7. PROSPECTIVE PIMONIDAZOLE STUDY

7.1 Introduction

Previous studies have assessed the tumour oxygenation status of head & neck and cervical cancers and yielded promising results (Section 1.6.3). For example, Kaanders et al showed the hypoxic fraction of head and neck cancers predicted outcome in patients undergoing radiotherapy (Kaanders et al. 2002). They recruited 43 patients who received a 500 mg/m^2 infusion of pimonidazole prior to tumour biopsy under general anaesthesia. Assessment of pimonidazole staining was carried out using image acquisition and analysis software. The density of pimonidazole staining in tumours was calculated and patients stratified into two groups by the median value. The 2-year local recurrence (p=0.01) and disease free (p=0.04) survival were significantly worse in the patients with a higher fraction of pimonidazole binding. There are, however, no published studies of the measurements of oxygenation status in oesophagogastric adenocarcinoma**.**

Oxygen electrodes have proved useful in a number of tumour types (Evans et al. 2004; Nordsmark et al. 2003; Parker et al. 2004), but are limited to accessible tumours such as breast, head & neck and cervical tumours. This would be impractical in oesophagogastric tumours due to inaccessibility, thus direct measurements would have to be performed intra-operatively as oxygen electrode probes which can be used endoscopically have yet to be developed. However, there are a number of other ways of measuring hypoxia in human tumours (Table 1.14). In this Chapter, the hypoxia-specific marker pimonidazole (Nordsmark et al. 2003) was chosen to investigate the oxygenation status of a prospective series of patients with oesophagogastric adenocarcinoma. The aim was to assess whether the density of pimonidazole staining can be used to predict prognosis. It would also be important to correlate oxygenation results with the potential hypoxia related markers (HIF-1α, Glut-1, CA-9, Epo, VEGF) to gain further insight into their functional roles in oesophagogastric cancer. Several studies have carried out this work in other tumour sites (Table 7.1). This chapter details the work that went in to setting up this study, some preliminary results and a discussion of future aims.

Table 7.1 Previous studies that have used pimonidazole to assess hypoxia in human tumours.

Correlation	Tumour location	Sub-type	No.	Findings	Reference
Positive	Skin or bladder cancer	SCC/ TCC	6/14	Statistical correlation between pimonidazole staining and CA-9 expression	(Wykoff et al. 2000)
	Head & neck or cervical cancer	SCC	84	Statistical correlation between pimonidazole staining and involucrin expression	(Raleigh et al. 2000)
	Bladder cancer	TCC	21	Statistical correlation between Glut-1 and CA-9 expression and pimonidazole staining	(Hoskin et al. 2003)
	Cervical cancer	SCC	42	Statistical correlation between Glut-1 and CA-9 expression and pimonidazole staining	(Airley et al. 2003)
	Cervical cancer	SCC	99	Weak statistical correlation between HIF-1α and pimonidazole staining	(Hutchison et al. 2004)
	Cervical cancer	SCC/ Adeno	N/A	Hypoxic fraction measured by the comet assay	(Olive et al. 2001b)
	Head & neck	SCC	42	Worse 2-year locoregional control (p=0.01) and disease-free survival (p=0.04).	(Kaanders et al. 2002)
Negative	Cervical cancer	SCC	28	No correlation between oxygen status measured by oxygen electrodes and pimonidazole	(Nordsmark et al. 2001)
	Head & neck or cervical cancer	SCC	18	No correlation between pimonidazole staining and VEGF expression	(Raleigh et al. 1998)
	Head & neck or cervical cancer	SCC	84	No correlation between pimonidazole staining and metallothionine expression	(Raleigh et al. 2000)
Inverse	Cervical cancer	SCC	5	PCNA	(Kennedy et al. 1997)
	Head & neck	SCC	20	Blood vessels	(Begg et al. 2001)

SCC = squamous cell carcinoma; TCC = transitional cell carcinoma; Adeno = adenocarcinoma; PCNA = proliferating cell nuclear antigen; N/A = not available; CA-9 = carbonic anhydrase-9; Glut-1 = glucose transporter-1; VEGF = vascular endothelial growth factor; HIF = hypoxia-inducible factor.

