κ B pathways, enhancing the migration and invasion capacity of HCC cells(87). Overexpression of both interleukin-8 and NT in HCC has been associated with portal vein invasion and worse prognosis(87). Furthermore, co-expression of NT and NTSR1, found in 50 percent of HCC, has also been found to be correlated with poor prognosis and alteration of the Wnt/ β -catenin pathway(87).

Fibrolamellar hepatocellular carcinoma (FL-HCCs) is a rare form of HCC which tends to happen in teenagers and young adults, without underlying liver disease(87). The underlying pathogenesis is due to an overexpression of the fusion protein DNA-PKAc and the PKAc kinase activity which depends on cyclin AMP (cAMP)(87). NT and its receptors, NTSR1 and NTSR2, are a potential source of cAMP which in turn increases PKA activity(87). In-vitro studies shows that NT and NTSR1 act as co-mitogens on EGF signalling by promoting EGFR phosphorylation. Plasma neurotensin is used clinically to monitor disease and detect recurrence

1.4.3 NEUROTENSIN AND NTSR RECEPTORS IN COLORECTAL CANCER

Neurotensin and its receptors as described above has been investigated in a wide variety of cancers. Although neurotensin has physiological actions, its main receptor, NTSR1 is not normally expressed on normal colonic epithelium. However, 40 percent of human colon cancer cell lines express NT receptors(104). In human colon cancer cell lines, addition of NT resulted in intracellular calcium mobilisation which leads to activation of the MAPK/ERK signalling pathway(104). Depending on the cell line investigated, the NT/NTSR1 signalling involves multiple signalling pathways which lead to cancer cell growth and proliferation(104).

Most studies investigated the NT/NTSR1 signalling pathway but the role of the NTSR3 is less clearly defined. NTSR1 and NTSR3 which exist as a heterodimer on HT29 colon cancer cell surface become internalised upon activation to exert its mitogenic effects (105). Blocking NTSR signalling in xenografted animal models with human colon cancer cell lines, slowed growth and aggressiveness of the cancer(104, 106, 107).

Studies looking at NTSR1 expression in patients with adenomas, inflammatory bowel conditions and adenocarcinomas, showed an increased percentage of NTSR1-positive epithelial cells in the adenocarcinomas compared to the inflammatory conditions and adenomas(108, 109).

Section 1.4.4 will discuss our research group's work so far which lead to this thesis' hypothesis.

1.4.4 SUMMARY OF WORK ON NEUROTENSIN PRIOR TO THIS STUDY AND RATIONALE FOR THESIS

An initial pilot study of 56 patients and 15 healthy controls had plasma neurotensin and IL-8 measured using enzyme-linked immunosorbent assay(110). The value of plasma neurotensin ≤ 54.47 pg/ml had a 77 percent sensitivity and 90 percent specificity in predicting CRC(110).

In 2015, a prospective study to look at the role of NT as a novel biomarker in the endoscopic screening of high-risk population for developing colorectal neoplasia was conducted(111). Twenty-six patients in total, of which 12 were control and 14 had a colonic pathology (13 had adenomatous polyps with high grade dysplasia, 1 had adenocarcinoma) were recruited. Pathology in the colon was associated with a 3.7-fold increase in NT levels. The value of 12.93pg/ml was associated with 87.5 percent sensitivity and 91.7 percent specificity for discriminating colonic pathology from normal colons(111).

Subsequently to this study, a larger prospective study with 165 participants was conducted to investigate neurotensin's diagnostic capabilities. Of these participants, 46 had polyps or CRC. Patients with colonic neoplasia had significantly higher NT levels compared to patients without neoplasia(112). Plasma NT had a sensitivity of 60.4 percent and specificity of 71.6 percent for diagnosing CRC and polyps(112).

This was then followed up with characterisation of NT and NTSR1 and 3 expressions in human CRC(113). A prospectively collected cohort of 64 patients had immunostaining of the above markers and there was significantly higher expression of NT in cancer tissue with higher T stage ($p < 0.01$), N stage ($p = 0.03$) and AJCC clinical stage ($p = 0.04$)(113). There was also significantly higher expression of NT, NTSR1 and NTSR3 in cancer tissue compared to adjacent colonic epithelium(113). Finally, there was significantly shorter DFS in patients with CRC who had high levels of NT expression compared to those with lower levels (35.8 months vs 46.4 months, 95% CI 42.2-50.5 months $p = 0.02$)(113). Above median NT expression in cancer tissue was a significant risk factor for disease recurrence (HR 4.10, 95% CI 1.14-14.7, $p = 0.03$)(113).

1.5 HYPOTHESIS, FOCUS, AND AIMS OF THE THESIS

1.5.1 HYPOTHESIS

Neurotensin, NTSR1 and NTSR3 expression increases from benign polyps to increasing stages of colorectal cancer.

1.5.2 FOCUS

This thesis has 3 main areas of focus:

1. Assess NT, NTSR1 and NTSR3 expression on abnormal colorectal pathology (polyps and colorectal cancer) and correlation to stage of disease (AJCC stage I- IV).

2. Correlate between plasma NT expression and tissue expression of NT, NTSR1 and NTSR3.
3. Assess the diagnostic accuracy of plasma NT in distinguishing polyps and colorectal cancer from normal/benign colonic disease.

1.5.3 FOCUS I: Assess NT, NTSR1 and NTSR3 expression on abnormal colorectal pathology (polyps and colorectal cancer) and correlation to stage of disease (AJCC stage I- IV).

- Primary aim:
 - Qualitative measurement of NT, NTSR1 and NTSR3 expression on polyps and colorectal cancer.
 - Determine any difference in expression between polyps and CRC.
 - Determine if there is any difference in expression between different stages of colorectal cancer.
- Secondary aims:
 - To establish correlation if any between NT, NTSR1 and NTSR3 expression and patient BMI, smoking status and alcohol intake
 - To establish correlation, if any, between tissue NT, NTSR1 and NTSR3 with T stage, N stage, M stage, lymphovascular invasion, EMVI presence, differentiation of tumour, presence of mucin, CDX2 positivity and microsatellite instability.

1.5.4 FOCUS II: Correlate between plasma NT expression and tissue expression of NT, NTSR1 and NTSR3.

- Primary aim:
 - To determine whether there is, if any, correlation between plasma NT level and tissue expression of NT, NTSR1 and NTSR3.

- To establish whether the level of NT correlates with stage of disease.
- Secondary aims:
 - To establish whether there is, if any, correlation between plasma NT and patient's BMI, smoking and alcohol intake.
 - To establish correlation, if any, between plasma NT and T stage, N stage, M stage, lymphovascular invasion, EMVI presence, differentiation of tumour, presence of mucin, CDX2 positivity and microsatellite instability.

1.5.5 FOCUS III: *Assess the diagnostic accuracy of plasma NT in distinguishing polyps and colorectal cancer from normal/benign colonic disease.*

- Primary aim:
 - To establish the diagnostic accuracy of plasma NT for colonic polyps and colorectal cancer as compared to normal.

CHAPTER 2: MATERIALS AND METHODS

2.1. REGULATORY AND ETHICS COMMITTEE APPROVAL

The project was granted ethical approval from the Human Research Authority on the 8th of November 2017.

Study title: *Expression pattern and clinicopathological relevance of Neurotensin and its receptors in colorectal cancer*

REC reference: 17/EE/0379

IRAS Project ID: 223925

Data from plasma samples which were prospectively collected by one of the research group members for his PhD (Mr Shengyang Qiu, SQ) was also used.

The study title for this study was '*Evaluating the role of Neurotensin as a diagnostic marker for colonic polyps and colorectal cancers*'

REC reference: 16/SC/0523

IRAS project ID: 208209. His samples were collected between November 2016 and July 2017.

Additionally, I collected one hundred plasma samples from patients who fulfilled the inclusion criteria were identified and included in the analysis from the Imperial College Healthcare Trust Tissue bank (ICHTB, Human Tissue Authority Licence: 12275, Research Ethics Committee Approval:12/WA/0196, Tissue Bank Application number R16007). These were also stored in -80°C freezers and were collected between February 2015 and January of 2017. SQ performed ELISA on 95 of these patients as the rest only had haemolysed samples or inadequate volume for analysis.

2.1.1 STUDY DESIGN

2.1.2 ENDPOINTS

Primary endpoint

- Measure the expression of Neurotensin, Neurotensin Receptors 1 and 3 on abnormal pathology (polyps and CRC) and quantify level of expression at different stages of invasion.

Secondary endpoints

- Correlate between plasma NT expression and tissue expression of NT, NTSR1 and NTSR3.
- Assess the diagnostic accuracy of plasma NT in distinguishing polyps and colorectal cancer from normal/benign colonic disease.

2.1.3 INCLUSION AND EXCLUSION CRITERIA

Inclusion criteria

- Patients aged 18 year or older
- Referral for colonoscopy
- Written informed consent obtained
- New diagnosis of colorectal cancer

Exclusion criteria

- Medical or psychiatric conditions impairing ability to give informed consent
- Patients currently on cancer treatment
- Patients who are pregnant, lactating or undergoing fertility treatment
- Patients undergoing immunosuppressive treatment or treatment for a malignant tumour

- Patients with another malignant tumour

Withdrawal criteria

- Patients can withdraw from the study at any point they wish to

2.1.4 NUMBERS CALCULATION

Hypothesis:

Neurotensin, NTSR1 and NTSR3 expression increases from benign polyps to increasing stages of colorectal cancer.

- Study design:
 - Prospective cohort study
- Sample size and power calculation:
 - Given the lack of data of sensitivity of the NT, NTSR1 and NTSR3 markers on colonic tissue, an accurate power calculation cannot be achieved.
 - Given a 10 percent and 20 percent detection rate of cancer and polyps respectively, we will aim to include 30 patients with polyps and 80 patients with cancer.
- Sample collection:
 - Samples collected are anonymised and blinded to avoid bias during sample analysis.
 - Data unblinded after all samples are analysed.
- Clinical data collection:
 - Patient paper clinical record forms were kept in a secure folder in the study centre.

- The hospital number, date of birth, BMI, smoking status and alcohol intake were recorded.
- All the data was anonymised and given a sample ID which only the research team had access to trace back to the individual patient.
- Table 2.1 contains all the clinical data collected for each patient.

2.2 TECHNIQUES USED IN THIS STUDY

2.2.1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Enzyme-linked immunosorbent assay is a quantitative technique first described in 1971 which detects the presence of a ligand in a liquid sample through the use of antibodies(114).

Endpoint	Variable type	Options
Age	Continuous	
Gender	Categorical	1. Female 2. Male
BMI	Continuous	
Bowel prep	Categorical	1. Yes 2. No
Smoking status	Categorical	1. Smoker 2. Non-smoker 3. Ex-smoker
Alcohol intake	Categorical	1. None 2. Little (<10 units/ week) 3. Moderate (10-20 units/week) 4. Heavy (>20 units/ week)
Diagnosis	Categorical	1. Normal 2. Polyp 3. Cancer 4. Recurrence 5. Diverticular disease 6. Colitis
Site of disease	Categorical	1. Right colon 2. Left colon

		3. Rectum 4. Multisite 5. Not applicable
Differentiation	Categorical	1. Well-differentiated 2. Moderate 3. Poor 4. Not applicable
T stage (initial)	Categorical	1. T0/is 2. T1 3. T2 4. T3 5. T4 6. Normal 7. Other 8. Recurrence
T stage (final)	Categorical	1. T0/is 2. T1 3. T2 4. T3 5. T4 6. Normal 7. Other 8. Recurrence
AJCC stage (initial)	Categorical	1. Stage 0 2. Stage I 3. Stage II 4. Stage III 5. Stage IV 6. Normal 7. Other 8. Recurrence
AJCC stage (final)	Categorical	1. Stage 0 2. Stage I 3. Stage II 4. Stage III 5. Stage IV 6. Normal 7. Other 8. Recurrence
Lymph node involvement	Categorical	1. Yes 2. No 3. Not applicable
Metastases	Categorical	1. Yes 2. No
Presence of mucin	Categorical	1. Yes 2. No 3. Not applicable

EMVI presence	Categorical	1. Yes 2. No 3. Not applicable
Lymphatic invasion	Categorical	1. Yes 2. No 3. Not applicable
CDX2 positivity	Categorical	1. Positive 2. Negative 3. Not applicable 4. Not done
MMR competence	Categorical	1. Competent 2. Incompetent 3. Not applicable
Neoadjuvant CRT	Categorical	1. Yes 2. No 3. Incomplete or reduced regime 4. Not applicable

Table 2.1 Data endpoints collected for each patient. BMI= Body Mass Index, CDX2= Caudal-type homeobox 2, CRT= chemoradiotherapy

The principles of ELISA involve the use of chromogenic substrates that produce a change in colour which indicates the presence of antigen. It relies on antibodies to detect a target antigen using highly specific-antigen interactions. Usually the antigens investigated are peptides, proteins, antibodies and hormones.

A 96-well plate is used and the antigen (or antibody) is immobilised on the walls of the plate. An enzyme-conjugated antibody is then added and this binds to target molecule attached to the wall of the plate. Any unbound antigen (and antibody) is washed away. The most commonly used enzyme labels are horseradish peroxidase (HRP) or alkaline phosphatase, or avidin-biotin complex (ABC).

The bound enzyme-conjugated antibody then catalysed a colour change in a substrate. The amount of colour change is then detected using a plate reader. Then the antigen concentration in a sample is plotted on a standard curve using antigens of a known concentration. The sample antigen concentration is then calculated using the optical density.

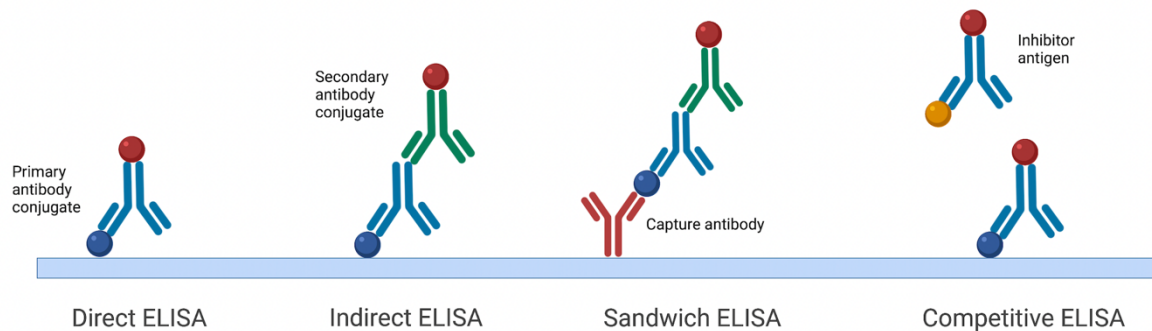


Figure 2.1 Summary of ELISA techniques

There are now many ELISA types of analysis and the main ones are: Direct ELISA, Sandwich ELISA, Competitive ELISA and Reverse ELISA (figure 2.1).

For this thesis, the Abbexa NTS ELISA kit (abx152510) was used as a competitive sandwich enzyme-linked immunosorbent assay (see appendix section). It was used for this thesis for 2 main reasons: the research team has used it previously and hence results could be compared and combined. Furthermore it has high sensitivity and specificity for human neurotensin in plasma, serum, and other biological fluids. The intra-assay precision was tested by the manufacturer for intra-assay precision by testing 3 samples with low, medium and high NT levels. The coefficient of variation (CV) was calculated by:

$$CV (\%) = (\text{Standard Deviation} / \text{Mean}) \times 100$$

The intra-assay CV was <10%, and the inter-assay CV was <12%.

Blood sample collection, preparation and storage

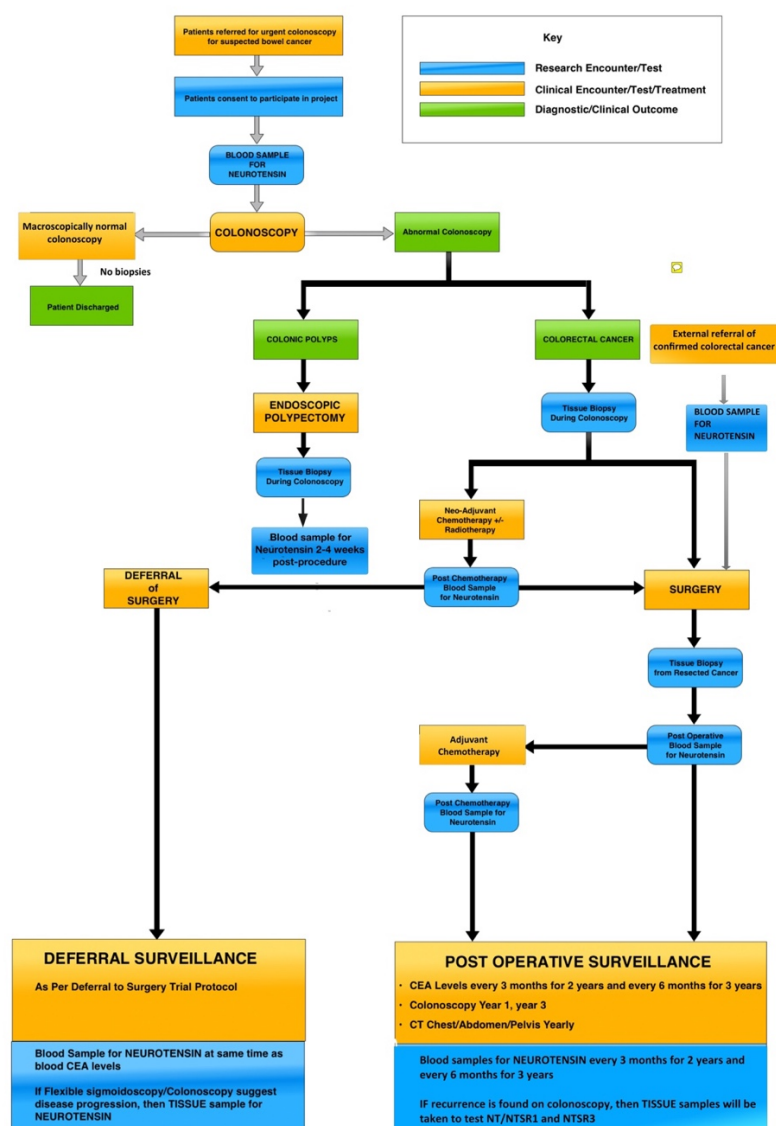


Figure 2.2 Study protocol for this thesis

After I consented the patients for participation in this study, I collected 4-8 mls of fasting peripheral venous blood sample prior to either the colonoscopy or surgery. The blood was collected in a standard 'purple'-top bottle containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (BD Vacutainer K2 EDTA 10ml tube). The time of collection and

fasting period prior to venepuncture were noted. The sample was taken to the laboratory and centrifuged for 10 minutes at 1500xg at 6°C within 30 minutes of collection. Samples collected from West Middlesex Hospital were kept in a cooler until they were processed and stored.

