Group Project #16
Biostats 517

SUMMARY

Difluoromethylornithine (DFMO) has shown promise as an agent for prevention of cancer, possibly through its suppression of the synthesis of the polyamines putrescine, spermidine, and spermine. A recent randomized, double-blind, placebo-controlled trial investigated the action of DFMO on polyamines in the colon tissue. One hundred and fourteen (114) patients with a history of colon polyps received either placebo or one of three DFMO doses (0.075, 0.2 or 0.4 g/m^2/day) for 12 months. Putrescine, spermidine, and spermine levels were measured at baseline, after 6 months of treatment, after 12 months of treatment, and 3 months after the discontinuation of treatment. The present analysis addressed the questions of whether DFMO treatment leads to a decrease in polyamine levels in the colon tissue and whether any effect is sustained following cessation of treatment. We divided the sample into placebo and DFMO treatment groups, collapsing the three dose groups into one DFMO group. We then compared polyamine levels in these groups during the treatment period using the mean of month 6 and month 12 measurements, and following the discontinuation of treatment using the month 15 measurement. Mean spermidine level was found to be significantly lower in the DFMO group compared to the placebo group during the treatment period (mean difference = -0.64 µmole/mg protein; 95% CI: -1.09,-0.20; two-sided p = 0.006). The mean ratio of spermidine to spermine was also found to be significantly lower in the DFMO group during the treatment period (mean difference = -0.13; 95% CI: -0.22, -0.04; two-sided p = 0.006). No significant differences were found in these measures three months after the discontinuation of treatment. There were no significant differences in putrescine levels between the placebo and DFMO groups during or after the treatment period. Further studies are needed to explore the action of DFMO on polyamines and its use as part of cancer prevention strategies.

Comment [A1]: Sex, age?

Comment [A2]: Somewhere in here you want to talk about dropout, which was somewhat does dependent in this study and therefore an important result.

Comment [A3]: While this might be acceptable for a primary analysis, you do not want to do this for absolutely every analysis. Hence you would not want to word it this way in the abstract.

Comment [A4]: It is just as important to show estimates and CI for nonsignificant results.

BACKGROUND

Putrescine, spermidine and spermine are polyamines present in eukaryotic and prokaryotic cells. They are highly regulated players in cellular proliferation and differentiation in normal and neoplastic cells. The decarboxylation of ornithine, the precursor of putrescine, by ornithine decarboxylase (ODC) is the rate-limiting step in this biosynthetic pathway [1]. Putrescine's reaction is triggered by ODC, which is followed by the production of spermidine and spermine. The induction of ODC in mice models has been shown to lead in the accumulation of polyamines, which is thought to result in tumor progression [2]. In other words, inhibition of ODC leads to the suppression of polyamine synthesis. Such inhibition could result in the reduction of cell proliferation, so compounds that target this reaction could implicate cancer treatment potential.

Research aimed at understanding how polyamines relate to the cell cycle yield a consensus that putrescine, spermidine and spermine all increases in a biphasic fashion during cellular growth with high levels observed during late G_1 and the onset of S phase of the cell cycle. In fact, spermidine in *in vitro* studies shows a linear correlation with specific growth rate which may indicate that spermidine accumulation is an event primarily associated with the process of cell replication [3].

Difluoromethylornithine (DFMO) is an inhibitor of polyamine synthesis that selectively targets ODC by forming an irreversible covalent bond, and thus, stops the proliferation of cells *in vivo* [4]. The inhibition of ODC by DFMO causes marked cell depletion of putrescine and

spermidine [3]; therefore, scientist hypothesized that reducing these polyamines are key in decreasing cell proliferation. To further support the notion that polyamines are closely tied to cell growth, several animal studies were performed to show that inhibition of ODC leads to decreased putrescine and spermidine, and halt proliferation *in vivo*. Furthermore in one study, when exogenous polyamines were reintroduced, cell growth was reinitiated substantiating the role of polyamines in cell growth [3].

The interaction between ODC and DFMO in the intestinal growth process has been recently investigated by Luk *et al*. [1], who confirmed reductions of ODC and polyamines in intestinal cells after DFMO treatment. Additionally a study by Celano *et al*. [5] showed that polyamine suppressed cells decreased the expression of c-myc, a proto-oncogene. These studies suggest that ODC inhibition may have the potential of inhibiting the expression of proto-oncogene in human intestinal cells.