7.2 Aims

The primary aim of this chapter was to establish a prospective study to examine hypoxia in oesophagogastric adenocarcinoma. Although some preliminary results will be discussed this prospective study has yet to accrue its target number of patients for final analysis. The future aims of this research are to:

- Assess tumour hypoxia as measured by pimonidazole administration and immunohistochemistry of adduct staining in oesophagogastric cancer
- Assess whether tumour hypoxia as measured by pimonidazole can be used to yield prognostic information
- Perform RNA extraction on the biopsy samples collected for cDNA microarray analysis; with a final aim to analyse the samples to find a 'hypoxia' profile for oesophagogastric cancer.

7.3 Setting up of the study

7.3.1 Sample size and statistical power calculations

Before ethical submission, discussions were held with a statistician to calculate the sample size required to assess whether pimonidazole staining can be used to predict prognosis in this group of patients. An estimate of survival in the relevant group of patients is given in Figure 7.1. This Figure excludes post operative deaths, known to be about 10% and so the derived sample size will need to be inflated accordingly.

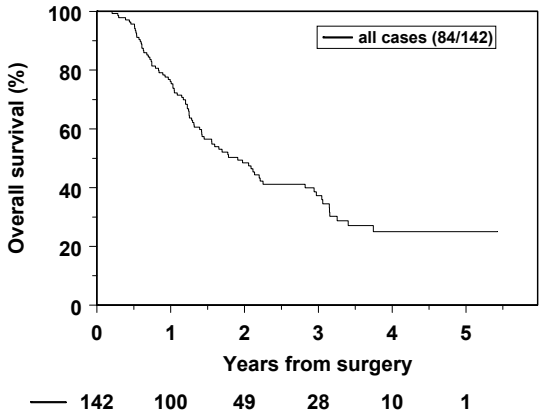

Figure 7.1 Kaplan-Meier estimate of survival from a previous cohort of patients with oesophagogastric adenocarcinoma treated with surgery at SMUHT.

It is thought that the measured marker will be prognostic and it is intended to split the cases at the median value and estimate and compare the survival experiences. Notionally this should lead to two equally sized groups, one with somewhat better outcome than depicted in Figure 7.1 and the other with somewhat worse outcome. Hazard ratios of 2/3 and 3/2 were taken relative to Figure 7.1 (giving a hazard ratio of 2.25 relative to each other). Modelling by piecewise exponential distributions gives the anticipated survival curves shown in Figure 7.2.

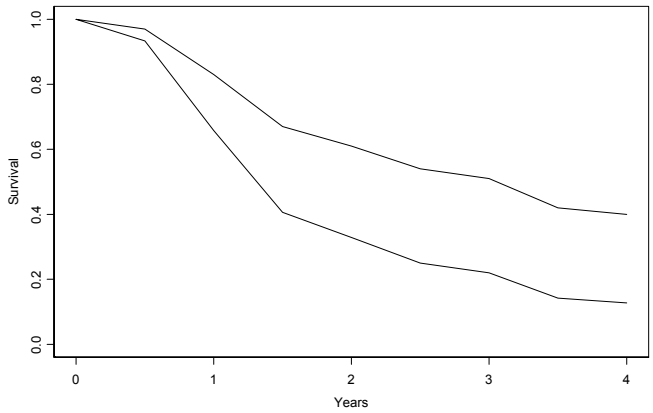

Figure 7.2 Piecewise exponentials from which the stimulations have been drawn

The plan is to accrue for 2.5 years at a constant rate of 40 per year (yielding 100 patients in total – approximately 90 for the prognostic analysis after removal of post-operative deaths). The final patient will be followed up for eighteen months and an analysis performed at four years.