Once the sample was centrifuged, it was carefully removed and using a 3ml plastic pipette, the supernatant was removed from the top layer, without disturbing the blood cells settled at the bottom of the tube. The plasma cells were aliquoted into 1.8ml Cryotubes (Nunc Biobanking and Cell Culture Cryogenic Tubes, 1.8mL, Internal threaded, Catalogue number 377267, Thermo Fisher Scientific, Massachusetts, USA). Depending on the volume of blood collected from each patient, at least 2 cryotubes were filled. The cryotubes were then anonymised and labelled with the study ID, date collected. If the sample was haemolysed, this was noted in the sample log (only 1 sample haemolysed). The samples were then stored in an -80°C freezer which was alarm protected to ensure a constant temperature.

ELISA protocol

1. An antibody specific to NT was precoated onto the 96-well ELISA microplate by the manufacturer.
2. Standard solutions were made with known concentrations of NT using serial dilution purified NT with 0.01mol/L PBS solution (pH= 7.0-7.2). Standard solutions were made between 0 and 3000 pg/ml.
3. Fifty microlitres (µL) of the standard solutions and plasma samples were added into the wells of the microplate. Fifty microlitres (µL) of standard diluent buffer solution was placed into 2 wells as control well. Each standard and sample were assayed twice in 2 adjacent wells to increase the consistency and accuracy of measurements.

4. Fifty μL of biotin labelled NT were immediately aliquoted into each well to set up the competitive inhibition reaction between this and the unbound NT in the study samples. Care was taken that the pipette tips did not touch the solution in the wells.
5. The plate was then sealed and was allowed to incubate for 1 hour at 37°C . A competitive inhibition reaction was launched between biotin and labelled NT and unlabelled NT with the precoated antibody specific to NT.
6. After incubation, the solution was discarded and thoroughly washed with wash buffer ($350\ \mu\text{L}$ per well) to remove the unbound conjugates.
7. One hundred μL of Avidin conjugated to HRP was added to each well and incubated for 30 minutes at 37°C .
8. The solution was then discarded, and unbound conjugates were washed away.
9. Ninety μL of TMB substrate solution were added to each well, and the plate was sealed in an opaque bag and incubated for 15 minutes at 37°C . The well which contained bound NT produced a blue colour product.
10. The stop solution was then added, which stopped the reaction and changed the solution into a yellow colour. The intensity of yellow was proportional to the NT amount of sample captured in each well.
11. The plate was then placed in a microplate reader (BioTek ELX800 Absorbance Reader, BioTek Instruments, Inc, Vermont, USA) and the optical density (OD) was measured spectrophotometrically at 450nm . The data was automatically exported as a Microsoft excel spreadsheet.

The protocol and antibody used was consistently the same in our research group, hence we could combine the results of the previous PhD student (Mr Shengyang Qiu) for the sensitivity of NT in distinguishing cancer from non-cancer.

I performed the collection, storage and analysis of all the blood samples in this thesis.

2.2.3 IMMUNOHISTOCHEMISTRY

Since immunohistochemistry's introduction in 1942, it has now become an essential tool in diagnostic and research laboratories. Once the IHC slides have been prepared, they can be viewed by either light or fluorescence microscopy.

Tissue collection, sample processing and preparation

On the day of surgery or colonoscopy, patients were consented prior to retrieval of cancer or polyp specimens. The specimens were fixed immediately in 10% formalin solution and transferred to the histopathology laboratory. Selected tissue samples were further examined by the histopathologist and were infiltrated with paraffin wax following standard protocols and then embedded in paraffin blocks.

Polyp and colorectal cancer tissue specimens were fixed immediately in 10% (volume to volume) neutral buffered formalin at room temperature (RT) for at least 24 hours. Within 72 hours, the specimens were processed into paraffin blocks using SAKURA Tissue-Tek® VIPTM Vacuum Infiltration Processor (Sakura Finetechnical Co. Ltd, Tokyo 103, Japan). One paraffin-embedded tumour block from each subject was cut into 5µm-thick paraffin sections and mounted on polysine coated slides. Prior to staining, one haematoxylin and eosin slide from each subject was assessed for morphological structures.

Immunohistochemical staining protocol

1. The sections were deparaffinized, hydrated and then heat mediated antigen retrieval was performed in EDTA based pH 9.0 solution for 30 minutes for NT and NTSR1 and 20 minutes for NTSR3.
2. The endogenous peroxidase was quenched with 3% hydrogen peroxide. Sections were incubated with rabbit polyclonal anti-Neurotensin antibody (1:100 dilution, Abcam, ab233107), rabbit polyclonal anti-Neurotensin Receptor1 antibody (1:400 dilution, Abcam, ab117592), rabbit polyclonal anti-Neurotensin Receptor3 (Sortilin1) antibody (1:200 dilution, GeneTex, GTX110857) and subsequently incubated with anti rabbit IgG conjugated with polymeric horseradish peroxidase linker (Leica Bond Polymer Refine Detection, DS9800).
3. DAB (LEICA BOND polymer refine detection), was used as the chromogen for 10 minutes and the sections were then counterstained with hematoxylin and mounted with DPX mounting medium.
4. Positive control tissues: Human liver for NT IHC, human CRC for NTSR1 IHC and human endometrium for NTSR3

Immunostaining quantification

Immunostaining scoring can be broadly divided into qualitative, semi-quantitative and quantitative.

Qualitative scoring is when the strength of staining is subjectively assessed by a trained histopathologist. The strength would vary from no staining (0) to strong staining (+++). If the strength is assigned a numeric value, then this becomes semi-quantitative scoring.

By using image analysis, for example using Image J software, the signal from the region of interest can be measured resulting in a more quantitative analysis. This can be challenging as background noise, uneven illumination can exaggerate the signal intensity. Use of

immunofluorescence, is an alternative but again the intensity will vary depending on the distance of the target, degree of staining, and excitation intensity which can obscure results.

Combinative semiquantitative scoring systems convert subjective perception of a certain marker's expression on tissue into quantitative data which can be used in statistical analysis. To reduce subjectivity, it is recommended to have more than one observer in the study(115). Three examples of combinative semiquantitative scoring systems are Allred score, immunoreactive score (IRS) and H-score.

The Allred score combines the percentage of positive cells and the intensity of the reaction in most examined fields(115). The two scores are then added together to a possible 8 values: 0-2 are considered as negative, 3-8 are considered positive (table 2.1).

Proportion score A	Positive cells, %	Intensity	Intensity score B
0	0	None	0
1	<1	Weak	1
2	1 to 10	Intermediate	2
3	11 to 33	Strong	3
4	34 to 66		
5	>= 67		
Final score range (A+B): 0-8			

Table 2.2 Allred scoring system

The immunoreactive score (IRS) has a score from 0 to 12, which results from multiplying positive cells proportion (0-4) and staining score (0-3) (table 2.2).

A (% of positive cells)	B (intensity of staining)	IRS score (multiplication of A and B): 0-12
0= no positive cells	0= no colour reaction	0-1= negative
1= <10% of positive cells	1= mild reaction	2-3 = mild
2= 10-50% of positive cells	2= moderate reaction	4-8= moderate
3= 51-80% of positive cells	3= intense reaction	9-12= strongly positive
4= >80% of positive cells		

Table 2.3: Immunoreactive score (IRS)

The H-score is determined by sum of the results of multiplication of the percentage of cells with staining intensity (score 0 to 3)

All three scores are designed to score cellular staining and are not useful for extracellular staining(115).

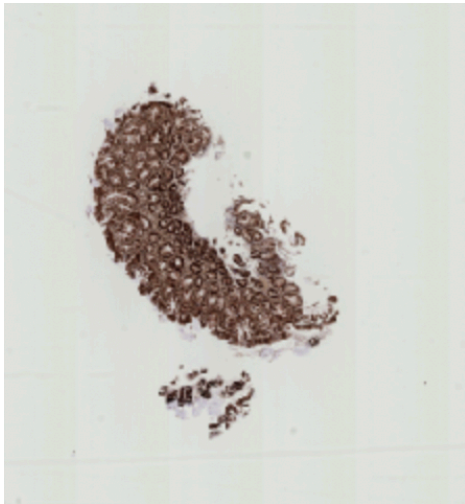
For Histoscore (H-score) assessment, 10 fields were chosen at random at x200 magnification and the staining intensity in the malignant cells was scored as 0, 1, 2 or 3 corresponding to negative, weak, intermediate and strong intensity, respectively. The total number of cells in each field and the percentage of positive cells in each field was counted. The **H score** was calculated using the following formula:

$$= [1 \times (\% \text{ of cells stained at } 1+) + 2 \times (\% \text{ of cells stained at } 2+) + 3 \times (\% \text{ of cells stained at } 3+)]$$

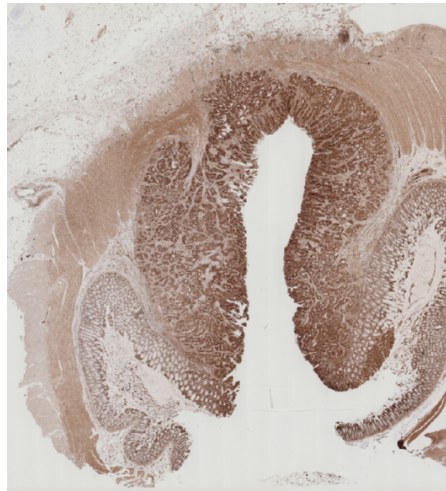
All samples were reviewed and scored by an experienced histopathologist who was blinded to background of each patient and hypothesis. SN was present during the scoring and was trained to identify cancer and polyp epithelial cells. SN then individually scored the same

samples (from scanned images using Fiji software) and was blinded to the patient background and to the scoring of the experienced histopathologist. The scores were subsequently checked and there was 90 percent interobserver agreement.

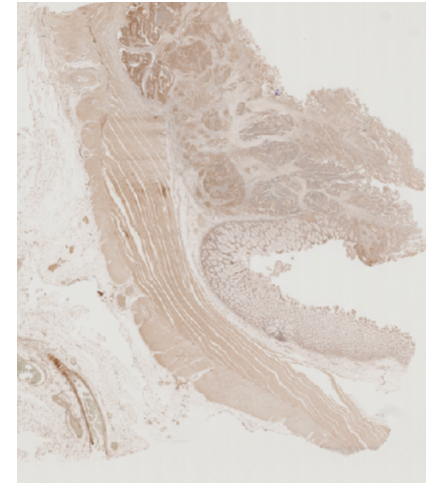
Scores used were as described above from 0 to 3+. Figure 2.3 shows the different types of straining strength.



A. Hyperplastic polyp 3+ NT strength



B. Rectal cancer with 2+ NT strength



C. Rectosigmoid cancer with 1+ NTSR1 strength

Figure 2.3 Histology slides demonstrating the strength of staining.

2.3 STATISTICAL ANALYSIS

All statistical analyses were carried out using SPSS 27 (IBM, USA). Prior to each step, the data was explored to determine their distribution which determined the type of analysis used (i.e. parametric versus non-parametric data). Missing values were denoted with the number -99 and were noted.

Table 2.4 below details the tests used for the analysis.

Objective	Statistical test/Method of analysis
Semi-qualitative measurement of NT, NTSR1 and NTSR3 expression on polyps and colorectal cancer	Histascor (H-score) = $1 \times (\% \text{ of cells } 1+) + 2 \times (\% \text{ of cells } 2+) + 3 \times (\% \text{ of cells } 3+)$
Determine any difference in NT, NTSR1 and NTSR3 expression between polyps and CRC	Kruskal Wallis and Mann Whitney tests (non-parametric data)
Determine any difference between tissue NT, NTSR1 and NTSR3 expression between different T stages (initial and final T stage)	Kruskal Wallis and Mann Whitney U tests (non-parametric data)
Determine if there is any difference in expression between different AJCC stages of colorectal cancer	Kruskal Wallis and Mann Whitney U tests (non-parametric data)
To establish correlation if any between NT, NTSR1 and NTSR3 expression and patient BMI, smoking status and alcohol intake	Kruskal Wallis and Mann Whitney U tests (non-parametric data)
To establish correlation, if any, between tissue NT, NTSR1 and NTSR3 with T stage, N stage, M stage, lymphovascular invasion, EMVI presence, differentiation of tumour, presence of mucin, CDX2 positivity and microsatellite instability, site of cancer	Spearman's Rank Correlation Kruskal Wallis and Mann Whitney U tests

To determine whether there is, if any, correlation between plasma NT level and tissue expression of NT, NTSR1 and NTSR3	Spearman's Rank Correlation
To establish whether the level of plasma NT correlates with stage of disease	Kruskal Wallis test
To establish whether there is, if any, correlation between plasma NT and patient's BMI, smoking and alcohol intake	Kruskal Wallis test
To establish correlation, if any, between serum NT and T stage, N stage, M stage, lymphovascular invasion, EMVI presence, differentiation of tumour, presence of mucin, CDX2 positivity and microsatellite instability	Kruskal Wallis test
To establish the diagnostic accuracy of plasma NT for colonic polyps and colorectal cancer as compared to normal	ROC curve analysis Optimal cut-off for best combined sensitivity and specificity determined by the Youden Index

Table 2.4 Objectives of the thesis and the statistical tests used for each objective.

**CHAPTER 3: CHAPTER 3: RESULTS - TISSUE NT AND NTSR 1 AND 3 EXPRESSION IN
COLORECTAL CANCER AND POLYPS**

3.1 TISSUE NT, NTSR1 AND NTSR3 EXPRESSION

3.1.1 PATIENT COHORT DEMOGRAPHIC AND HISTOPATHOLOGICAL CHARACTERISTICS

One hundred and six patients were prospectively recruited from the Royal Marsden Hospital and Chelsea and Westminster NHS Foundation Trust between December 2017 and June 2019. Both, plasma neurotensin and tissue samples were analysed from all participants after appropriate consent was obtained. Two of the patients were excluded because histology showed lymphoma and lipoma respectively. The total number of patients included in this study is 104.

The patient demographics for all the recruited participants are described in table 3.1.

Out of the 104 patients included in the study: 78 had colorectal adenocarcinoma (21 right-sided colon cancer, 11 left-sided colon cancer, 45 rectal cancers and 1 synchronous right and left colonic cancer), 17 had polyps (5 hyperplastic, 11 adenomatous with low grade dysplasia and 2 adenomatous with high grade dysplasia), 4 had recurrence of colorectal cancer and 5 patients had normal colons. Table 3.2 summarises the diagnoses and table 3.3 to 3.5 summarise the demographic, histopathological and treatment characteristics of patients with cancer and recurrence, polyps and normal colonoscopies.

Figure 3.1 demonstrates the plasma NT, NT H-score, NTSR1 H-score and NTSR3 H-score distribution. The median plasma NT concentration for all the participants was 1732.7 pg/mL (IQR: 1180.9 – 2598.6 pg/mL). The mean was 2044.3 pg/mL (standard deviation: 1276.5

pg/ml). Plasma NT was not normally distributed, and the data was positively skewed (Kolmogorov-Smirnov $p < 0.01$). The median NT H-score was 200 (IQR:199.6 – 243.6). The mean NT H-score was 221.6 (Standard deviation 103). The median NTSR1 H-score was 160

	Median	IQR	Mean	SD
Age (years)	66	53 – 74.7500	64.2019	14.55981
BMI	25.7877	23.0044 – 28.0700	25.7345	4.15692
	Category	Frequency (n)	Frequency %	
Gender	Female	42	40.4	
	Male	62	59.6	
Smoking status	Smoker	7	6.7	
	Ex-smoker	35	33.7	
	Non-smoker	61	58.7	
	Unknown	1	1.0	
Alcohol intake	None	30	28.8	
	Little (<10 units/week)	46	44.2	
	Moderate (10-20 units/week)	10	9.6	
	Heavy (>20 units/week)	14	13.5	
	Unknown	4	3.8	
Bowel preparation	Yes	84	80.8	
	No	12	11.5	
	Unknown	8	7.7	

Table 3.1 Prospective cohort demographics. BMI= Body Mass Index

Diagnosis	Frequency (n)	Percentage (%)
Normal	5	4.8
Hyperplastic polyp	3	2.9
Adenomatous polyp with LGD	11	10.6
Adenomatous polyp with HGD	2	1.9
Sessile serrated polyp	1	1.0
Cancer	78	75
Recurrence	4	3.8
Total	104	100.0

Table 3.2 Summary of diagnoses

(IQR: 115.4 – 157.4) and the mean was 136.4. Finally, the median NTSR3 H-score was 200 (IQR: 168.0 – 204.6) and mean was 186.3. All H-scores were not normally distributed and the data were positively skewed (Kolmogorov-Smirnov $p < 0.01$).