Previous DFMO clinical trials have demonstrated dose-limiting toxicities at varying concentrations ranging from 1.75 g/m^2 three times per day to 3.0 g/m^2/day [6-8] . These trials investigated DFMO as a treatment for cancer patients either with or without an additional chemotherapeutic agent. Levels of putrescine and spermidine were suppressed with DFMO treatment. None of these studies showed improvement in the underlying cancer. Here we report the results of a randomized clinical trial to assess the use of low dose DFMO as an agent to prevent colon cancer in patients diagnosed with colon polyps.

QUESTIONS OF INTEREST

This statistical analysis aims to address the following questions:

1. Does treatment with DFMO lead to a decrease in polyamine levels within colon polyp tissue?
2. Is any effect of DFMO on colon polyp polyamine levels apparent after treatment is stopped?

The client requested that we address if safe doses of DFMO result in polyamine inhibition. However, from the data provided in this study it was not possible to address this question. We therefore, focused this analysis on questions 1 and 2 above.

Comment [A5]: Good to note.

SOURCE OF THE DATA

The present analysis employs data from a Phase IIb randomized, double-blinded, placebo-controlled trial to test DFMO suppression of the polyamines spermidine, spermine, and putrescine. The study was conducted at the University of California Irvine, and participants were 114 patients (17 F, 97 M), ages 45-80, with a history of colon polyps. Patients received either placebo or one of three DFMO doses (0.075, 0.2 or 0.4 g/m^2/day). They were followed for a total of 15 months, consisting of a 12-month treatment period followed by a 3-month off-treatment period. Colon polyp biopsies were acquired at baseline, month 6, month 12, and month 15 and were assayed for spermidine, spermine, and putrescine (micromole/mg protein).

We do note the following limitations of the data set. Patterns of missing data were observed, with a greater percentage of missing polyamines measurements occurring in the 0.4 g/m^2/day DFMO dose group than in other treatment groups. This may indicate that the higher dose is not well-tolerated, resulting in a higher dropout rate. Also, the study sample consists of a disproportionate number of male patients (85%). The high representation of males in this sample

Comment [A6]: I would put this in Results, especially because we are worried that it is toxicity, and it may be polyamine associated toxicity. (Or at least we need to worry that it might be, in which case our results are biased.)

may prevent generalization of study results to females if DFMO effects are modified by sex. However, no evidence was found in the literature to suggest that this is the case. Random assignment to treatment group has been implemented to prevent confounding by age, sex, or by unmeasured variables.

Comment [A7]: Yes. (About half the patients from a VA hospital)

STATISTICAL METHODS

Treatment condition was the predictor of interest in the present analysis. Since the dose response relationship was not of primary interest, we dichotomized treatment condition into two levels, placebo and DFMO treatment, to reduce the total number of comparisons performed. The DFMO treatment group included all patients on DFMO doses of 0.075, 0.2 or 0.4 g/m^2/day.

Comment [A8]: It was of interest, or we would not have bothered. That having been said, we do sometimes consider a primary analysis collapsed across treatment groups just in order to demonstrate a response. But descriptively, we would not want to limit all analyses to this.

The outcomes of interest included levels of spermidine, spermine, putrescine, and the ratio of spermidine to spermine, measured both while subjects were on treatment and after discontinuation of treatment. On-treatment levels were compared between DFMO and placebo groups using the mean of the 6 and 12-month measurements; off-treatment levels were compared using the 15-month measurements. All correlations between baseline and follow-up measurements within the treatment groups were less than 0.5; therefore, we did not attempt to adjust for baseline values by analyzing the difference between baseline and follow-up measures.

Comment [A9]: You would make this decision before looking at the data. You would need to guess the most appropriate approach.

Descriptive statistics were generated to assess the distributions of the outcomes variables and to detect the presence of outliers. Two-sided two-sample t-tests, assuming unequal variances, were conducted to test for differences between the placebo and DFMO treatment groups. Comparisons between these groups were done for both on-treatment and off-treatment levels of spermidine, spermine, putrescine, and the ratio of spermidine/spermine. Thus, a total of eight t-tests were conducted. A Bonferroni procedure was used to maintain the overall probability of Type 1 error at $\alpha = 0.05$. Only subjects with available measurements at the various time-points were included in the analysis; that is, no attempt was made to impute missing values. All analyses were performed using Stata 10 (StataCorp LP, College Station, TX).

Comment [A10]: Which multiple comparisons were you most interested in? How many comparisons?