Statistical power calculation

Using a simulation approach with 1000 repeats 22 deaths are expected in the good prognosis group and 34 deaths in the poor prognosis group with 56 deaths in total by the time of the analysis if the above model holds. In this situation, the log-rank test of equality of survival curves with a 5% two-sided significance level will have approximately 85% power.

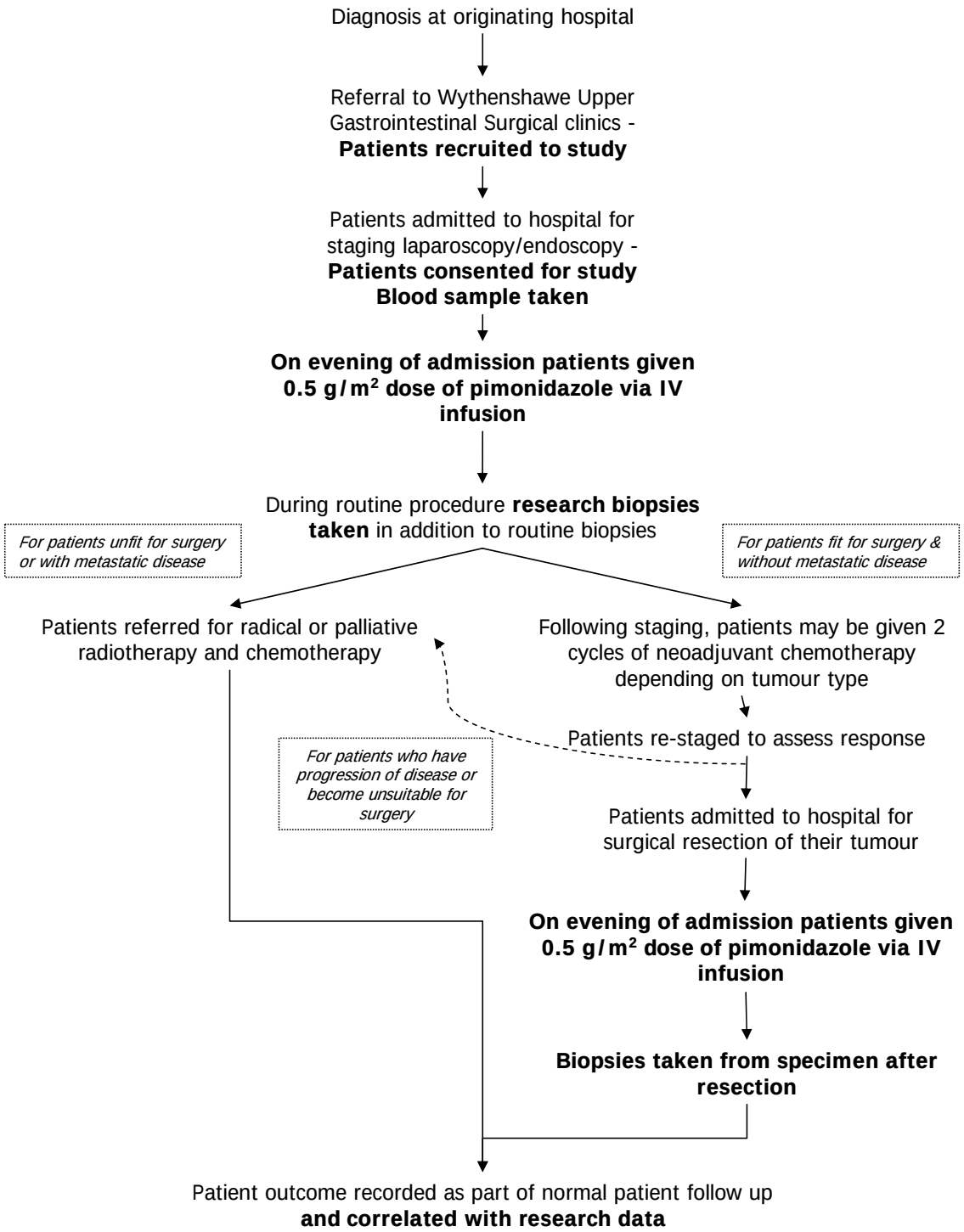

Figure 7.3 Diagram of the patient pathway

7.3.2 Protocol development

The study protocol was produced and agreed by December 2004. Figure 7.3 shows the diagram of the patient pathway and illustrates how the research study links in with routine patient management. Ethical, MHRA and R&D approval was obtained (Appendix II). Ethical approval was granted by South Manchester Local Research and Ethics Committee in March 2005 (Appendix II). An example of the patient information sheet used in the study is provided in Appendix II. The first patient was recruited in to the study in July 2005. Between July 2005 and January 2006 10 patients were recruited.

7.3.3 Summary of patients recruited

Fifteen patients were approached and nine patients agree to take part in the study. Eight patients had pimonidazole administered prior to staging laparoscopy and research biopsies were taken at staging endoscopy. Table 7.2 summarises the details of these patients. Three of the patients proceeded to surgical resection where additional research biopsies were taken from the resected surgical specimen (after additional doses of pimonidazole). In addition, one other patient had pimonidazole administered prior to surgical resection (this patient was recruited into the study having previously had staging laparoscopy prior to the study commencing). The details of the four patients who received pimonidazole prior to surgical resection are shown in Table 7.3. No adverse events were observed during or after pimonidazole administration.