	Frequency (n)	Percentage (%)
Age	Median 61 years (IQR 58.8 – 65.2) Mean 62.0 (S.D. 14.4)	
Gender	29 Female 53 Male	35.4 64.6
BMI	Median 25.6 (IQR 24.7 – 26.6) Mean 25.7 (S.D. 4.1)	
Bowel prep	65 Yes 10 No 7 Missing	79.3 12.2 8.5
Smoking status	3 Smokers 54 Non-smokers 23 Ex-smokers 1 Missing	4.9 65.9 28 1.2
Alcohol intake	27 None 33 Little (<10 units/week) 7 Moderate (10-20 units/week) 11 Heavy (>20 units/week) 4 Missing	32.9 40.2 8.5 13.4 4.9
Diagnosis	78 Cancer 4 Recurrence	95.1 4.9
Site of disease	21 Right colon 11 Left colon 46 Rectum 3 Recurrence 1 Synchronous (Right and left colon)	25.6 13.4 56.1 3.7 1.2
Differentiation	66 Moderate 11 Poor 3 Not applicable	80.5 13.4 3.7

	2 Missing	2.4
T stage (initial)	1 T0 6 T1 13 T2 36 T3 17 T4 2 Recurrences	1.2 7.3 15.9 43.9 20.7 2.4
T stage (final)	5 T0 2 T1 10 T2 44 T3 15 T4 2 Recurrences 3 Not mentioned	6.1 2.4 12.2 53.7 18.3 2.4 4.8
AJCC stage (initial)	1 Stage 0 11 Stage I 13 Stage II 40 Stage III 12 Stage IV 1 Recurrence 4 Missing	1.2 13.4 15.9 48.8 14.6 1.2 4.9
AJCC stage (final)	5 Stage 0 9 Stage I 33 Stage II 21 Stage III 13 Stage IV 1 Recurrence	6.1 11 40.2 25.6 15.9 1.2
Lymph node involvement	26 Yes 50 No 6 Missing	31.7 61 7.3
Metastases	13 Yes 69 No	15.9 84.1
Presence of mucin	21 Yes 60 No	25.6 73.2
EMVI presence	26 Yes 51 No 1 Not applicable 4 Missing	31.7 62.2 1.2 4.9
Lymphatic invasion	16 Yes 60 No 1 Not applicable 5 Missing	19.5 73.2 1.2 6.1
CDX2 positivity	16 Yes 60 No 1 Not applicable 5 Missing	19.5 73.2 1.2 6.1

MMR competence	57 Competent	69.5
	11 Incompetent	13.4
	1 Not applicable	1.2
	13 Missing	15.9
Neoadjuvant CRT	22 Yes	26.8
	18 No	22
	7 Incomplete or reduced regimen	8.5
	5 Missing	6.1

Table 3.3 Cancer and recurrent cancer patient cohort demographic, histopathological and treatment characteristics. AJCC staging= American Joint Committee on Cancer staging, CRT= Chemoradiotherapy, EMVI= extramural vascular invasion, MMR= Mismatch repair system

	Frequency (n)	Percentage (%)
Age	Median 74 years (IQR 66.4 – 79.1) Mean 72.8 years (S.D. 12.4)	
Gender	10 Female 7 Male	58.8 41.2
BMI (kg/m²)	Median 26.4 (IQR 23.7 – 28.4) Mean 26.0 (S.D. 4.6)	
Bowel prep	14 Yes 2 No 1 Missing	87.5 12.5 5.9
Smoking status	3 Smoker 5 Non-smoker 9 Ex-smoker	17.6 29.4 52.9
Alcohol intake	3 None 10 Little (<10 units/week) 3 Moderate (10-20 units/week) 1 Heavy (>20 units/week)	17.6 58.8 17.6 5.9
Diagnosis	3 Hyperplastic polyps 11 Adenoma with LGD 2 Adenoma with HGD 1 Sessile serrated polyp	17.6 64.7 11.8 5.9
Site of disease	4 Right colon 2 Left colon 9 Rectum 2 Multisite	23.5 11.8 52.9 11.8

Table 3.4 Polyp patient cohort demographic and histopathological characteristics. BMI=

Body mass index, HGD= high grade dysplasia, LGD= low grade dysplasia

	Frequency (n)	Percentage (%)
Age (years)	Median 74 (IQR 57.0 – 86.2) Mean 71.6 (S.D. 11.8)	
Gender	3 Female 2 Male	60 40
BMI (kg/m²)	Median 24.7 (IQR 18.6 – 32.0) Mean 25.4 (S.D. 4.2)	
Bowel prep	5 Yes 0 No	100
Smoking status	0 Smokers 2 Non-smokers 3 Ex-smokers	0 40 60
Alcohol intake	0 None 3 Little (<10 units/week) 2 Heavy (>20 units/week)	0 60 40

Table 3.5 Patient demographic characteristics who had normal colonoscopies

3.1.2 PATIENT COHORT HISTAScore (H-SCORE) TISSUE EXPRESSION

Out of the 104 patients included in the study, 5 patients had normal colonoscopies and the remaining 99 patients had colorectal cancer or polyp(s) or recurrent neoplastic disease.

From the 99 patients with colonic pathology, 1 had very advanced disease and only peritoneal fluid was obtained, whilst 6 had complete pathologic response so were not tested. Out of the remaining 92 patients, 87 of the patients recruited eventually had IHC for NT, NTSR1 and NTSR3. The NT, NTSR1 and NTSR3 H-scores were not normally distributed, and the data were positively skewed ($p < 0.01$, Kolmogorov-Smirnov test).

There was no significant difference between NT, NTSR1 and NTSR3 scores between male and female and status (see table 3.6). There was a significant difference between the NT H-

score in the alcohol intake cohort suggesting an effect of alcohol excess on the NT tissue expression but not its receptors.

The distribution of NT, NTSR1 and NTSR3 was also observed and there was no difference between cytoplasmic and cell membrane expression.

		Median NT H-score (IQR)	P value	Median NTSR1 H-score (IQR)	P value	Median NTSR3 H-score (IQRQ)	P value
Gender	F	200 (171.6 – 247.2)	0.39*	180 (111.7 – 188.6)	0.47*	200 (147.7 – 214.1)	0.87*
	M	200 (201.5 – 256.6)		140 (102.9 – 153.1)		200 (167.4 – 211.8)	
Smoking status	Smoker	200 (174.7 – 293.3)	0.90**	100 (-24.1 – 224.2)	0.59**	200 (178.4 – 205.6)	0.25 **
	Non-smoker	200 (185.8 – 244.8)		140 (103.0 – 164.8)		200 (146.6 – 198.7)	
	Ex-smoker	200 (189.5 – 270.5)		170 (113.1 – 174.8)		200 (173.3 – 232.3)	
Alcohol intake	None	300 (235.2 – 301.3)	0.02**	180 (113.0 – 208.3)	0.15**	200 (156.0 – 216.2)	0.23 **
	Little	200 (148.0 – 220.2)		100 (82.0 – 143.4)		200 (137.1 – 200.9)	
	Moderate	200 (168.7 – 342.4)		180 (141.1 – 207.8)		200 (198.9 – 279.0)	
	Heavy	200 (165.8 – 277.6)		85 (44.2 – 173.3)		200 (138.7 – 249.6)	

Table 3.6 Tissue H-score and demographic characteristics of the patient cohort. * Mann-Whitney U test; ** Kruskal Wallis test

3.1.2.1 SEMI-QUANTITATIVE MEASUREMENT OF NT, NTSR1 AND NTSR3 EXPRESSION ON POLYPS, COLORECTAL CANCER AND RECURRENT CANCERS

By comparing the expression of NT, NTSR1 and NTSR3 across the different diagnoses: there was a borderline significant increase in the NT H-score in cancer compared to polyps ($p=0.05$) and a significant difference across all the groups of diagnoses for NT and both its receptors (Kruskal Wallis analysis $p < 0.05$) (Figure 3.1). By comparing the NTSR1 and NTSR3 H-score between polyps and cancer, there was no significant difference in expression ($p= 0.468$ and $p = 0.463$ respectively, Kruskal Wallis analysis).

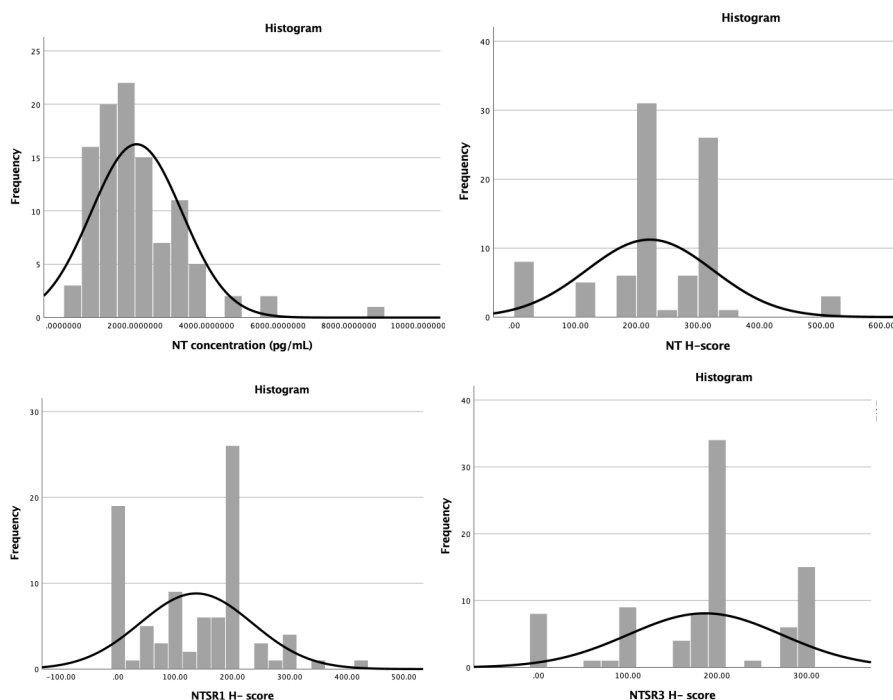


Figure 3.1 Histograms of plasma neurotensin, tissue Neurotensin H-score, tissue NTSR1 H-score and tissue NTSR3 H-score distribution.

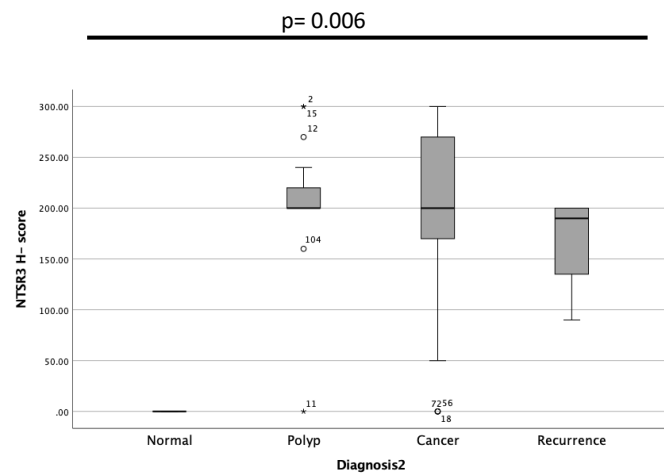
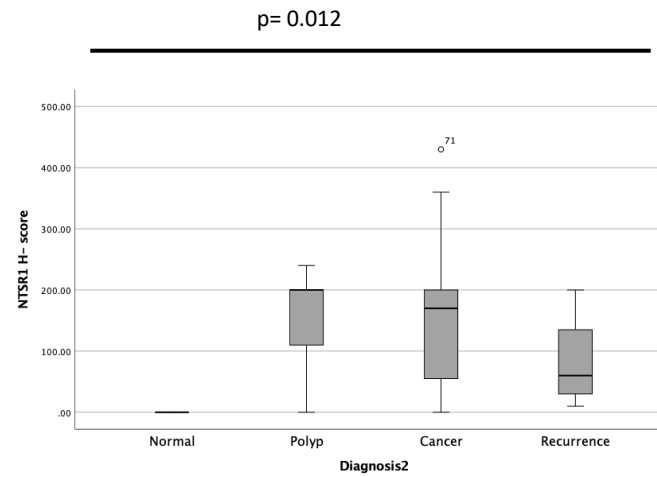
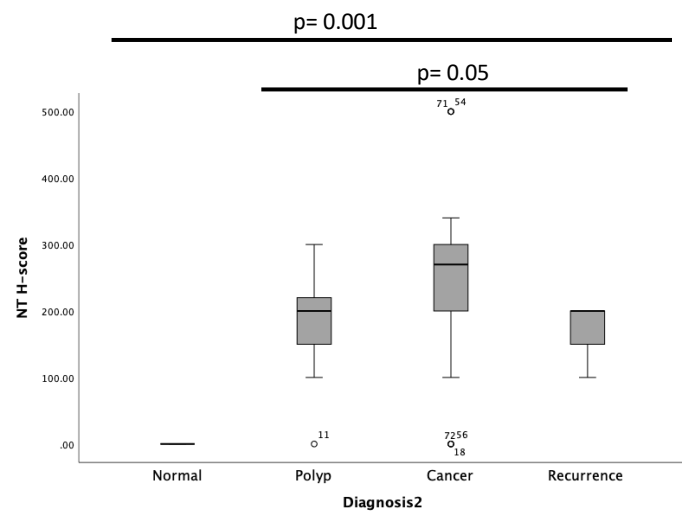


Figure 3.2 H-score for NT, NTSR1 and NTSR3 across the 4 types of diagnoses. Kruskal Wallis analysis.

3.2.2 CORRELATION BETWEEN NT, NTSR1 AND NTSR3 TISSUE EXPRESSION AND HISTOPATHOLOGICAL CHARACTERISTICS

The results comparing the NT, NTSR1 and NTSR3 H-score across initial and final stage of disease, initial and final T stage, lymph node involvement, presence of metastases, grade of differentiation, presence of mucin, extramural vascular invasion, lymphatic invasion, CDX2 positivity, and MMR status are summarised in figures 3.3 – 3.9. Initial stage implies stage prior to any treatment or surgery and final stage incorporates histology.

There was a significant difference in the NT H-score across the T1 to T4, recurrence and 'not applicable' group ($p=0.04$) but not if the analysis was made across T1 to T4 only ($p= 0.369$).

There was almost a significant difference across the T1 to T4 final stage, 'not applicable' and recurrent cases for the NT H-score with a p value of 0.05.

NT H-score was significantly different in patients in terms of lymph node involvement, AJCC initial and final stage disease (I-IV, normal and recurrent disease), presence of mucin, EMVI presence, lymphatic invasion, MMR incompetence and CDX2 positivity, although the latter only had 2 CDX2 negative specimens and 16 cancers were not tested at all.

NTSR1 H-score showed a significant difference across the AJCC initial and final stages (I-IV, normal and recurrent disease) and the neoadjuvant chemoradiotherapy treatment.

NTSR3 H-score showed a significant difference across the AJCC initial stages (I-IV, normal and recurrent disease) and presence of mucin.

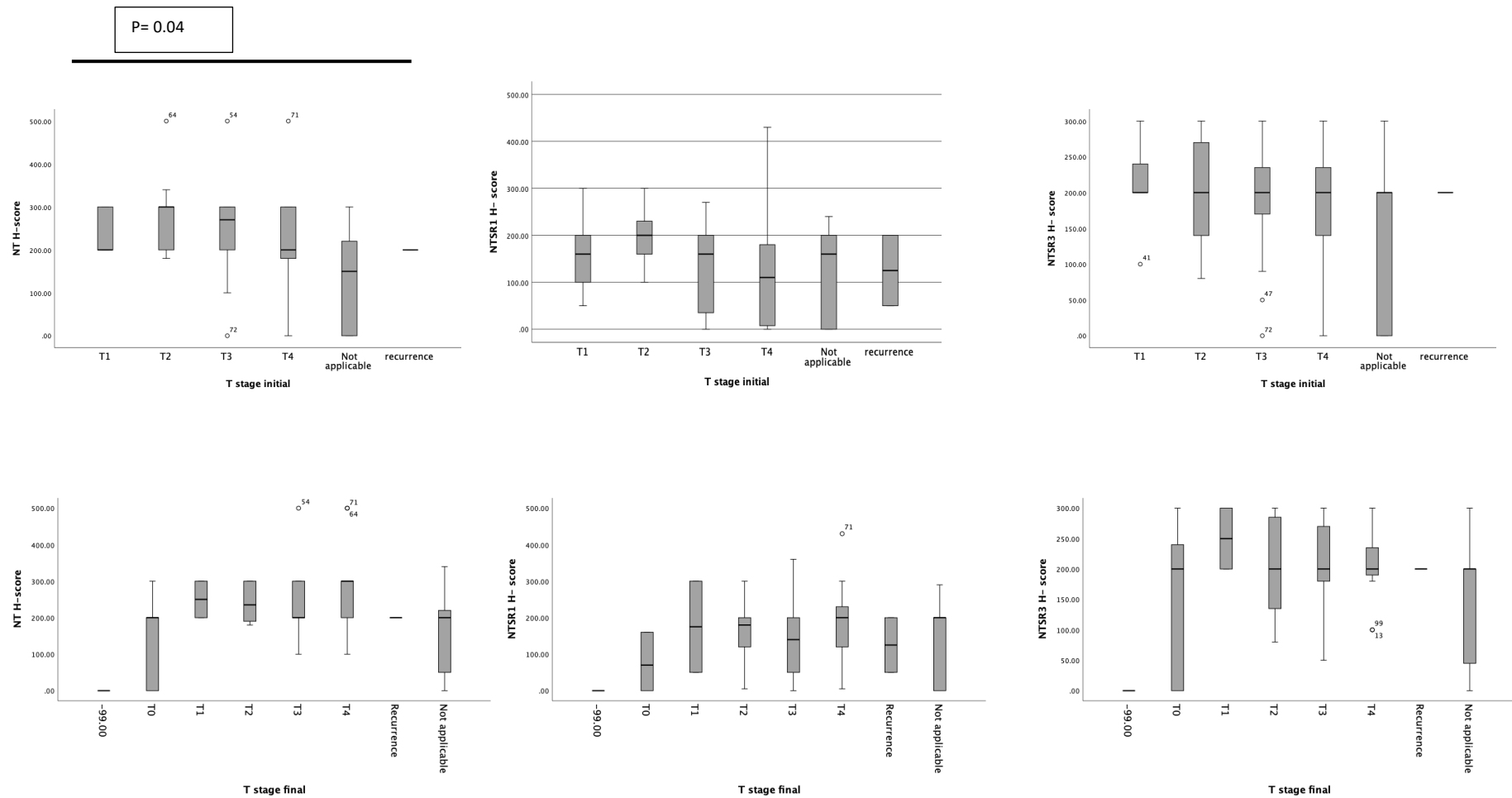


Figure 3.3: Tissue NT, NTSR1 and NTSR3 H-score across the initial and final T stages. Only significant p values noted on the chart.

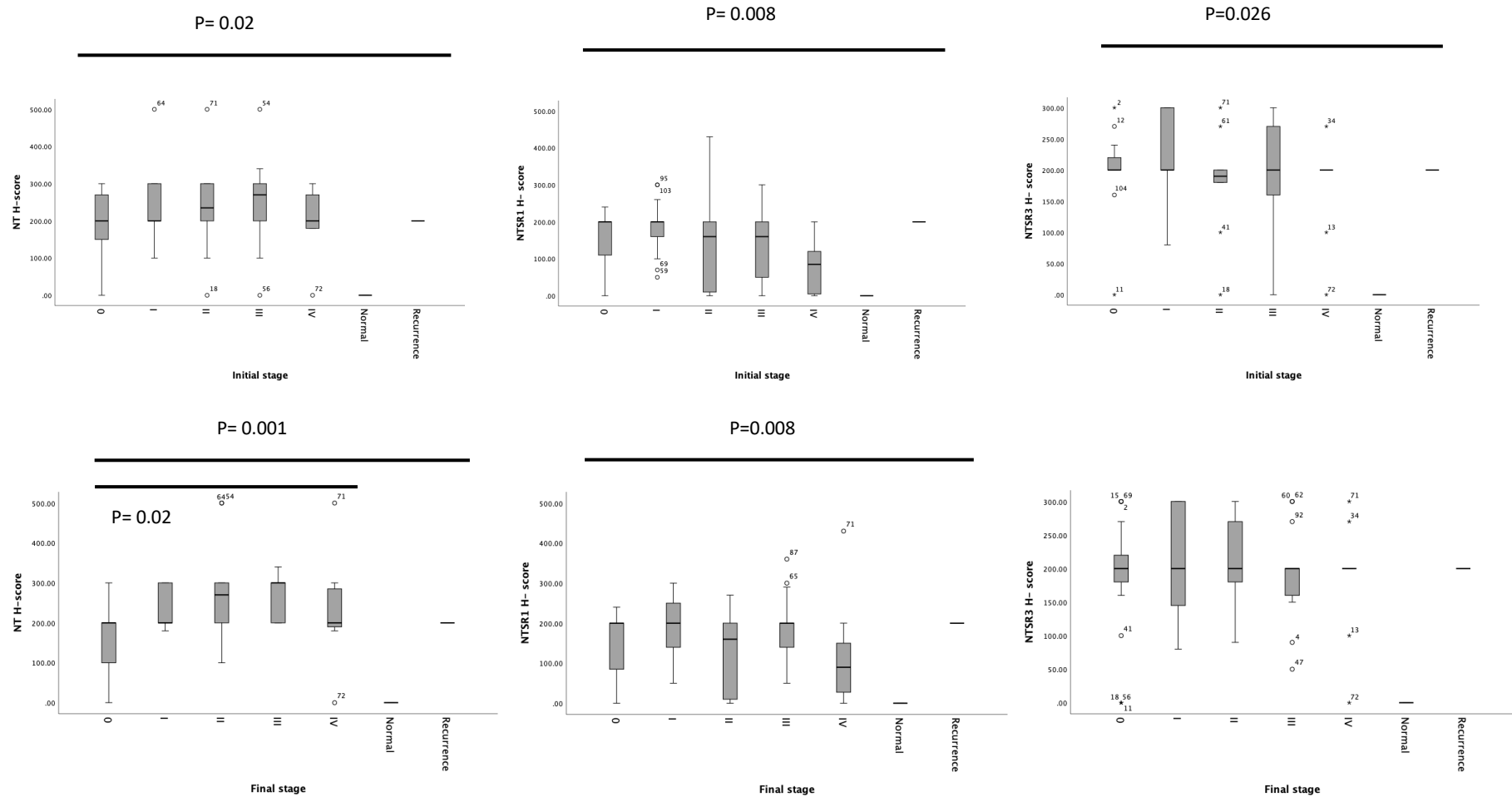


Figure 3.4 Tissue NT, NTSR1 and NTSR3 H-score across initial and final AJCC stages. Only significant p values noted on the chart.