Table 7.2 Summary of the patients recruited so far who had research biopsies taken at staging laparoscopy

Study code	Age, Sex	Site of tumour	Sub-site	Findings at staging laparoscopy	Proceeded to surgical resection, surgical procedure performed
GOP 001	68, F	Stomach	Body (linitis plastica)	Peritoneal metastases and ascites	No
GOP 002	66, M	Stomach	Proximal third	Clear	Yes, total gastrectomy with D2 lymphadenectomy
GOP 003	77, M	Stomach	Pylorus	Clear	No (unfit for surgery), gastro-duodenal stent inserted as palliation
GOP 004	82, M	Stomach	Incisura	Evidence of serosal invasion, otherwise clear	Yes, subtotal gastrectomy
GOP 005	69, M	Oesophagus	GOJ	Clear	OEO-2 neo-adjuvant chemotherapy followed by surgery
GOP 006	69, M	Oesophagus	Lower third	Clear	No, bone metastases on PET imaging
GOP 008	64, M	Oesophagus	GOJ	Clear	OEO-2 neo-adjuvant chemotherapy; awaiting surgery
GOP 009	74, F	Stomach	Fundus	Clear	Yes, total gastrectomy, splenectomy and D2 lymphadenectomy

Table 7.3 Summary of the patients recruited so far who had research biopsies taken from the surgical specimen after resection

Study code	Site of tumour	Sub-site	TNM stage	Diff	Involvement of the resection margins
GOP 002R	Stomach	Proximal third	T3 N3 M0	Poor	No
GOP 005R	Oesophagus	GOJ	T3 N1 M0	Poor	Yes (CRM+)
GOP 007R	Oesophagus	GOJ	T3 N1 M0	Poor	No
GOP 009R	Stomach	Fundus	T4 N1 M1	Poor	No

7.3.4 Pimonidazole adduct staining and scoring

Formalin fixed tumour tissue obtained in this study was stained for pimonidazole adducts. Section 2.8.4 details the immunohistochemical methods used for specimen staining.

Descriptive histopathology

Figure 7.4 (1a) shows strong focal staining in an adenocarcinoma specimen. Figure 7.4 (2a and 3b) shows strong focal staining in a metastatic lymph node deposit. Note in this section that the normal rim of lymph node tissue does not stain with pimonidazole (Figure 7.4 [2a]). Figure 7.4 (3a) shows strong pimonidazole staining in cells adjacent to necrosis. Figure 7.4 (5a) shows inflammation associated with areas of positive pimonidazole staining.

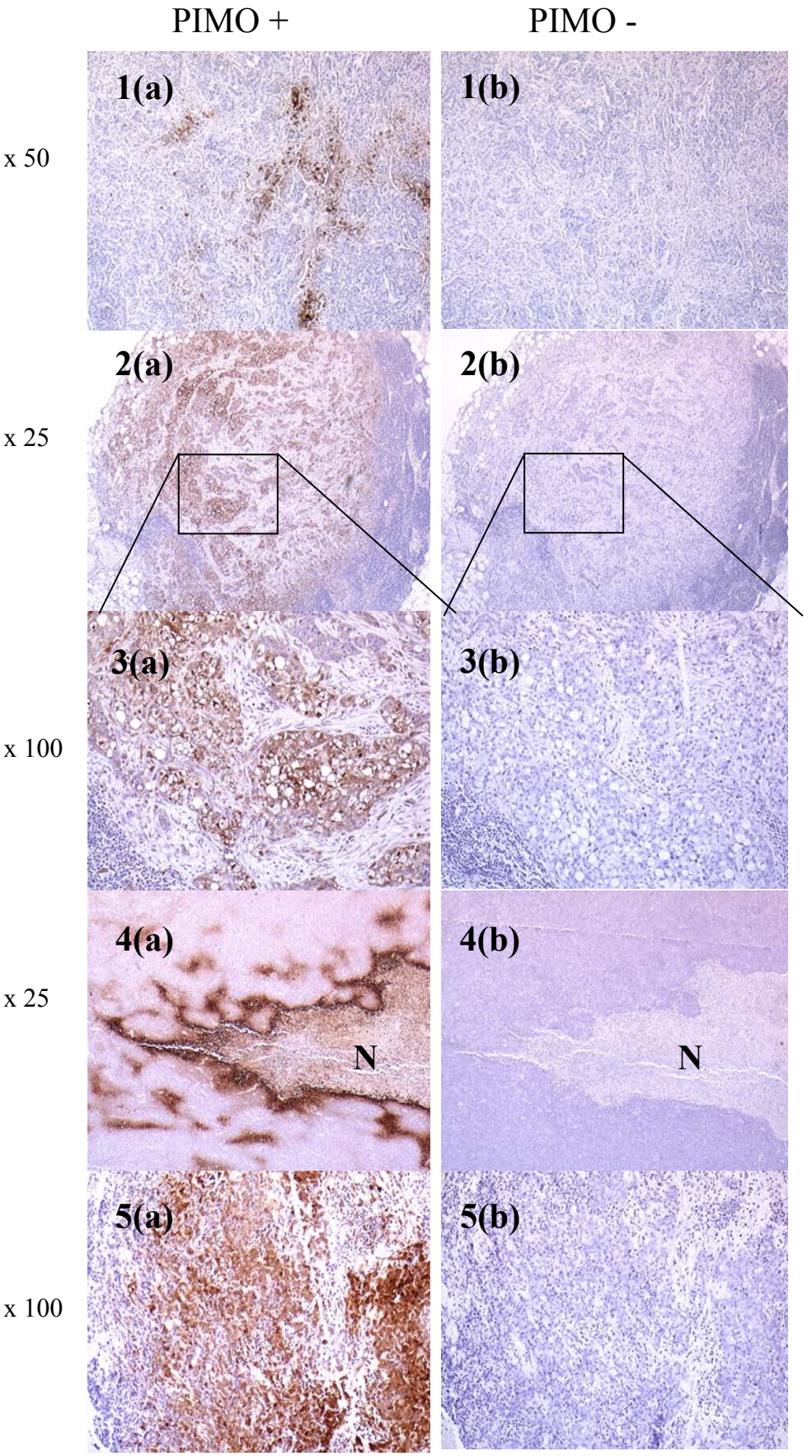

Figure 7.4 Photomicrographs of representative sections of pimonidazole adduct staining.