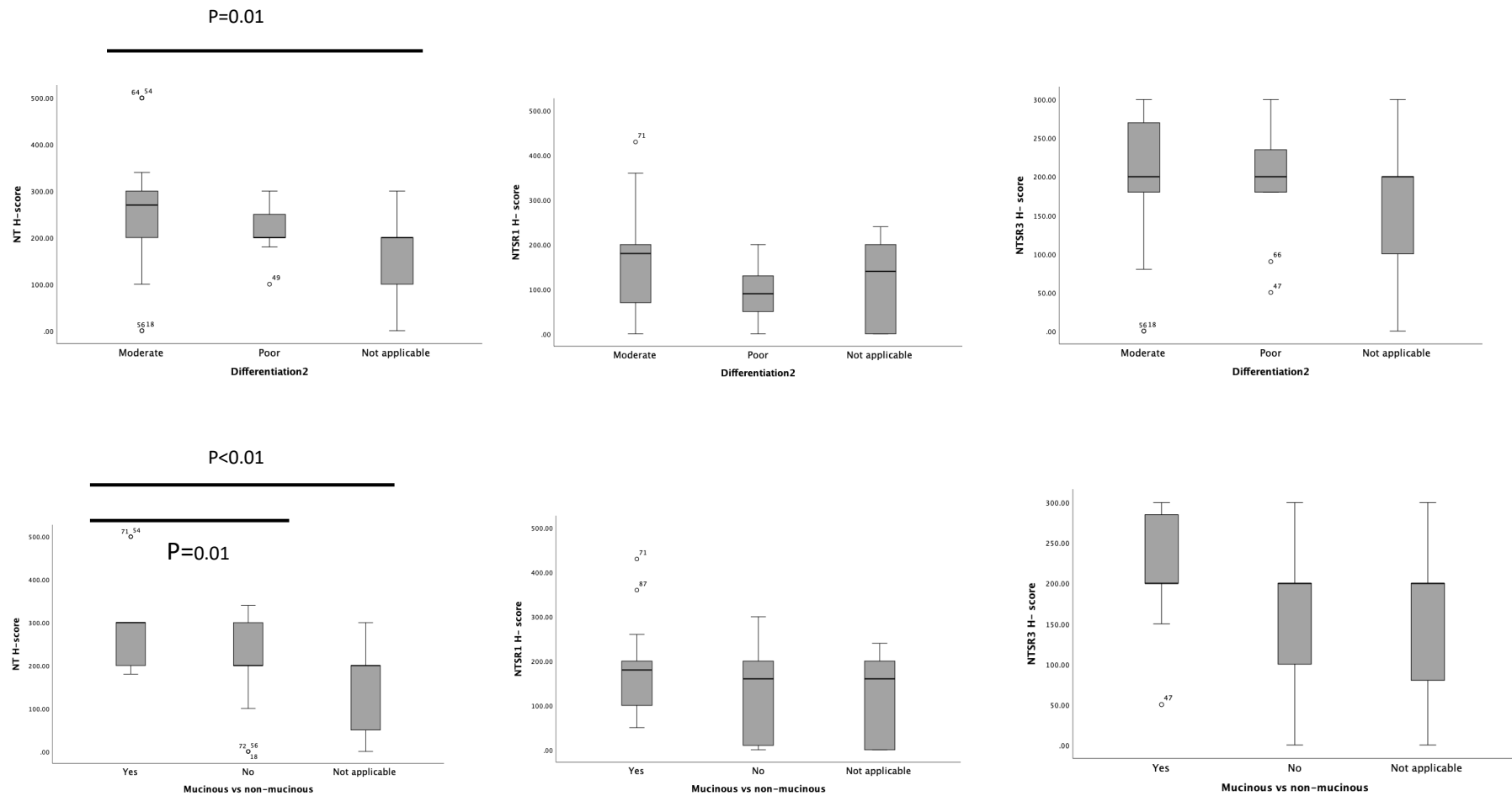


Figure 3.5 Tissue NT, NTSR1 and NTSR3 H-score according to differentiation of tumour and presence of mucin. Only significant p values noted.

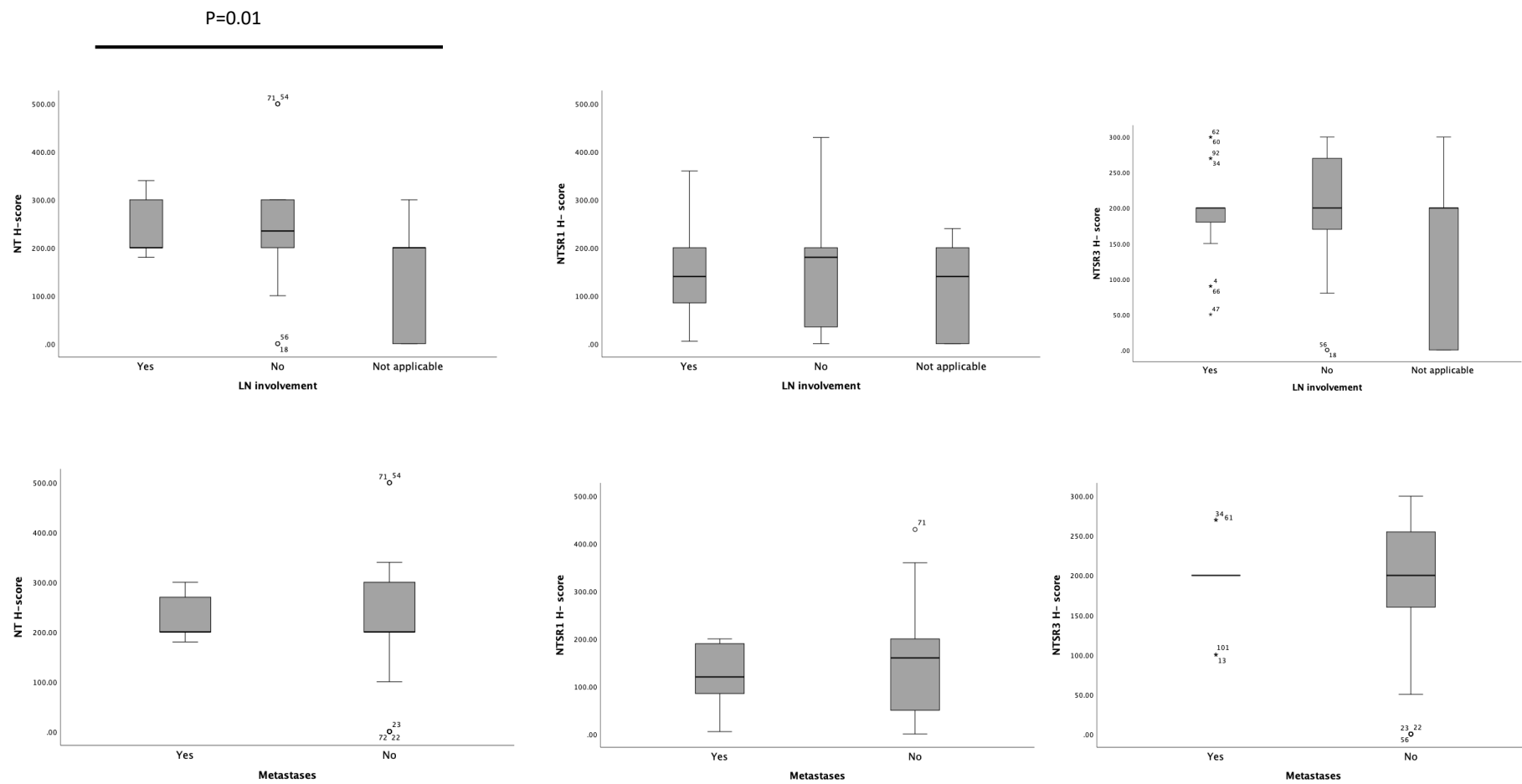


Figure 3.6 Tissue NT, NTSR1 and NTSR3 H-score according to lymph node involvement and presence of metastases

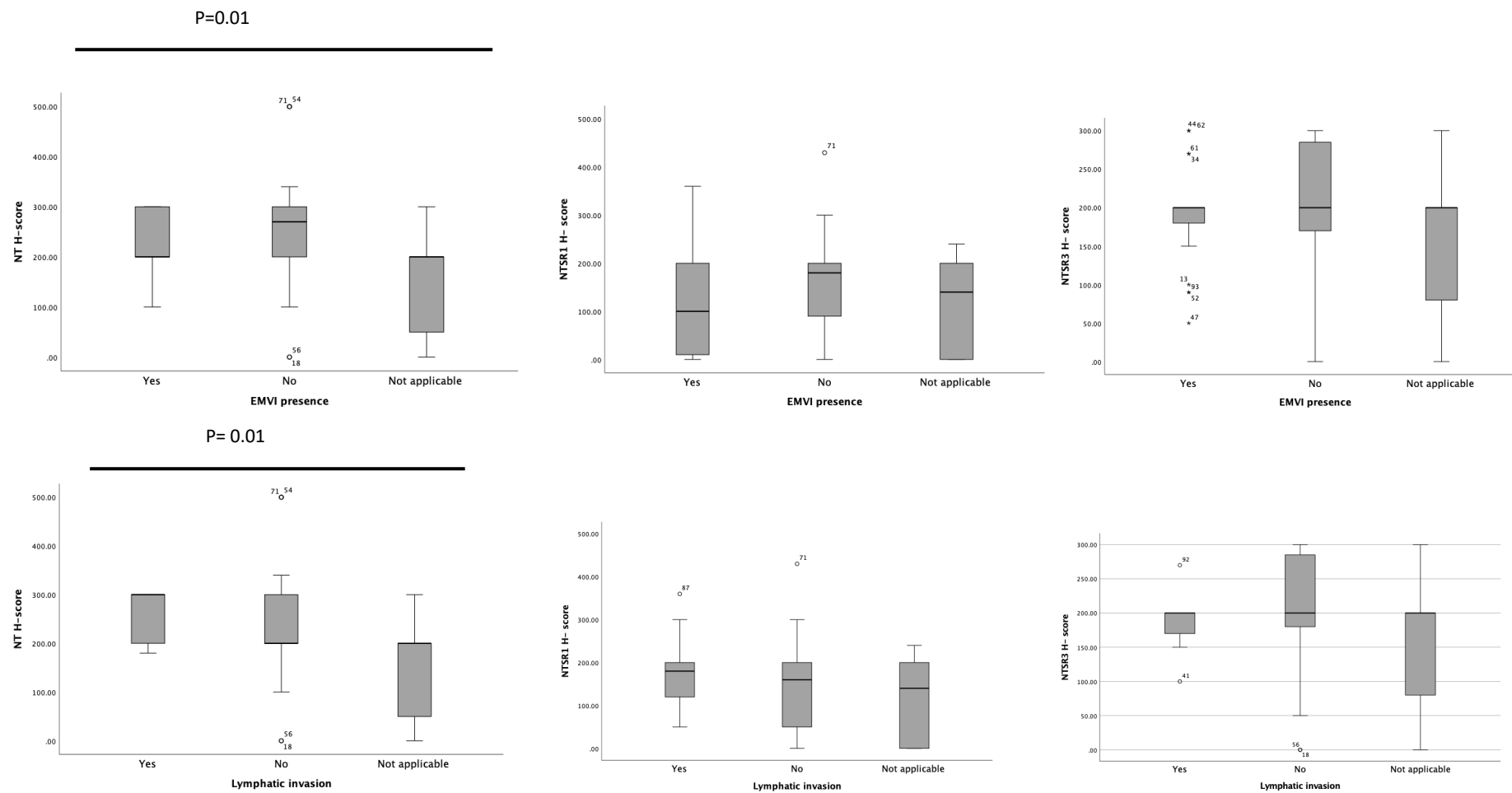


Figure 3.7 Tissue NT, NTSR1 and NTSR3 H-score according to EMVI and LI presence on histology. Only significant p values noted.

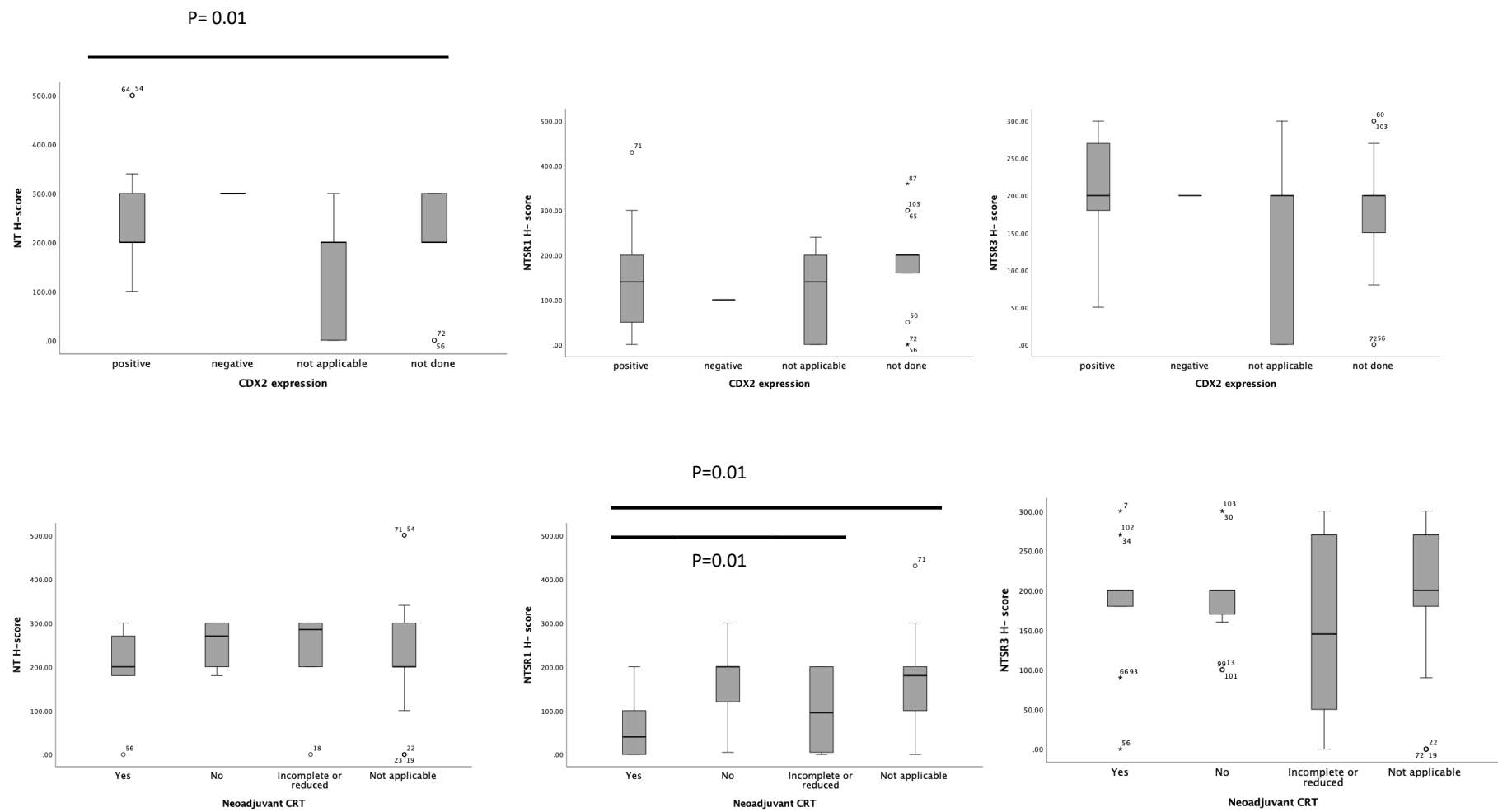


Figure 3.8 Tissue NT, NTSR1 and NTSR3 H-score according to CDX2 expression and neoadjuvant chemoradiotherapy.

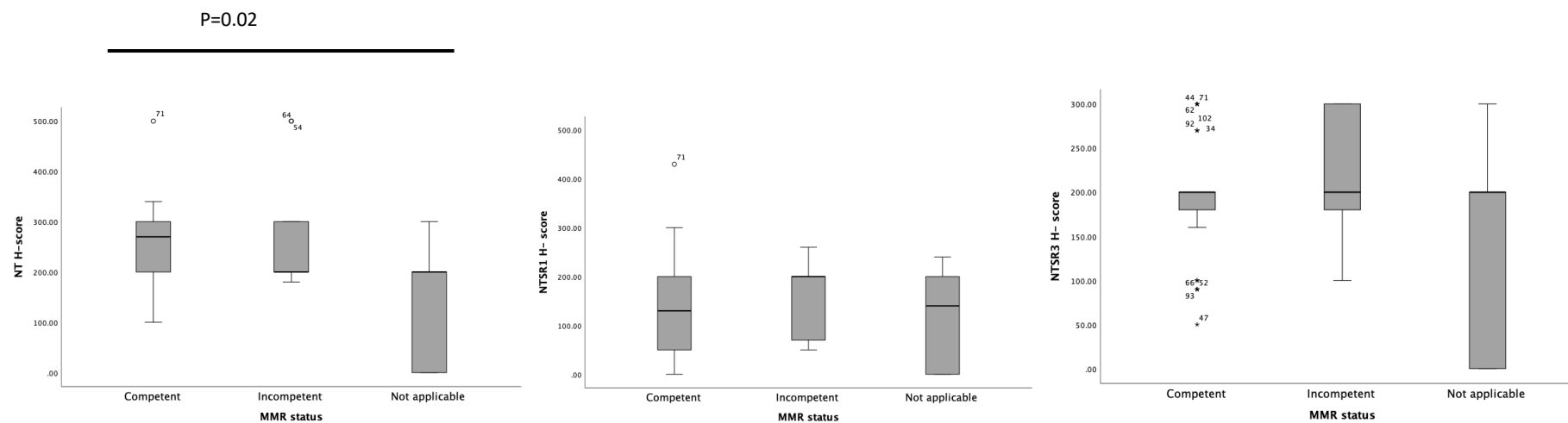


Figure 3.9 Tissue NT, NTSR1 and NTSR3 H-score association with MMR status. Only significant p values noted.