Postive (Pimo +) and the corresponding negative (Pimo -) control slides are shown; N = necrosis.

Pimonidazole scoring

Eight endoscopic biopsy specimens were stained for pimonidazole adducts. Table 7.4 shows the intensity and percentage of pimonidazole staining found in these biopsies. Two out of the eight biopsies had strong (10% & 30%) staining for pimonidazole (25%); whereas the remaining biopsies were negative. One biopsy obtained contained no tumour material.

All tumour material from three patients who received pimonidazole prior to surgical resection was stained for pimonidazole adducts. Material from GOP005R has yet to be processed. The intensity and percentage scores from each tumour block number from each patient are shown in Table 7.5. GOP002R had a median pimonidazole staining of 1 (0 to 10%); this is in agreement with the initial endoscopic biopsy from this patient which was negative for pimonidazole. GOP007R and GOP009R had more extensive pimonidazole staining; with median pimonidazole staining of 40% (range 0 - 90%) and 20% (5 - 95%) respectively. Heavy staining in GOP009R blocks were found in areas of metastatic lymph node, liver metastasis and tumour invading the pancreas. The preceding endoscopic biopsy of GOP009 had evidence of pimonidazole staining.

Table 7.4 Pimonidazole adduct scoring on the endoscopic biopsies specimens

	Pimonidazole staining	
Study code	**Intensity**	**Percentage**
GOP001	Negative	0
GOP002	Negative	0
GOP003	Negative	0
GOP004	*	*
GOP005	Negative	0
GOP006	Strong	10%
GOP008	Negative	0
GOP009	Strong	30%

* no tumour visualised

Table 7.5 Pimonidazole adduct scoring on the resected tumour specimens

GOP 002R			GOP 007R			GOP 009R		
Block No.	**Intensity**	**%**	**Block No.**	**Intensity**	**%**	**Block No.**	**Intensity**	**%**
A1	Neg	0	A8	Strong	75	A5	Strong	20
A2	Weak	2	A9	Strong	40	A7	Strong	10
A3	Weak	1	A10	Strong	50	A8	Strong	25
A4*	Strong	5	A11	Strong	40	A9	Strong	40
A5	Mod	5	A12	Strong	10	A10	Strong	30
A10	Neg	0	A14	Mod	60	A11	Strong	20
A12	Weak	2	A17	Mod	60	A12*	Strong	60
A13	Strong	<1	A18	Strong	10	A13	Strong	20
A14	Neg	0	A19	Strong	2	A17	Strong	5
A15	Neg	0	A20	Strong	95	B†	Strong	95
A16	Strong	10	A21	Mod	40	C‡	Strong	20
A17	Weak	<1	A22	Mod	10			
A18	Neg	0	A23	Mod	10			
B1	Strong	<1	A24	Neg	0			
			B1	Strong	75			
			C2	Strong	50			
			C3	Strong	80			

* = metastatic lymph node; † = liver metastasis; ‡ = pancreatic invasion

7.3.5 RNA extraction

Section 2.8.5 details the method of RNA extraction from normal and tumour tissue obtained in this study. Figure 7.5 shows an example of an extraction trace which is produced using the Nanodrop spectrophotometer used to assess RNA concentration and general quality. Five μg of total RNA free from genomic DNA is required for successful microarray analysis. Table 7.6 shows the extraction values of total RNA that were isolated using Trizol with further cleaning using a Qiagen RNeasy minikit. For the sample to be suitable for microarray analysis the minimum RNA concentration is 0.6 μg/ul and the 260/280 nm ratio of the Total RNA should be 1.9 – 2.1. A ratio of ~ 2.0 is generally accepted as 'pure' for RNA. Prior to microarray analysis the Agilent Bioanalyser is used to assess RNA quality.

The success rate for RNA extraction was high; there were only three inadequate samples: two tumour and one normal tissue sample all taken from small endoscopic biopsy specimens (Table 7.6). All samples had an A260/280 ratio between 1.9 to 2.1, indicating that they would be suitable for successful microarray analysis.

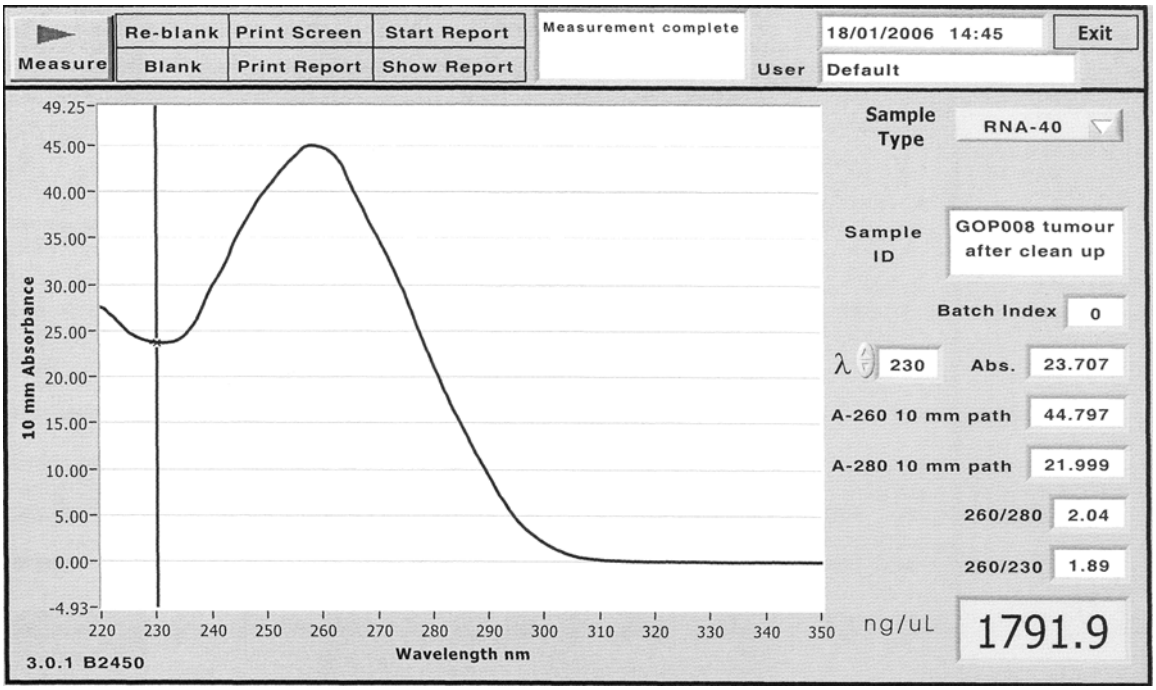

Figure 7.5 Example of an RNA extraction trace which is produced using the Nanodrop spectrophotometer

Table 7.6 Table of RNA extraction results in the patients recruited so far in to the prospective study