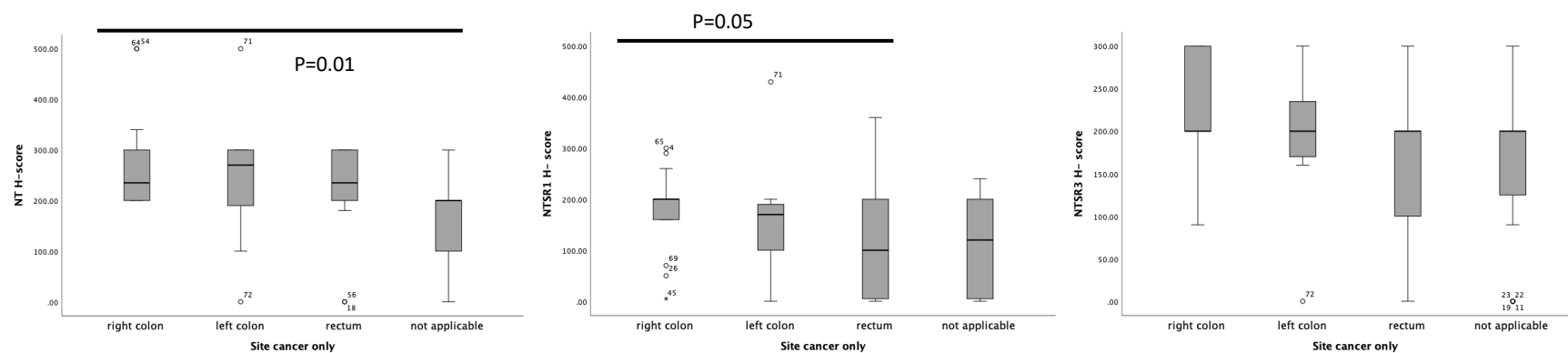


Figure 3.10 Tissue NT, NTSR1 and NTSR3 H-score expression according to site of cancer

3.1.3 DISCUSSION OF RESULTS AND LIMITATIONS

The primary aim of this thesis was to assess the expression of NT, NTSR1 and NTSR3 on abnormal colorectal pathology (polyps and cancer) and correlate this expression to stage of disease.

From the results presented so far, NT and its receptors show a significant difference between AJCC stages across all groups including patients that were in the 'normal' group where we assume there is zero expression of NT and NTSR1 (which is confirmed from literature)(109, 113). The only receptor which did not show any significant difference across the final AJCC stage of disease, was NTSR3. It is important to note that analysis where the 'normal' group was excluded, only showed significant change across the stages in NT H-score but not for the receptors. Inclusion of more 'normal' tissue biopsies, as well as more cancer in stages I, II and IV may show a more significant difference. The challenge of an ethics approval for biopsies taken when there is no clinical need was the reason for excluding this from the project's aims.

Another reason where the difference between NTSR1 expression may not be as evident could be because of the effect of chemoradiotherapy on these receptors. Figure 3.8 demonstrates that NTSR1 receptors are significantly downregulated in patients who had either partial or full course CRT. This may also explain the difference of NTSR1 expression between colon and rectal cancers.

NT expression was significantly higher in moderately differentiated tumours compared to poorly differentiated ones and the 'not applicable' group, which includes patients who had benign polyps. The difference could be attributed to the degree of differentiation but the presence of mucin could affect the level of NT expression as 6 out of 14 poorly differentiated adenocarcinomas in this cohort were mucinous versus the 14 of a total of 66 moderately differentiated adenocarcinomas. Presence of mucin showed a significantly higher NT H-score in patients with mucinous adenocarcinomas and a borderline difference ($p=0.05$, Kruskal Wallis analysis) for NTSR3 expression.

Other poor prognostic markers also showed significant differences in NT H-score. EMVI presence was interestingly associated with a lower NT H-score but the presence of lymphatic invasion showed a higher NT expression compared to the patients without. Only 2 patients had CDX2 negative tumours and hence a statistical analysis to detect a difference between CDX2 positive and CDX2 negative was invalid. The same applies to the MMR competent tumours which in this study showed that they had a higher NT H-score compared to the MMR incompetent tumours.

There was a significant difference between NTSR1 expression between colon and rectum and again this could be due to the effect of CRT in rectal cancers. Although there is a significant difference between all sites and the 'not applicable' group, once the latter is removed, the difference is no longer significant between right, left colon and rectum for NT expression. The same analysis for polyps showed no difference across all sites.

3.2 SERUM NT AND TISSUE NT, NTSR1 AND NTSR3 EXPRESSION IN PATIENTS WITH COLORECTAL CANCER AND POLYPS

3.2.1 PATIENT COHORT NT EXPRESSION

3.2.1.1 SERUM NT CORRELATION AND TISSUE NT, NTSR1 AND NTSR3 EXPRESSION

Table 3.7 summarises the plasma NT expression and the patient cohort demographic details.

Figures 3.11 shows the correlation between the plasma NT and the different diagnoses groups (normal versus polyps versus cancers and recurrent cancers).

		Median NT concentration (pg/mL)	IQR	P value
Gender	F	1732.7	1624.7 – 2476.7	0.929*
	M	1723.8	1730.0 – 2350.6	
Smoking status	Smoker	2004.9	1168.5 – 2405.8	0.09**
	Non-smoker	1804.1	1908.0 – 2651.1	
	Ex-smoker	1486.2	1362.7 – 2018.0	
Alcohol use	None	1649.7	1541.4 – 2800.0	0.977**
	Little	1648.3	1634.3 – 2246.0	
	Moderate	1734.0	964.6 – 3162.4	
	Heavy	1909.0	1409.2 – 2567.4	

Table 3.7 Plasma NT concentrations and cohort demographic characteristics. * Mann-Whitney U test, ** Kruskal Wallis test

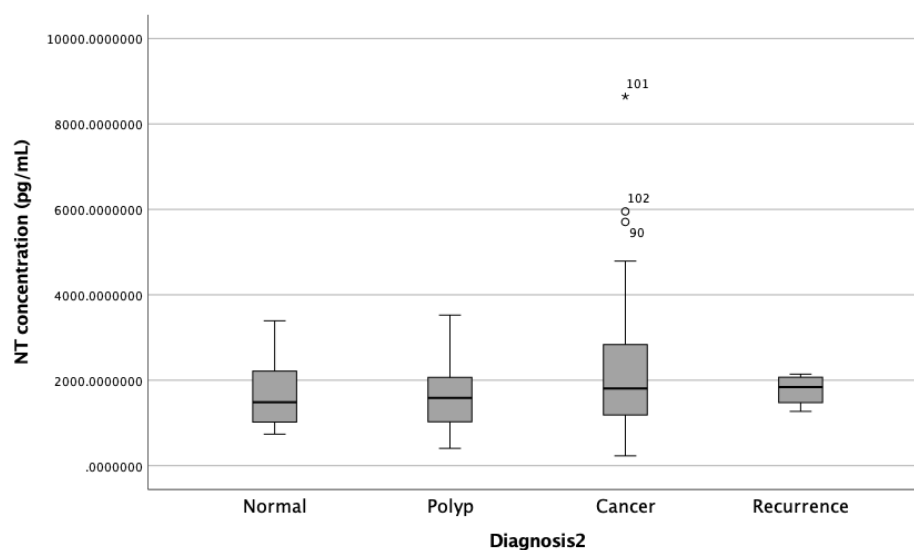


Figure 3.11 Plasma NT concentration (pg/mL) across the different diagnosis groups (p=0.53 Kruskal Wallis analysis).

3.2.1.2 PLASMA NT CORRELATION WITH NT, NTSR1 AND NTSR3 H-SCORE

Table 3.8 summarises the correlation between plasma NT and tissue NT and its receptors.

There is strong correlation between tissue NT and NTSR1 expression (Spearman coefficient, $\rho = 0.472$, $p < 0.01$)

	Plasma NT	NT H-score	NTSR1 H-score	NTSR3 H-score
Plasma NT	x	$\rho = 0.120$ $P = 0.27$	$\rho = 0.127$ $P = 0.24$	$\rho = -0.114$ $P = 0.30$
NT H-score	$\rho = 0.120$ $P = 0.27$	x	$\rho = \mathbf{0.472}$ $P < \mathbf{0.01}$	$\rho = \mathbf{0.352}$ $P < \mathbf{0.01}$
NTSR1 H-score	$\rho = 0.127$ $P = 0.24$	$\rho = \mathbf{0.472}$ $P < \mathbf{0.01}$	x	$\rho = \mathbf{0.305}$ $P = \mathbf{0.01}$
NTSR3 H-score	$\rho = -0.114$ $P = 0.30$	$\rho = \mathbf{0.352}$ $P < \mathbf{0.01}$	$\rho = \mathbf{0.305}$ $P = \mathbf{0.01}$	x

Table 3.8 Correlation between plasma NT and tissue NT and NTSR1 and NTSR3. Correlation between tissue NT and its receptors.

3.2.1.2 CORRELATION BETWEEN PLASMA NT AND HISTOPATHOLOGICAL DISEASE CHARACTERISTICS

Plasma NT concentration was compared to AJCC stage of disease (initial and final), T stage (initial and final), lymph node involvement, metastases, grade of differentiation, presence of mucin, EMVI and lymphatic invasion, CDX2 positivity, MMR competence and neoadjuvant CRT. There was no significant difference across any of these groups. Figure 3.12 demonstrates these findings.

3.3 DISCUSSION OF RESULTS

3.3.1 IMPLICATION OF RESULTS

The correlation between the plasma NT concentration and NT, NTSR1 and NTSR3 expression has been negative in this study. This could be because there is no correlation between circulating NT and colorectal cancer/polyp tissue expression of NT and its receptors.

Another possibility could be the type of assay we have used and the possibility of degradation of the protein prior to analysing the results which could have affected the results.

The first part of the results support the findings that tissue NT expression is inversely correlated with advancing initial stage of disease (Spearman correlation -0.324, $p < 0.01$), lymph node involvement ($\rho = -0.318$, $p < 0.01$), grade of differentiation ($\rho = -0.330$, $p < 0.01$), mucin presence ($\rho = -0.400$, $p < 0.01$), lymphatic invasion ($\rho = -0.338$, $p < 0.01$) and MMR status ($\rho = -0.361$, $p < 0.01$). There is a weak inverse correlation between tissue NT expression and CDX2 positivity ($\rho = -0.236$, $p = 0.03$) and EMVI presence ($\rho = -0.223$, $p = 0.04$).

3.3.2 LIMITATIONS

The prospective cohort group was not as homogenous as the initial aim of the study intended. The stage of disease was mostly stage III as the centres the patients were recruited from, specialise in advanced colorectal disease. Many these patients underwent chemoradiotherapy prior to being recruited in the study which can affect the molecular make-up of the tissue.

Furthermore, the number of polyps fell short of the required 30. There was a weak correlation between NT H-score and diagnosis ($\rho = 0.251$, $p = 0.02$), but the correlation may have changed with a larger number.

Analysis involving the 'non-applicable' or normal group, made a difference in the correlation between the NT H-scores and known poor prognostic criteria.

3.4 CONCLUSION

There is some evidence to support that NT H-score expression increases with advancing stage of disease. However, the number of patients we recruited with early stage and the fact that 29 patients out of the 78 who had cancer received neoadjuvant chemoradiotherapy needs to be considered as a significant limitation in interpreting our results.

Tissue NT expression is correlated with NTSR1 expression but not NTSR3. Further study into the role of NTSR3 in colorectal cancer may show why there is a difference.

Poor prognostic factors (lymphovascular invasion, presence of mucin) were associated with a higher NT expression.

Chemoradiotherapy significantly decreases NTSR1 expression but has no effect on NT and NTSR3 tissue expression.

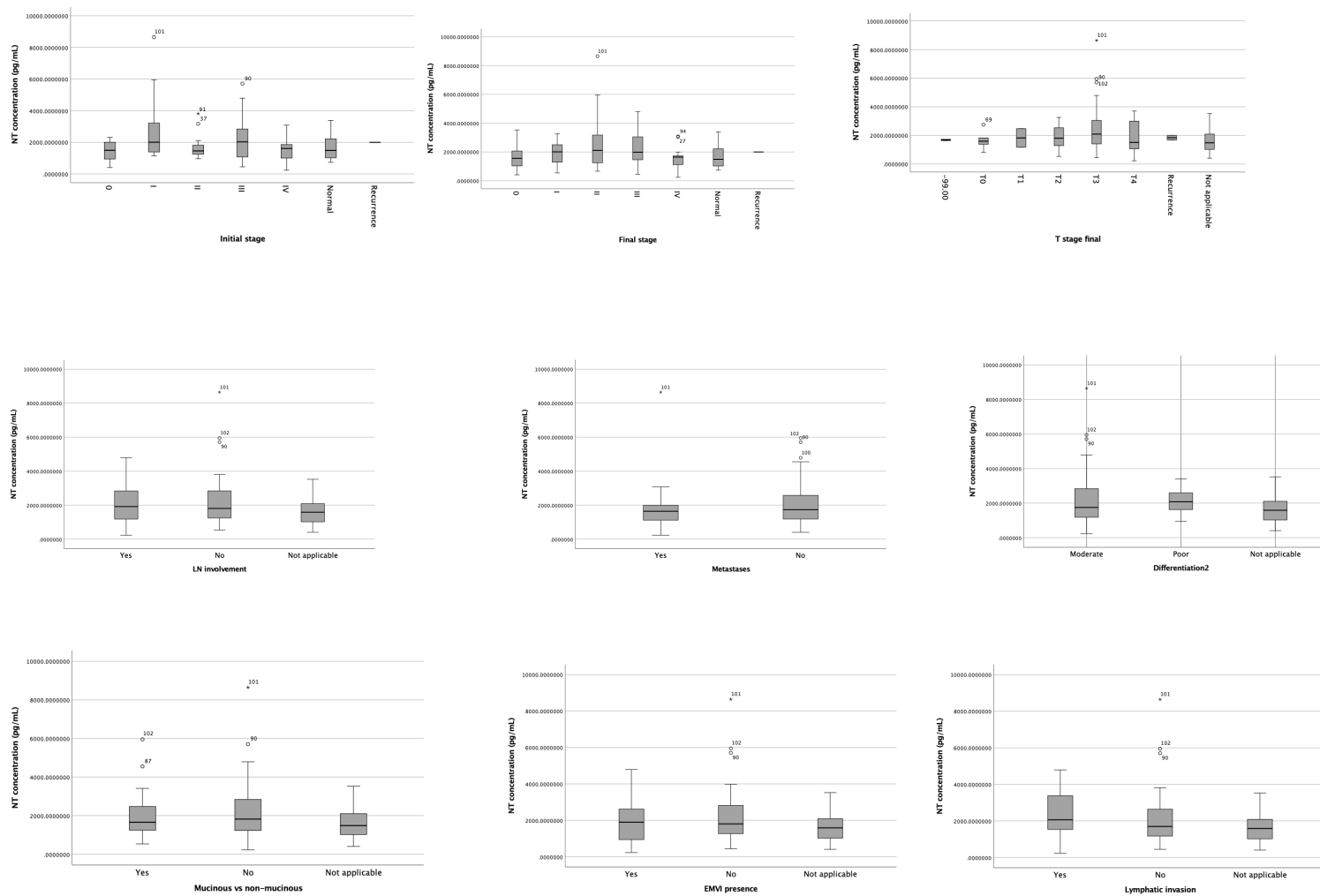


Figure 3.12 Plasma NT expression (pg/mL) and correlation with histopathological characteristics

CHAPTER 4: RESULTS – DIAGNOSTIC ABILITY OF PLASMA NT IN DISTINGUISHING COLORECTAL CANCER FROM NORMAL/BENIGN DISEASE

4.1 PLASMA NT EXPRESSION

4.1.1 COMBINED COHORT DEMOGRAPHIC AND HISTOPATHOLOGICAL CHARACTERISTICS

A previous PhD student who is part of our research group (SQ) prospectively recruited 165 patients who were under the two-week wait rule for colonoscopy. His aim was to assess the diagnostic ability of NT. In his analysis, he included 95 patients from the Imperial College Tissue Bank.

SQ's cohort consisted of 84 patients who had normal colonoscopies, 19 had diverticular disease, 20 patients had colorectal cancer, 34 had polyps and 8 had colitis. The validation cohort from the Imperial College Tissue bank included 95 samples for analysis. Forty-six of the patients had normal colonoscopies, 8 had diverticular disease, 7 had colitis, 10 had cancer and 24 had polyps. The blood sample collection, processing, storage and analysis were identical to this thesis' described method in chapter 2.

Table 4.1 and 4.2 summarises the combined characteristics and diagnoses of the 3 patient cohorts. In total, there were 364 patients, with 135 normal colonoscopies, 75 polyps, 108 patients with cancer, 4 with recurrence, 27 with diverticular disease and 15 with colitis.

	Median	IQR	Mean	SD
Age (years)	62.7	52.6 – 73.0	62.1	14.8
BMI	24.9	22.2 – 28.0	25.4	5.0
	Category	Frequency (n)	Frequency %	
Gender	Female	185	51	
	Male	178	49	
Smoking status	Smoker	34	9.3	
	Ex-smoker	35	9.6	
	Non-smoker	197	54.1	
	Unknown	98	26.9	
Bowel preparation	Yes	235	64.6	
	No	121	33.2	
	Unknown	8	2.2	

Table 4.1 Combined patient cohort demographics

Diagnosis	Frequency (n)	Percentage (%)
Normal	135	37.1
Polyps	75	20.6
Cancer	108	29.7
Recurrence	4	1.1
Diverticular disease	27	7.4
Colitis	15	4.1
Total	364	100

Table 4.2 Diagnoses distribution for the combined patient group

4.2 DIAGNOSTIC ABILITY OF PLASMA NT FOR COLONIC POLYPS AND COLORECTAL CANCER

SQ's results showed that at the cut-off value of >365.6pg/ml, the sensitivity of detecting colorectal abnormalities, adenomas and cancers, was 75% and specificity 60.3%, respectively. For detecting CRC, at the cut-off of >422.0pg/mL, the sensitivity was 70% and specificity was 63.5%. Figure 4.1 is taken from SQ's results from his PhD thesis.

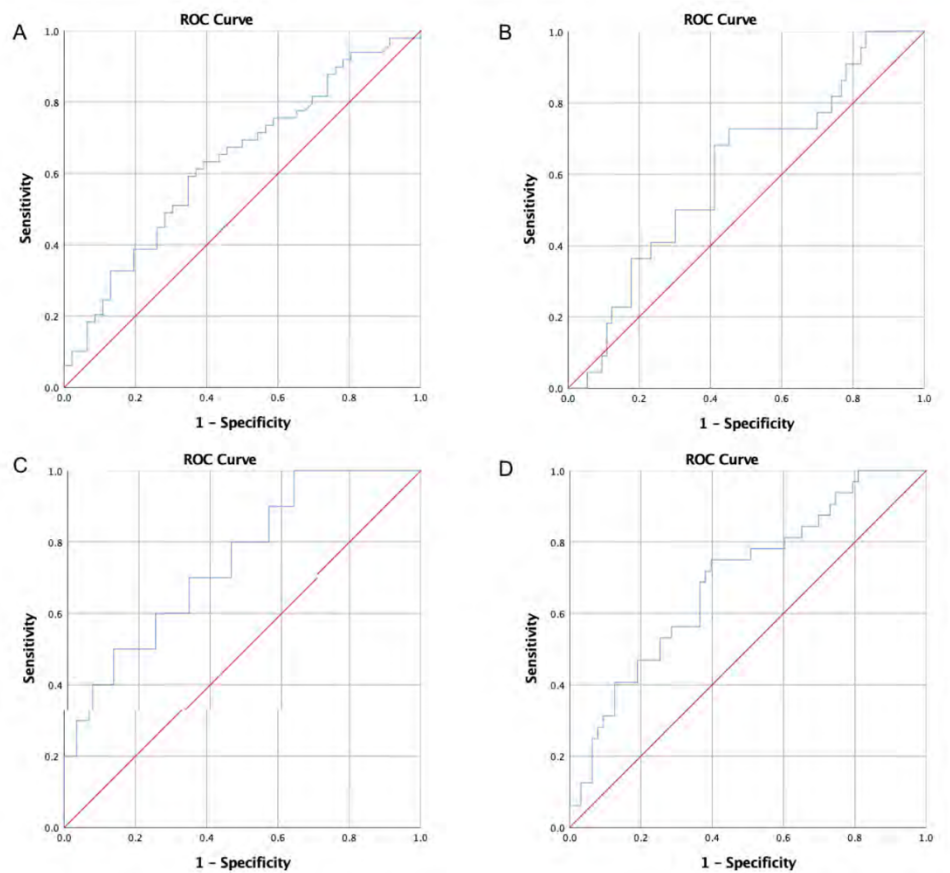


Figure 4.1 Receiver operator characteristic (ROC) curves for diagnostic ability of plasma neurotensin. A: all colorectal pathologies, B: polyps, C: cancer, and D: neoplasia. (Taken from Mr Shengyang Qiu's PhD thesis)

By combining SQ's analysis and the cohort of patients from this study, the number of patients increased to 364 with 108 cancers and 135 normal. ROC curve analyses were performed to determine the optimal cut-off and the sensitivity and specificity of plasma NT for distinguishing normal from cancer, normal from presence of polyps, early from late colorectal cancer and normal from all colonic pathology. Figure 4.2 shows the ROC curve graphs for each category. Table 4.3 summarises the results.

	Optimal cut-off (pg/mL)	Area under the curve (AUC)	p	Sensitivity (%)	Specificity (%)
Normal vs cancer	>637.9	0.863	<0.01	80.6	76
Normal vs Polyps	>460.8	0.697	<0.01	69.3	62
Early vs late disease (Early= polyps and T0 – 2; Late= T3 - T4)	>885.9	0.714	<0.01	70.1	65.1
Normal vs abnormal	>573.7	0.744	<0.01	62.9	73.6

Table 4.3 Summary of ROC curve analysis of different subgroups from the patient combined cohort

4.3 GENERAL DISCUSSION OF RESULTS

4.3.1 SUMMARY OF IMPORTANT FINDINGS AND IMPLICATIONS

The diagnostic ability of plasma neurotensin in distinguishing between normal colonoscopies and cancer shows a sensitivity of 80.6 percent and specificity of 76 percent. Although the specificity may not be as high as the currently available screening stool test (FIT has a reported specificity of 91 – 97 percent), its sensitivity in detecting adenomas, is 69.7% whilst FIT has a 20 to 30 percent sensitivity for detecting advanced adenomas(116). The overall sensitivity of detecting cancer compared to normal is comparable to FIT test.

From this study's results, NT's ability to distinguish between early and late cancer has a 71.4% sensitivity compared to the reported 20 – 40 percent for FIT test in a recent meta-analysis(117).

Table 4.4 summarises the main blood and stool biomarkers in use for CRC.

Biomarker	Sensitivity (%)	Specificity (%)	Clinical application
NT	80.6	76	Research
CEA	30 - 63	63 - 100	Surveillance
CA19 – 9	18 – 52	79 - 100	Surveillance
Sept 9	76	87	Commercially available
Guaic stool test	11 - 64	91 - 98	Screening
Faecal Immunochemical test (FIT) stool	70 - 90	91 -97	Screening

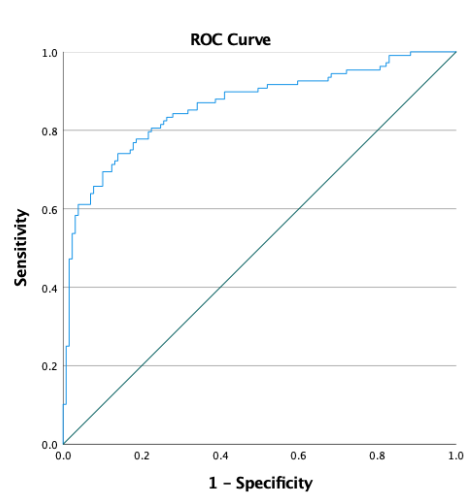
Table 4.4 Summary of blood and stool biomarkers and their sensitivity/specificity currently available compared to NT sensitivity/specificity from this study

4.3.2 LIMITATIONS

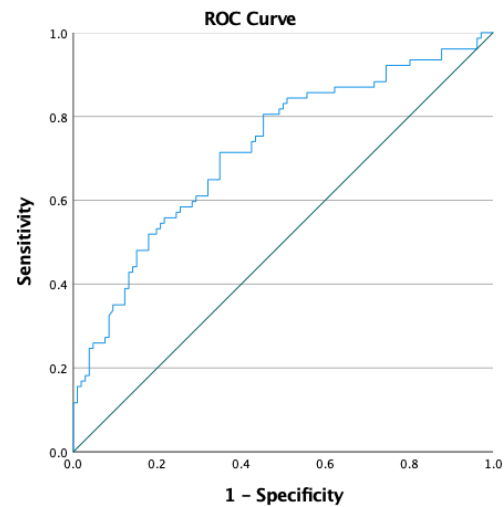
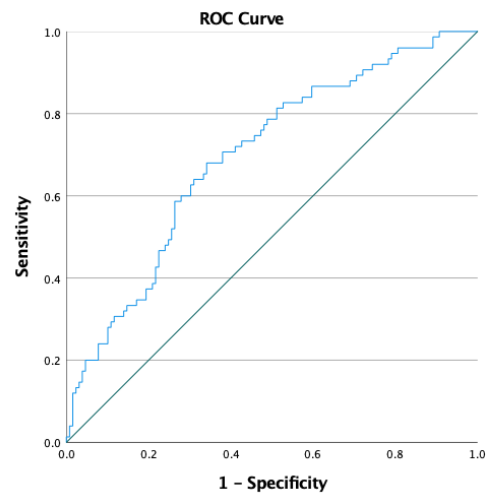
Despite the overall large number of patients included in the study, the samples collected prospectively came from a heterogeneous group of patients. From SQ's analysis, patients recruited had no treatment prior to their colonoscopy, whilst a significant number of patients recruited for my thesis had some form of treatment (either chemoradiotherapy or previous surgery if recurrent disease).

4.4 CONCLUSION

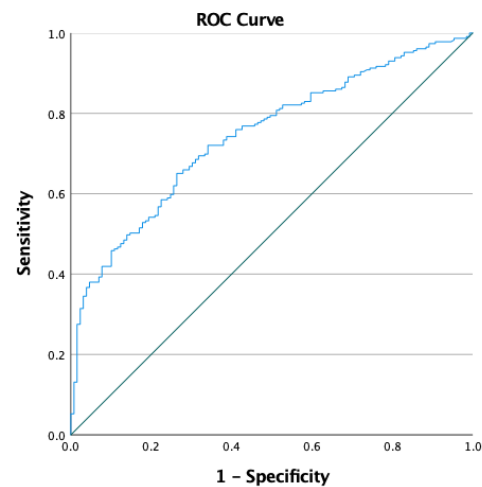
Plasma NT has shown encouraging diagnostic ability in detecting CRC which is comparable in sensitivity to FIT test and has a better sensitivity in detecting polyps compared to the currently available markers.



B.



C.



D.

Figure 4.2.

A. ROC curve analysis for normal vs cancer

B. ROC curve analysis for normal vs polyps

C. ROC curve analysis early vs late disease

D. ROC curve analysis for normal vs all abnormal colonic pathology

CHAPTER 5: GENERAL DISCUSSION, FUTURE WORK, AND CONCLUSION

5.1 SUMMARY OF RESULTS FROM THIS THESIS AND IMPORTANCE IN COLORECTAL CANCER

This study has investigated the expression of NT, NTSR1 and NTSR3 in colorectal cancer and polyps. Although there is an increasing expression of NT from polyps to stage IV disease, the expression of its receptors does not show a significant difference.

Presence of several poor prognostic factors are associated with higher NT expression i.e. mucin presence and lymphovascular invasion. Our research group has shown that there is a significantly reduced disease-free survival in patients with high NT expression ($p\ 0.02$)(113). These findings support the potential role of NT as a prognostic marker and its role in CRC pathogenesis.

Our study has also shown that there is a strong correlation between the NT and NTSR1 expression and a moderate correlation between NT and NTSR3 expression. Mechanistic studies support this finding and have shown that the NTSR1/NTSR3 exists as a heterodimer bound to the cell membrane. Once NT binds to this heterodimer, and is then internalised, it leads to activation of the ERK1/2 pathway. ERK1/2 activation by NT is lower in cells expression both NTSR1 and sortilin compared to cells expressing only NTSR1(118). This implies that not all colorectal cancer cells express both NTSR1 and NTSR3, and expression of the latter on the cell membrane may have an additional role in the cancer cell proliferation. Internalisation of NTSR1/NTSR3 does not affect the cell membrane NTSR3 expression but does reduce the NTSR1 expression due to the conformational changes resulting from binding onto this receptor(118).

In this study we did not find a difference in the receptors and NT expression between the cell membrane and cytoplasm of the colorectal cancer epithelial cells.

There was a significant difference of NTSR1 expression between rectum and colon but this could be due to the effect of neoadjuvant chemoradiotherapy in patients with rectal cancer.

Plasma NT did not show any correlation with tissue NT, NTSR1 and NTSR3 expression. It is however showing promising prospects as a diagnostic marker with a sensitivity of 80.6 percent and 76 percent specificity. Even more importantly, its sensitivity of detecting early cancer and polyps is higher compared to the currently available biomarkers.

5.2 FUTURE WORK

5.2.1 PLASMA NEUROTENSIN AS A DIAGNOSTIC BIOMARKER

The Cancer Research UK diagnostic biomarker roadmap (appendix 1) shows the path towards developing a diagnostic biomarker. Our research group has completed the biomarker discovery stage 1 and 2 as well as the qualification stage 1.

Prior to clinical application, further study into the effect of bowel preparation to plasma neurotensin is needed. Neurotensin is a gut hormone and bowel preparation causes an osmotic effect with a resulting increased gut motility which could affect the plasma NT level. It is also important to recruit patients who have not had prior chemoradiotherapy as it could also affect the level of plasma NT.

Finally, during the recruitment of patients for this thesis, qFIT testing was not available to our patient cohort. In future studies, direct comparison of qFIT testing and plasma NT in symptomatic patients as well as combination of the two tests could further validate NT as a potential biomarker and/or an additional marker for further triaging patients in need of more invasive investigations for the diagnosis of CRC.

5.2.2 NEUROTENSIN EXPRESSION AS A PROGNOSTIC MARKER

The prognostic/predictive biomarker pathway as summarised by the CRUK is included in appendix 2.

The assay development stage 1, Discovery stage 1, assay development stage 2 and discovery stage 2 steps have been completed by our research group. The next step is to validate the correlation between NT and clinical outcome in a prospective study cohort. An additional tool and valuable guide to reporting studies on prognostic biomarkers is the REporting recommendations for tumour MARKer prognostic studies(REMARK) (119). This is a twenty-point checklist which provides a framework of guidelines on study design, pre-planned hypotheses, patient and specimen characteristics, statistical methods and data presentation and validation(120).

An important aspect of the checklist highlights the need to compare neurotensin to other biomarkers in use for example CEA and ct DNA to determine its prognostic ability.

The use of H-score as a quantification method is a validated scoring system which allows statistical evaluation of strength and percentage of cells staining in a research

setting. Despite the care to reduce inter-observer and intra-observer errors, the method does have significant reproducibility issues if more widely used. Alternative methods include image analysis software (for example use of J image) with marking of region of interest and use of software to measure the signal. Additionally, it would be interesting to look at the expression of NT near the invasive border of aggressive cancers and compare them to more indolent ones.

The results have shown that NT expression has promising potential as a prognostic marker with increasing expression with stage of disease. More early stage disease patients are needed to establish the difference in NT staining between early and advanced cancers are needed. Also, access to initial biopsies prior to diagnosis would remove the confounding factor of chemoradiotherapy effect on the tissue removed during surgery.

5.2.3 MECHANISTIC STUDY – NANOSTRING ANALYSIS

Gene expression profiling using NanoString RNA analysis is an important next step in identifying mechanism by which neurotensin may drive the colorectal carcinogenesis. Each cartridge can assess up to 800 genes either known to be involved in carcinogenesis or can be tailored to look at specific pathways the researcher is interested in.

By assessing changes in the gene expression between cancers with low and high neurotensin expression who exhibit difference in prognosis can reveal which RNA,

DNA and protein markers are involved. These can then be used for identifying therapeutic targets in patients whose cancer may be driven by neurotensin.

5.3 FINAL CONCLUSION

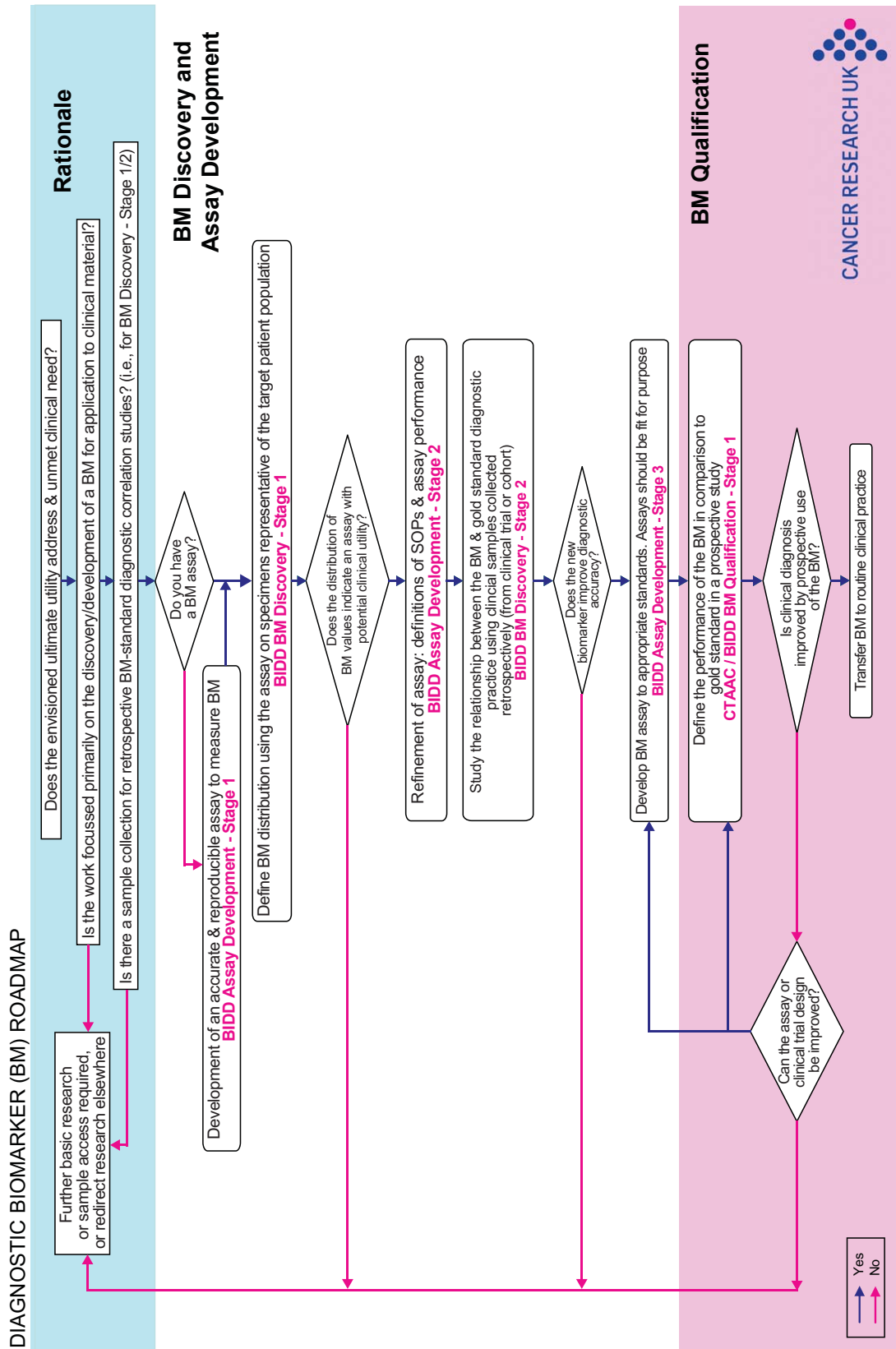
In this study, I conclude that protein NT expression in colorectal cancer is associated with worsening stage of disease and presence of mucin and lymphatic invasion.

Furthermore, plasma NT is a promising diagnostic marker for detecting colorectal cancer, as well as polyps compared to normal.