Study Code	Sample Type	Weight (mg)	Conc (ng/ul)	Vol (µl)	Total RNA (µg)	Yield (µg/mg)	A260/ 280 ratio†	A260/ 230 ratio
GOP 001	N	20	136.3	30	4.0 *	0.2	2.03	0.85
GOP 001	T	30	703.7	30	21.0	0.7	2.07	1.58
GOP 002	N	22	524.2	10	5.2	0.23	1.97	1.79
GOP 002	T	55	309.3	30	9.2	0.17	1.90	1.33
GOP 002	T (R)	43	414.4	30	12.4	0.3	1.89	0.97
GOP 003	N	-	1276.3	30	38.3	-	2.04	1.59
GOP 003	T	23	371.4	30	11.1	0.48	2.08	0.83
GOP 004	N	-	552.0	30	16.6	-	1.96	1.66
GOP 004	T	26	289.3	10	2.8 *	0.11	2.04	1.96
GOP 005	N	-	730.5	30	21.9	-	2.05	1.93
GOP 005	T	40	325.7	10	3.3 *	0.08	2.06	1.64
GOP 006	N	8	455.6	30	13.7	1.71	1.97	1.91
GOP 006	T	27	554.7	30	16.6	0.61	2.00	1.64
GOP 007	N (R)	-	1149.7	30	34.5	-	2.04	1.91
GOP 007	T (R)	33	248.8	30	7.5	0.22	2.14	0.63
GOP 008	N	-	686.0	30	20.6	-	2.05	1.76
GOP 008	T	30	1791.9	30	53.8	1.79	2.04	1.89
GOP 009	N	-	953.1	30	28.6	-	2.04	1.96
GOP 009	T	28	539.7	30	16.2	0.58	2.01	1.97
GOP 009	N (R)	-	1771.6	30	53.1	-	2.03	2.21
GOP 009	T (R)	-	1712.0	30	51.36	-	2.03	2.24
GOP 010	N (R)	-	2187.4	30	65.6	-	2.03	1.92
GOP 010	T (R)	-	3163.8	30	94.91	-	1.96	2.01

N = normal mucosa; T = tumour tissue; R = taken at resection; * = insufficient RNA for microarray analysis; † = range required for successful microarray analysis 1.9 – 2.1; Values shown are after cleaning using the Qiagen RNeasy mini-kit

7.4 Discussion and conclusion

Preliminary results suggest hypoxia is found in around a quarter of oesophagogastric cancers. This appears to be the first study showing hypoxia, as measured by pimonidazole staining, is present in these tumours. Pimonidazole is the most validated exogenous marker of tumour hypoxia (Hutchison et al. 2004; Kaanders et al. 2002; Overgaard 1994). It undergoes reduction at low cellular oxygen tensions ($p0_2$ <10mmHg). It is thought to identify mainly chronic (diffusion-limited hypoxia) rather than acute hypoxia as around 30 min hypoxia is required to form these complexes. Therefore, expression is mainly found at a distance from blood vessels.

The pimonidazole data collected for this Chapter suggest inter and intra-tumour variability of hypoxia in oesophagogastric cancers, which has been reported in other tumour types studied (Carnell et al. 2006; Goethals et al. 2006a). The preliminary findings presented in this chapter broadly agree with a recent study carried out in resected colorectal adenocarcinoma (Goethals et al. 2006a). It showed that hypoxia was present in all 20 tumours with percentage pimonidazole staining ranging 2 to 38%, median 17%. Similar ranges have been found for head and neck (range 0 to 17%, median 6%) and cervical cancer (1-14%, median 4%) (Kaanders et al. 2002; Olive et al. 2001a). In prostate adenocarcinoma (n=37) the majority stained for pimonidazole [34 of 37 (92%)] compared with the 25% observed here (Carnell et al. 2006). Direct comparison is difficult as studies have used different scoring methods. Nevertheless, although the dataset is small, there appears to be less of a spread of hypoxia compared with other cancers where hypoxia has been studied using pimonidazole. Only 25% of oesophagogastric tumours studied were hypoxic. The early results from the endoscopic biopsies suggest a dichotomous pattern with hypoxia either present or absent.

It would be anticipated that the 25% of patients with pimonidazole expression will have a poor prognosis, but final analysis will be performed after final target accrual. Since leaving the department in February 2006, a further 3 patients have been recruited. The aim is to recruit a further 87 patients over the next 2 years, to assess whether percentage of pimonidazole staining can be used to predict prognosis.