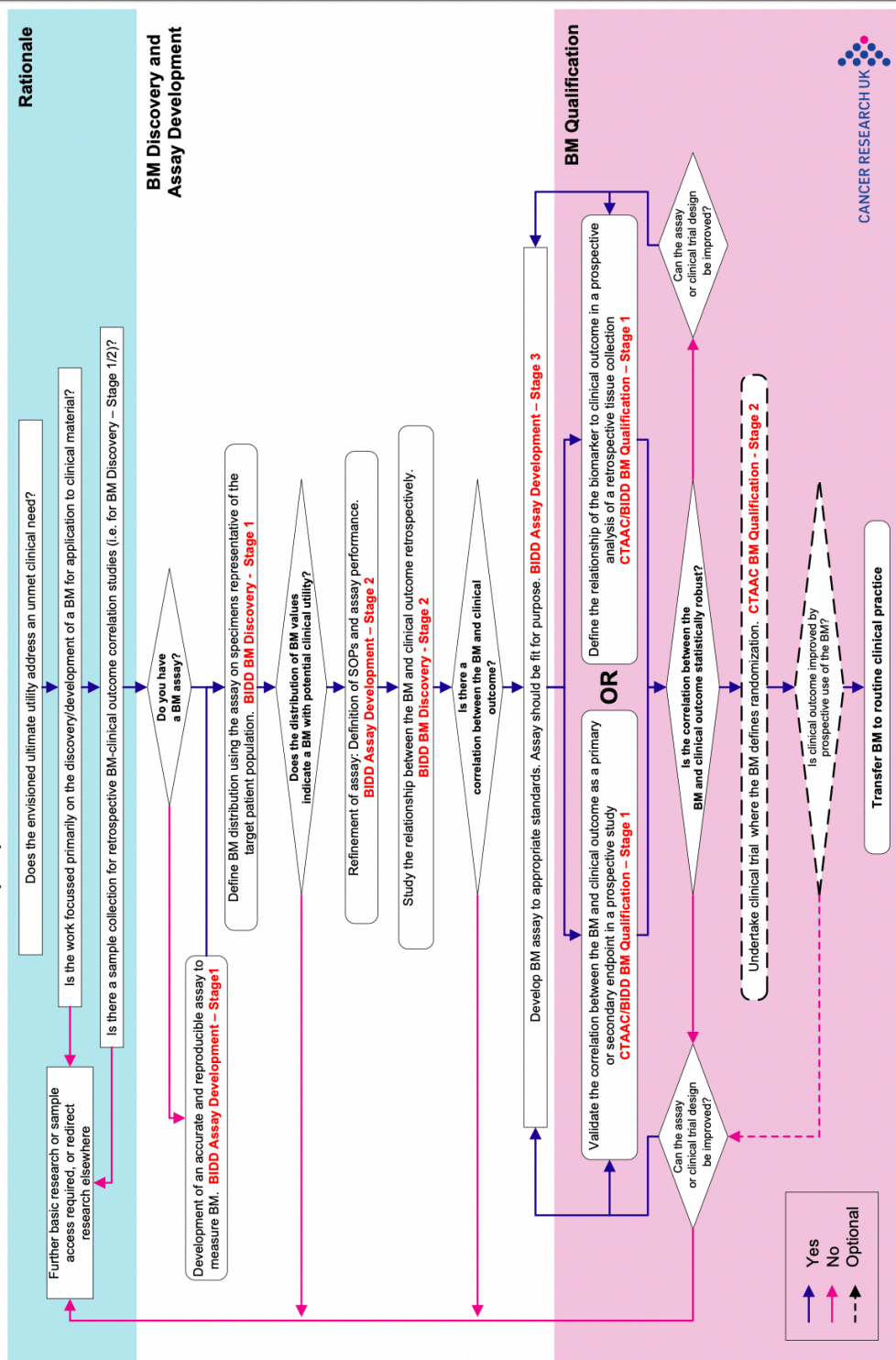
Future work is required to investigate the pathway through which neurotensin drives carcinogenesis which can lead to identification of therapeutic targets.

APPENDIX

1. CRUK roadmaps



PROGNOSTIC/PREDICTIVE BIOMARKER (BM) ROADMAP



3. Neurotensin antibody ELISA kit



Abbexa Ltd, Innovation Centre, Cambridge Science Park, Cambridge, CB4 0EY, UK
Telephone: +44 (0) 1223 755950 - Fax: +44 (0) 1223 755951 - E-Mail: info@abbexa.com

DATASHEET

Human Neurotensin (NTS) ELISA Kit

Catalogue No.: abx152510



Human Neurotensin ELISA Kit is a competitive ELISA kit for use with Serum, plasma and other biological fluids. This assay has high sensitivity and excellent specificity for detection of Neurotensin (NT)

No significant cross-reactivity or interference between Neurotensin (NT) and analogues was observed.

Please note that this kit is also available as a CLIA Kit [abx490378](#).

Target: Neurotensin (NT)

Reactivity: Human

Tested Applications: ELISA

Recommended dilutions: Optimal dilutions/concentrations should be determined by the end user.

Test Range: 37 pg/ml - 3000 pg/ml

Sensitivity: < 13.2 pg/ml

Validity: The validity for this kit is 6 months.

Storage: Shipped at 4 °C. Upon receipt, store the kit according to the storage instruction in the kit's manual.

Stability: The stability of the kit is determined by the rate of activity loss. The loss rate is less than 5% within the expiration date under appropriate storage conditions. To minimize performance fluctuations, operation procedures and lab conditions should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same user throughout.

Swiss Prot: [P30990](#)

GeneID: [4922](#)

Gene Symbol: NTS

4. REC approval



Health Research Authority

East of England - Essex Research Ethics Committee

The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

08 November 2017

Professor Paris Tekkis
Area D
Chelsea and Westminster Campus
Imperial College London
SW10 9NH

Dear Professor Tekkis

Study title:	Expression pattern and clinicopathological relevance of Neurotensin and its receptors in colorectal cancer
REC reference:	17/EE/0379
IRAS project ID:	223925

Thank you for your letter of 3 November 2017, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact please contact hra.studyregistration@nhs.net outlining the reasons for your request.

Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.



Health Research Authority
South Central - Oxford B Research Ethics Committee

Whitefriars
Level 3, Block B
Lewin's Mead
Bristol
BS1 2NT

Telephone: 0117 342 1389

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

12 October 2016

Prof. Paris Tekkis
Professor of Colorectal Surgery, Faculty of Medicine, Department of Surgery & Cancer
Imperial College
Royal Marsden Hospital
Fulham
SW3 6JJ

Dear Prof. Tekkis

Study title:	Evaluating the role of Neurotensin (NT) as a diagnostic marker for colonic polyps and colorectal cancers
REC reference:	16/SC/0523
Protocol number:	CR4556
IRAS project ID:	208209

Thank you for your letter of 10 October 2016, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Mr Stephan Ramey, nrescommittee.southcentral-oxfordb@nhs.net. Under



Imperial College Healthcare Tissue Bank
Department of Surgery and Cancer,
Hammersmith Hospital,
Du Cane Road,
London W12 0HS
Tel: +44 7711 701382
Email: gerry.thomas@imperial.ac.uk

ICHTB HTA licence: 12275
REC Wales approval: 12/WA/0196

Professor Paris Tekkis
Department of Academic Surgery
Chelsea and Westminster Campus
369 Fulham Rd
London SW10 9HN

16 March 2016

Dear Paris,

Re: Tissue Bank application number R16007;
Project title: Evaluating the role of neurotensin as a novel biomarker in
colorectal

I am pleased to confirm that the Tissue Management Committee of the ICHTB has approved your application for access.

In order to satisfy HTA tracking requirements, would you please ensure the samples you use are reported to the tissue bank via the online database and sub-collection, along with any publications you produce, that should contain the acknowledgment wording as per the signed MTA.

Yours sincerely,

Professor Gerry Thomas
DI, Hammersmith HTA Research Licence.

5. Original data

74	2	38	1	2	28.27160494	1	3	6	3	5	3	4	3	2131.320885	1	1	1	1	-99	3	5	-99	4	2	2	2	2	1	1	2	2	200	0	180
75	1	71	2	2	31.20256067	1	3	6	1	5	1	2	2	3258.474157	1	1	1	4	2	2	6	3	3	2	2	2	2	2	2	2	200	200	300	
76	1	57	3	1	27.10824824	1	3	6	3	5	3	2	2	793.3556182	1	1	1	4	4	3	6	4	4	2	2	2	1	1	2	2	200	20	200	
77	2	75	3	3	27.03703104	1	2	3	3	3	4	7	4	1469.138629	3	1	3	4	1	1	6	6	7	3	2	3	3	2	2	2	100	120	200	
78	2	46	2	2	26.97036864	1	3	6	2	5	2	2	2	1557.845266	1	1	1	4	5	5	6	4	4	1	1	2	2	2	2	200	80	200		
79	1	80	3	4	25.62916058	1	3	6	2	5	2	2	2	1626.020431	4	1	1	1	4	2	4	6	2	1	1	2	2	2	2	1	300	160	200	
81	2	63	2	1	30.34607438	1	3	6	1	5	1	2	2	2534.524787	1	1	1	4	4	2	6	4	3	2	2	2	2	2	2	2	300	180	90	
82	2	59	2	3	25.88181902	1	4	7	6	5	4	5	3	1998.961173	4	-99	-99	1	9	8	6	7	6	-99	2	1	-99	-99	-99	-99	200	200	200	
83	2	40	2	1	25.86344284	1	3	6	3	5	3	2	2	3657.548098	1	1	1	1	4	3	6	4	4	2	2	2	1	1	2	2	270	60	180	
84	2	58	3	2	21.60498827	1	3	6	3	5	3	2	2	2316.78717	1	1	1	2	4	1	2	6	4	2	2	2	1	2	2	2	300	200	180	
85	2	61	3	4	25.91087241	1	3	6	3	5	3	2	2	3222.765024	1	1	1	1	4	3	-99	5	4	4	2	2	2	2	2	2	180	0	180	
86	2	51	1	2	31.15198578	1	3	6	3	5	3	2	2	2844.520336	1	1	1	1	4	3	4	4	4	2	2	2	2	2	2	2	270	0	180	
87	1	83	2	1	17.04032978	-99	3	6	3	5	3	-99	-99	1643.129401	4	-99	-99	2	5	5	6	5	-99	-99	1	-99	-99	-99	-99	-99	-99	-99	-99	
88	1	62	2	1	24.54460627	1	3	6	3	5	3	3	2	4554.009416	4	-99	-99	-99	-99	4	4	-99	4	1	2	1	1	1	1	1	300	360	150	
89	2	69	3	2	25.78571346	1	3	6	3	5	3	3	2	2797.727468	4	-99	-99	1	-99	4	6	-99	4	1	2	1	2	2	2	2	200	200	200	
90	2	78	2	2	24.74270506	1	3	6	2	5	2	2	2	1458.124602	1	2	2	4	4	3	6	4	4	2	2	2	2	2	2	2	180	180	180	
91	2	39	2	2	30.08334548	1	3	6	2	5	2	2	2	2707.371954	1	1	1	4	4	3	6	3	4	2	2	2	2	2	2	2	300	100	200	
92	2	74	2	2	25.95058933	2	3	6	3	5	3	2	2	3807.347128	1	1	1	2	3	3	4	4	4	2	2	2	2	2	2	2	99	99	99	
93	2	56	2	1	27.99123448	1	3	6	3	5	3	5	3	3414.629562	1	1	1	3	4	4	4	4	5	5	1	2	1	2	2	1	1	300	140	270
94	1	71	2	1	22.37328839	1	3	6	3	5	3	2	2	2620.00084	1	1	1	1	4	3	6	5	4	2	2	2	1	1	1	1	-99	180	5	90
95	1	89	3	2	26.60955545	2	3	6	1	5	1	2	2	3080.80152	4	-99	-99	4	5	5	6	4	3	2	1	2	-99	-99	-99	-99	270	180	200	
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97	2	85	2	2	27.83200798	1	2	3	3	3	4	7	4	374.306987	3	1	3	4	2	1	6	2	7	3	2	3	3	2	3	2	200	200	200	
98	1	87	2	-99	24.63048929	-99	3	6	7	6	2	2	2	1737.25929	1	2	2	2	2	4	3	4	4	2	2	2	2	2	2	2	270	200	160	
99	1	62	2	1	17.98866291	1	3	6	3	5	3	4	3	1119.883232	1	1	1	1	5	5	6	5	4	1	2	2	1	1	1	1	180	90	200	
100	2	59	2	1	33.60929705	1	3	6	3	5	3	2	2	2711.893173	1	1	1	2	4	3	6	4	5	2	2	2	2	2	2	2	300	200	100	
101	2	54	3	1	26.01456816	1	3	6	2	5	2	2	2	4700.55474	1	1	1	4	4	4	4	4	4	1	2	2	1	1	1	1	300	100	200	
102	1	54	2	1	30.02150189	1	3	6	3	5	3	2	2	8646.060395	1	1	1	2	2	3	6	3	4	2	1	2	2	2	2	2	200	100	100	
103	2	50	2	3	31.58386374	1	3	6	3	5	3	3	2	1592.838387	1	1	1	1	2	3	-99	3	4	2	2	1	2	2	2	2	300	180	270	
104	2	66	2	2	25.93826547	1	3	6	3	5	3	2	2	1178.250704	4	-99	-99	2	2	2	-99	2	2	-99	2	2	2	2	2	2	300	200	300	
106	1	74	3	1	29.02059488	1	2	3	3	3	4	7	4	2068.651626	3	1	3	4	1	1	-99	6	7	3	2	3	2	3	2	2	200	240	160	

Shells all data 02.03.22

SampleID	Gender	Age	Smoking	Alcohol	BMI	Rowfreq	Diagnosis	Diag2	Site	Stenopy	Silencaser	Differentiation	Differentiation2	NT	CDX2	MMR2groups	MMR	NCRT	Initiastage	Finalstage	pTIR2	Tstage	Tlens	LIinvolvement	Metastases	Mucinosa	EMV	EMVx2	LI	Lx2	NTHscore	NTSR1Hscore	NTSR2Hscore	
1	2	68	3	2	28.14148227	1	3	6	3	5	3	2	2	1602.821087	1	1	1	1	1	4	2	3	3	3	2	2	2	2	2	2	-99	-99	-99	
2	2	88	3	3	27.28227418	1	2	3	1	1	4	7	4	946.4104741	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	3	300	200	300	
3	2	67	3	4	36.70720749	1	2	2	2	2	4	7	4	693.1663197	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	200	200	200	
4	2	84	3	2	21.65510814	-99	3	6	1	5	1	2	2	1185.892524	1	1	1	4	4	4	6	3	7	1	2	2	2	2	2	2	200	250	90	
5	1	75	3	1	27.23407575	1	2	3	2	2	4	7	4	965.46847015	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	300	200	200	
6	2	73	2	4	29.57256688	1	3	6	3	5	3	2	2	1785.01181	1	1	1	3	4	3	4	4	4	2	2	2	2	2	2	2	300	200	300	
7	2	50	3	2	28.87055242	1	3	6	3	5	3	3	2	538.534347	1	1	1	1	4	2	-99	3	3	2	2	1	2	2	2	2	300	100	300	
8	1	40	2	1	31.73626073	1	3	6	3	5	3	2	2	1023.383247	4	1	1	2	1	1	6	1	7	2	2	2	2	2	2	2	-99	-99	-99	
9	1	32	1	3	31.03421487	2	3	6	3	5	3	2	2	970.2298917	1	1	1	3	4	4	3	5	5	1	2	2	2	2	2	2	-99	-99	-99	
10	2	86	2	4	24.54460790	-99	3	6	7	6	2	4	6	1491.134974	3	1	3	4	7	4	6	6	7	3	2	3	3	2	3	2	0	0	0	
11	1	74	2	2	22.86810772	1	2	2	3	3	3	7	4	1686.373381	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	0	0	0	
12	1	87	2	2	32.88137369	1	2	4	3	3	4	7	4	407.7328687	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	240	0	270	
13	2	51	2	1	24.74218446	1	3	6	3	5	3	2	2	889.6644963	1	1	1	2	5	5	5	5	5	2	1	2	1	1	2	2	300	200	100	
14	1	74	3	4	21.07719523	1	1	1	4	5	4	6	4	1486.167619	3	1	3	4	7	6	6	6	7	3	2	3	3	2	3	2	-99	-99	-99	
15	1	73	3	2	21.1659242	2	2	3	5	4	4	7	4	1728.614333	3	1	3	4	2	1	6	6	7	3	2	2	3	3	2	3	100	200	300	
16	1	68	1	1	29.71166208	1	2	2	3	3	4	7	4	2188.181547	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	-99	-99	-99	
17	2	53	3	2	18.36547291	1	2	5	1	1	4	7	4	2005.969897	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	200	200	200	
18	2	59	2	2	22.74600233	1	3	6	3	5	3	2	2	1161.619919	3	1	3	3	5	1	2	5	1	2	2	2	2	2	2	2	0	0	0	
19	1	68	2	2	30.71248447	1	1	1	4	5	4	6	4	4392.545789	3	1	3	4	7	6	6	6	7	3	2	3	3	2	3	2	0	0	0	
20	2	62	1	3	26.29757785	1	2	3	1	1	4	7	4	2004.888284	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	200			
21	1	86	2	2	24.34116705	1	2	3	1	1	4	7	4	1028.726939	3	1	3	4	2	1	6	6	7	3	2	3	3	2	3	2	100	200	300	
22	1	54	3	2	-99	1	1	1	4	5	4	6	4	4216.410181	3	1	3	4	7	6	6	6	7	3	2	3	3	2	3	2	0	0	0	
23	2	76	3	2	26.3	1	1	1	4	5	4	6	4	737.770305	3	1	3	4	7	6	6	6	7	3	2	3	3	2	3	2	0	0	0	
24	1	47	1	2	27.31148181	1	2	3	5	4	4	7	4	1264.119186	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	200	100	200	
25	1	81	2	1	21.64413201	1	3	6	1	5	1	2	2	2106.119347	1	1	1	4	3	3	6	3	4	2	2	2	2	2	2	2	300	200	180	
26	2	82	2	2	22.5827131	1	3	6	3	5	3	2	2	939.7944519	1	1	1	4	4	4	6	3	4	2	2	1	2	2	2	2	300	200	100	
27	2	56	3	3	28.58767107	1	3	6	3	5	2	4	7	3044.490155	1	1	1	4	6	5	5	5	4	2	1	2	2	2	2	2	200	100	200	
28	1	78	3	2	22.38631428	2	2	3	3	3	4	7	4	1594.500307	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	200	180	240	
29	2	84	3	3	21.81818182	1	3	6	3	5	3	3	2	1457.488616	1	1	1	2	3	4	6	3	3	-99	2	1	1	1	1	1	300	140	180	
30	2	59	2	2	23.26347372	1	3	6	1	5	1	2	2	1570.212537	1	1	1	2	4	3	3	4	4	2	2	2	2	2	2	2	300	160	300	
31	2	86	3	2	27.00740812	1	3	6	1	5	1	2	2	1247.720644	1	1	2	4	3	3	6	-99	4	2	2	1	2	2	2	2	200	200	200	
32	2	84	2	2	23.50806299	1	3	6	1	1	1	2	2	271.4270215	1	1	1	2	4	2	4	3	4	1	1	1	1	1	1	-99	-99	-99		
33	1	84	2	1	19.22222222	1	3	6	1	5	1	2	2	270.3509881	1	1	1	4	2	4	6	3	4	1	2	2	1	1	1	1	-99	-99	-99	
34	2	68	2	2	19.85048028	1	3	6	3	5	3	2	2	825.1891529	1	1	1	1	5	5	-99	4	3	1	1	2	1	1	1	2	180	5	270	
35	2	74	3	1	27.18235471	1	3	6	2	5	2	2	2	889.6644963	1	1	1	4	4	3	6	4	4	2	2	2	2	2	2	2	100	180	300	
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37	2	44	2	1	30.28010191	1	3	6	3	5	3	2	2	3710.48214	1	1	1	2	3	3	6	4	5	2	2	2	2	2	2	2	200	5	200	
38	1	87	2	2	23.84202154	1	3	6	3	5	3	2	2	1981.171775	1	1	1	4	4	4	6	3	4	2	2	2	2	2	2	2	200	200	200	
39	2	52	2	2	26.0639063	1	3	6	3	5	3	2	2	2183.32057	1	1	1	3	4	4	6	3	5	1	2	2	2	2	2	2	2	-99	-99	-99
40	2	87	2	2	26.36678201	1	2	4	3	3	4	7	4	1138.229183	3	1	3	4	2	1	6	2	4	2	2	3	3	2	3	2	200	100	200	
41	1	85	2	2	25.3272726	-99	3	6	1	5	1	4	7	1804.136714	1	1	2	4	3	1	6	2	4	2	2	2	2	2	2	1	1	200	200	200
42	1	86	2	1	19.28199303	1	3	6	3	5	3	2	2	1581.470152	2	1	1	1	5	5	4	4	4	1	1	2	2	2	2	2	300	100	200	
43	1	85	2	4	25.59491188	-99	3	6	1	5	1	4	7	1718.48773	1	1	1	4	4	4	6	3	4	2	2	2	2	2	2	2	200	100	0	
44	1	66	3	3	19.73789252	1	3	6	1	5	1	2	2	893.345669	1	1	1	4	4	3	6	5	4	2	2	2	2	2	2	2	200	180	300	
45	2	63	2	4	26.04166067	1	3	6	1	5	1	2	2	2092.05662	1	1	1	4	5	5	6	4	4	1	1	2	1	1	2	2	200	5	200	
46	1	49	2	2	22.95684114	1	3	6	3	5	1	3	2	1613.98793	1	1	1	4	4	4	6	4	5	1	2	2	1	1	1	1	-99	-99	-99	
47	1	47	2	2	17.10351752	1	3	6	7	7	5	3	2	3078.77949	1	1	1	3	4	4	3	4	4	1	2	1	1	1	1	2	200	50	50	
48	2	40	2	2	24.33314127	2	3	6	3	5	3	2	2	2501.88848	1	1	1	1	4	2	-99	4	4	2	2	2	2	2	2	2	-99	-99	-99	
49	2	52	2	2	30.48181654	1	4	7	2	3	4	7	4	1271.11584	1	1	1	4	4	4	6	4	4	2	2	2	2	2	2	2	100	200	80	
50	2	89	2	2	24.18162103	2	4	7	6	4	5	3	2	2802.44545	4	-99	2	5	5	6	7	6	-99	2	2	2	3	2	3	2	200	50	200	
51	2	49	2	4	36.78134903	1	3	6	2	5	2	2	2	3987.961802	1	1	1	4	4	4	6	4	4	1	2	2	2	2	1	1	200	200	160	
52	2	54	3	1	25.10284786	1	3	6	3	5	3	2	2	729.0984676	1	1	1	3	4	3	6	4	4	4	2	2	2	1	1	1				

Sheila all data 02.03.22

SampleID	Gender	Age	Smoking	Alcohol	BMI	Brewery	Diagnosis2	Diagnosis	Site	Sleeping	Silencer	Differentiation	Differentiation2	NT	CDX2	MMR2	gypa	MMR1	NCRT	Initiation	Finstage	pTSD	Tstage	Tclass	LInvolvement	Metastases	Mucinous	EMV	EMV2	LI	Lx2	NTHscore	NTSR1hours	NTSR3hours
1	2	68	3	2	28.144827	1	3	6	3	5	3	2	2	102.821087	1	1	1	1	1	4	2	2	3	3	2	2	2	2	2	2	2	-99	-99	-99
2	2	88	3	2	27.8228748	1	2	3	1	1	4	7	4	946.4104741	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	300	200	300	
3	2	67	3	4	36.70720419	1	2	2	2	2	4	7	4	693.1603197	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	300	200	300	
4	2	84	3	2	21.65510814	-99	3	6	1	5	1	2	2	1185.892824	1	1	1	4	4	4	6	3	7	1	2	2	2	2	2	2	340	290	90	
5	1	75	3	1	27.734075	1	2	3	2	2	4	7	4	469.6607015	3	1	3	4	1	1	6	6	7	3	2	3	3	2	2	2	300	200	300	
6	2	75	2	4	29.5725668	1	3	6	3	5	3	2	2	1765.01181	1	1	1	3	4	4	3	4	4	2	2	2	2	2	2	2	300	200	300	
7	2	50	3	2	28.8705242	1	3	6	3	5	3	3	2	538.5343347	1	1	1	1	4	2	-99	3	3	2	2	2	1	2	2	2	300	100	300	
8	1	40	2	1	31.73626073	1	3	6	3	5	3	2	2	1023.383247	4	1	1	2	1	1	6	1	7	2	2	2	2	2	2	2	-99	-99	-99	
9	1	32	1	3	21.03421487	2	3	6	3	5	3	2	2	970.2239317	1	1	1	3	4	4	3	5	5	1	2	2	2	2	2	2	-99	-99	-99	
10	2	86	2	4	23.15440779	1	1	1	4	5	4	6	4	1021.134374	3	1	3	4	7	6	6	6	7	3	2	3	3	2	3	2	0	0	0	
11	1	74	2	2	22.6610732	1	2	2	3	3	4	7	4	1038.372381	3	1	3	4	1	1	6	6	7	3	2	3	3	2	2	2	0	0	0	
12	1	87	2	2	32.86137369	1	2	4	3	3	4	7	4	407.7326667	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	240	0	270	
13	2	51	2	1	24.7421846	1	3	6	3	5	3	2	2	889.664963	1	1	1	2	5	5	5	5	5	2	1	2	1	2	2	300	200	100		
14	1	74	3	4	21.07719623	1	1	1	4	5	4	6	4	1486.107619	3	1	3	4	7	6	6	6	7	3	2	3	3	2	3	2	-99	-99	-99	
15	1	73	3	2	21.1502942	2	2	3	5	4	4	7	4	1726.614333	3	1	3	4	2	1	6	6	7	3	2	3	3	2	3	2	100	200	300	
16	1	68	1	2	21.1160508	1	2	2	3	3	4	7	4	2106.181347	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	-99	-99	-99	
17	1	53	3	2	18.36457391	1	2	5	1	1	4	7	4	2005.609697	3	1	1	3	4	1	1	6	6	7	3	2	3	3	2	2	200	200	200	
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19	1	68	2	2	30.71248447	1	1	1	4	5	4	6	4	3392.545769	3	1	3	4	7	6	6	6	7	3	2	3	3	2	3	2	0	0	0	
20	2	62	1	3	26.29737785	1	2	3	1	1	4	7	4	2004.888284	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	200	200	200	
21	1	86	3	2	24.23411065	1	2	3	1	1	4	7	4	1028.799334	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	100	200	200	
22	1	54	2	2	26.3	1	1	1	4	5	4	6	4	2016.410181	3	1	3	4	7	6	6	6	7	3	2	3	3	2	2	2	0	0	0	
23	2	76	3	2	26.3	1	1	1	4	5	4	6	4	737.770336	3	1	3	4	7	6	6	6	7	3	2	3	3	2	3	2	0	0	0	
24	1	47	1	2	27.31481481	1	2	3	5	4	4	7	4	1264.191956	3	1	3	4	1	1	6	6	7	3	2	3	3	2	3	2	200	100	200	
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27	2	56	3	3	28.08768107	1	3	6	2	5	2	4	2	3044.641055	1	1	1	4	5	5	6	5	4	2	1	2	2	2	2	2	200	120	200	
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30	2	59	2	2	23.35643772	1	3	6	1	5	1	2	2	1070.212537	1	1	1	2	4	3	3	4	4	2	2	2	2	2	2	2	300	160	300	
31	2	86	3	2	27.62047812	1	3	6	1	5	1	3	2	1247.762664	1	1	2	4	3	3	6	-99	4	2	2	1	2	2	2	2	200	200	200	
32	2	84	2	1	33.52082829	2	3	6	1	5	1	2	2	231.4370075	1	1	1	4	5	5	6	5	5	1	1	2	2	1	1	1	-99	-99	-99	
33	1	84	2	1	10.22222222	1	3	6	1	5	1	2	2	1503.926881	1	1	1	4	2	4	6	3	4	1	2	2	2	1	1	1	-99	-99	-99	
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35	2	74	3	1	27.1823471	1	3	6	2	5	2	2	2	889.664963	1	1	1	4	4	3	6	4	4	2	2	2	2	2	2	2	100	180	300	
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38	1	87	2	2	23.84630154	1	3	6	1	5	1	5	3	1981.917727	1	1	2	2	4	4	6	4	5	1	1	1	1	1	1	1	200	200	200	
39	1	52	2	2	26.39689691	1	3	6	3	5	3	2	2	1985.160527	1	1	1	4	3	3	6	2	4	1	2	2	2	2	2	2	-99	-99	-99	
40	2	87	2	2	26.39627691	1	2	4	1	3	4	7	4	1138.29183	3	1	3	4	2	1	6	2	7	3	2	3	3	2	3	2	200	100	200	
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42	1	66	2	1	19.28796335	1	3	6	3	5	3	4	4	1581.470152	2	1	1	1	5	5	4	4	4	1	1	2	2	2	2	2	300	100	200	
43	2	67	3	4	35.30864198	-99	3	6	3	5	3	4	4	2158.46773	1	1	1	1	4	3	3	4	4	2	2	2	2	2	2	2	200	0	200	
44	1	66	3	3	19.77899392	1	3	6	1	5	1	2	2	659.344699	1	1	1	4	4	3	6	5	4	2	2	2	2	1	1	2	200	160	300	
45	2	82	4	2	26.31166897	1	3	6	1	5	1	2	2	1992.566661	1	1	1	4	4	3	6	4	4	1	1	2	2	2	2	2	200	100	200	
46	1	49	2	2	22.95684114	1	3	6	1	5	1	3	2	1613.96739	1	1	1	4	4	4	6	4	4	1	2	2	1	1	1	1	-99	-99	-99	
47	1	47	2	2	17.10351752	1	3	6	3	5	3	5	3	2078.777349	1	1	1	3	4	4	3	4	4	1	2	1	1	1	2	2	200	50	50	
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49	2	52	2	2	30.58181054	1	4	7	3	5	4	4	3	1271.1094	1	1	1	-99	3	3	6	5	5	2	2	2	2	1	1	2	100	10	180	
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51	2	49	2	4	36.79134632	1	3	6	2	5	3	2	2	2987.561602	1	1	1	3	4	4	4	4	4	3	2	2	2	2	2	2	300	200	180	
52	2	54	3	1	25.16384786	1	3	6	3	5	3																							

84	1		1	5	1	60.75068803	1	72	1.68	0	25.61020408	1	26	1	1	1/26/2017	5	10/31/2017	10/31/2017	196.891	1	3	0	0	0	0	2	1	1	0	0	0			
84	0		1	2	1	82.47222222	3				0	0	2	0	4/30/2015	2	12/31/2016		145.825																
85	0		1	5	1	64.39729603	3	64	1.6	0	25	1	25	1	1/26/2017	2	9/6/2017	9/6/2017	236.278		2	2	0	0	0	0	1	0	2	0	1	0			
86	0		1	2	1	51.24931507	3	79	1.73	1	26.3958034	1	14	1	1/26/2017	5	10/31/2017	10/31/2017	362.069																
86	0		1	1	1	60.83333333	3				0	0	2	0	1/14/2016	2	12/31/2016		372.030																
87	0		1	3	1	55.12056795	3	75	1.78	0	23.67135363	1	24	1	1/26/2017	2	9/6/2017	9/6/2017	446.434																
87	0		1	2	1	61.66444444	2				0	0	2	0	1/14/2016	1	12/31/2016		846.305																
88	0		1	1	1	66.16712329	2	56	1.64	0	20.82069992	1	24	1	1/27/2017	2	9/6/2017	9/6/2017																	
88	0		1	1	1	70.01111111	2				0	0	2	0	1/14/2015	1	12/31/2016		122.545																
89	0		3	2	1	56.41100589	1	110	1.95	0	28.9833562	1	27	2	0	1/27/2017	2	9/6/2017	9/6/2017	212.33															
90	0		2	1	1	73.50277778	3				0	0	2	0	1/14/2015	1	12/31/2016		266.95																
90	0		2	7	2	72.54246668	3	76	1.73	1	25.39343112	1	26	1	1/27/2017	2	9/6/2017	9/6/2017	193.239																
90	0		5	2	1	70.80833333	3				0	0	2	0	1/14/2015	1	12/31/2016		302.335		1	4	1	1	0										
91	0		2	1	1	80.61399863	1	66	1.65	0	24.24242424	1	22	1	1/27/2017	5	10/31/2017	10/31/2017	516.201																
91	0		1	1	1	56.04444444	2				0	0	2	0	1/14/2015	1	12/31/2016		298.13																
92	0		1	1	1	47.16255556	1	67	1.53	0	24.67165032	1	26	1	1/27/2017	2	9/6/2017	9/6/2017	261.223																
93	0		1	1	1	55.16164384	4	95	1.73	0	31.74179889	1	24	1	1/27/2017	5	10/31/2017	10/31/2017	215.365																
93	0		1	1	1	70.88888889	3				0	0	2	0	1/14/2015	1	12/31/2016		221.245																
94	0		1	1	1	58.9745121	1	54	1.63	0	20.32443826	1	25	1	1/31/2017	2	9/6/2017	9/6/2017	215.071																
95	0		1	1	1	53.34246575	1				0	1	24	1	1/26/2017	5	10/31/2017	10/31/2017	197.295																
96	0		1	2	1	56.19444444	2				0	0	2	0	1/21/2016	1	12/31/2016		169.65																
96	0		1	1	1	34.48219178	2	54	1.6	0	21.08375	1	24	1	1/26/2017	2	9/6/2017	9/6/2017	188.719																
96	0	10	3	4	1	66.76644444	1				0	0	2	0	1/21/2016	1	12/31/2016		378.55																
97	0		3	2	1	72.05479452	2	0			0	1	36	2	0	1/26/2017	5	10/31/2017	10/31/2017	521.449															
97	0		1	1	1	70.70505056	3				0	0	2	0	1/21/2015	1	12/31/2016		182.11																
98	0		3	4	1	70.71908923	3				0	0	2	0	1/26/2017	5	10/31/2017	10/31/2017	264.279																
98	0		5	2	1	80.55555556	2	32	1.57	0	12.96227109	0	0	2	0	1/21/2015	1	12/31/2016		338.7		1	2	0	0	0									
99	1		1	5	2	78.86493151	3	0			0	1	25	1	1/26/2017	5	10/31/2017	10/31/2017	294.789		1	4	0	0	0	2	0	1	0	1	0	0	0		
99	0		5	1	1	82.20833333	1				0	0	2	0	1/21/2015	1	12/31/2016		480.87																
100	0		1	2	1	53.67942035	1	82	1.8	0	25.30864189	1	27	1	1/26/2017	1	9/6/2017	9/6/2017	255.308																
100	0		2	1	1	76.30277778	3				0	0	2	0	1/21/2015	1	12/31/2016		91.493																
101	0		1	1	1	68.1705691	2				0	1	26/2017	1	1/26/2017	2	9/10/2017	9/6/2017	744.071																
101	0		1	1	1	39.31944444	3				0	0	2	0	1/21/2015	1	12/31/2016		199.8																
102	0		2	1	1	68.20568904	1	83	1.75	0	27.1004082	1	27	1	1/26/2017	5	10/31/2017	10/31/2017	366.735																
102	0		2	1	1	72.9186667	3				0	0	2	0	1/21/2015	1	12/31/2016		600.72																
102	0		1	2	1	80.44388889	1	86	1.83	0	25.68037455	1	26	1	1/26/2017	2	9/6/2017	9/6/2017	321.142																
102	0		3	1	1	73.26444444	2				0	0	2	0	1/21/2015	1	12/31/2016		133.535																
104	0	0.3	1	4	2	47.01977808	2	78	1.82	0	23.54789609	1	26	1	1/26/2017	5	10/31/2017	10/31/2017	566.124																
104	0		5	1	1	61.95	2				0	0	2	0	1/21/2015	1	12/31/2016		772.87		1	3	0	0	0										
105	0		1	2	1	29.77292274	1	87	1.8	1	26.85185185	1	25	1	1/26/2017	2	9/10/2017	9/6/2017	642.542																
105	0		1	2	1	81.11944444	3				0	0	2	0	1/21/2015	1	12/31/2016		122.545																
106	0		2	1	1	64.70598904	1				1.63	0	1	1	1/26/2017	2	9/10/2017	9/6/2017	545.199																
106	0		1	1	1	59.14722222	2				0	0	2	0	1/20/2015	3	1/7/2017		347.545																
105	0		1	2	1	54.9726027	2	90	1.84	0	26.5831758	1	24	1	1/26/2017	1	9/10/2017	9/6/2017	513.381																
106	0		2	7	1	60.502137	1	65	1.75	0	21.2244989	1	34	1	1/30/2017	5	10/31/2017	10/31/2017	340.464																
106	0		1	1	1	52.9186667	1				0	0	2	0	1/22/2015	3	1/7/2017		299.68																
106	0		1	1	1	26.26573432	2	48	1.72	1	16.22496648	0	0	2	0	1/13/2017	5	9/10/2017	10/31/2017	698.582															
106	0		1	1	1	68.43888889	3				0	0	2	0	1/20/2015	1	12/31/2016		335.445																
110	0		1	2	1	89.00041096	3	86	1.67	0	30.83653053	1	25	1	1/13/2017	2	9/10/2017	9/6/2017	268.893																
110	0		1	2	1	60.5082182	3	68	1.71	0	23.25501864	1	24	1	1/13/2017	2	9/10/2017	9/6/2017	197.231																
111	0	100		4	2	46.60388889	1				0	0	2	0	1/22/2015	1	12/31/2016		236.33																
111	0	9.9	1	4	1	67.68493151	1	65	1.65	0	23.87511478	0	1	1	1/13/2017	3	9/10/2017	9/6/2017	266.142																
111	0		1	1	1	56.66111111	1				0	0	2	0	8/4/2015	1	12/31/2016		149.91																
111	0		1	2	1	68.46576342	3	83	1.88	0	23.48347669	1	24	1	1/13/2017	3	9/10/2017	9/6/2017	415.096																
111	0		3	2	1	73.76111111	1				0	0	2	0	8/4/2015	3	1/7/2017		433.67																

0

9	13	0	3	1	
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