A two-sample t-test tests the null hypothesis that the means of an outcome variable are equal in two independent groups. The test gives an estimate of the magnitude of the difference in means between the two groups, and a 95% confidence interval. The confidence interval can be interpreted in the following way: If the true difference were within the confidence interval, then the difference obtained in this data set would not be an unusual value to be obtained in repeated experiments (the value actually obtained is within the central 95% of the sampling distribution). Finally, the test provides a p value, which describes the probability that the observed data would be obtained if the null hypothesis were true; typically, a p value < 0.05 on a two-sided t-test provides evidence of a true difference in means between the groups. The interpretation of the confidence interval together with the p value, allow us 1) to accept or reject the null hypothesis of no difference in means and 2) to determine whether any apparent differences in means are of scientific interest. The Bonferroni procedure is applied when multiple comparisons are conducted to ensure that the overall probability of obtaining significant differences by chance does not exceed some fixed significance level (in this case, 0.05). When applying the Bonferroni adjustment, we reject the null hypothesis for a given comparison only when a p value less than $0.05/n$ is obtained, where n is the total number of comparisons conducted.

Comment [A11]: what is n?

RESULTS

Baseline Patient Characteristics

Table 1. Baseline subject characteristics and measurements.

	Placebo (32)			**DFMO Treatment** (82)		
	mean	sd	median	mean	sd	median
Age	65.87	(8.51)	66.37	62.68	(7.89)	63.45
% Female	18.8%	-	-	13.4%	-	-
Spermidine (μmole/mg protein)	3.26	(1.45)	2.93	3.47	(1.60)	2.96
Spermine (μmole/mg protein)	8.22	(5.54)	7.52	8.49	(6.06)	7.48
Putrescine (μmole/mg protein)	0.66	(0.44)	0.57	0.64	(0.50)	0.57

The 114 patients in this study were randomized to receive one of four doses of DFMO (0 0.075, 0.2, 0.4 g/sq^2/day). Since we are not examining dose response of DMFO in this analysis, the three groups receiving any DFMO have been aggregated into a single DFMO treatment group. When the sample is divided this way, there are 32 patients in the placebo group, and 82 in the DFMO group.

Table 1 shows the means, standard deviations and medians for age, sex, and levels of spermidine, spermine, and putrescine measured at the onset of the study. There are no missing data at baseline in either group. There are several outlying values among the spermine measurements (levels > 20 μmole/mg protein), but these are evenly distributed across the two treatment groups (1 in the placebo group and 3 in the DFMO group). The putrescine measurement also appears to have several outliers, although it is less obvious where outliers should be defined, in this case. Those greater than the 95th percentile of the distribution (putrescine levels > 1.5 μmole/mg protein) are proportionately overrepresented in the DFMO group (1 in the placebo group and 5 in the DFMO group).

The proportion of females is higher in the placebo group than in the DFMO group; however, the sex-specific sample sizes of this study make assessing treatment response by sex infeasible.

Post Randomization Patient Characteristics

Table 2 gives patient characteristics for subjects at baseline, while on-treatment, and post-treatment. Spermidine, spermine, and putrescine measurements from month 6 and month 12 have been averaged to obtain the measurements for the on-treatment period. For DFMO treatment and post treatment, the age measurement is that taken at baseline, but the statistics are calculated over those who received spermidine, spermine, and putrescine measurements at that time period. There is missing data with a higher frequency in the patients on drugs than on the placebo, indicating possible side effects of DFMO that result in a higher drop-out rate. Figure 1 shows polyamine values at baseline, on treatment, and post treatment, for the placebo group and the group receiving DFMO.

The outliers in spermine and putrescine levels at baseline could skew results if they dropped out of one dose group and not the other; for spermine, all four outliers had all measurements made at all time periods. For putrescine, of the 6 outliers, 2 of the outliers at baseline had missing data at later time points, both in the DFMO group. Because progress of

Comment [A12]: As noted above, present descriptive statistics at more levels than this.

Comment [A13]: Are these thresholds used consistently for defining "outliers". I would not refer to the extreme values this way. In any case, you should list such categorization in your methods.

Comment [A14]: I guess I am not very impressed by this result. There is roughly 3:1 randomization in this table.

Comment [A15]: You lose all chance to see a time effect as well. Might be a good thing just to show an effect, but descriptively you should present this info.

Comment [A16]: And might be biasing.

Comment [A17]: The polyamines do have a skewed distribution. But I would avoid repeatedly calling them outliers. That has a connotation of spurious data in some people's minds. I would instead just call them extreme values.

outliers during treatment may be indicative of drug success, and the baseline outliers stay well represented throughout the study, they have been included in our analysis.

Comment [A18]: Hopefully you would never consider deleting such values.

Figure 1. Polyamine measurements at baseline, on treatment, and post treatment, for placebo group and on DFMO group.

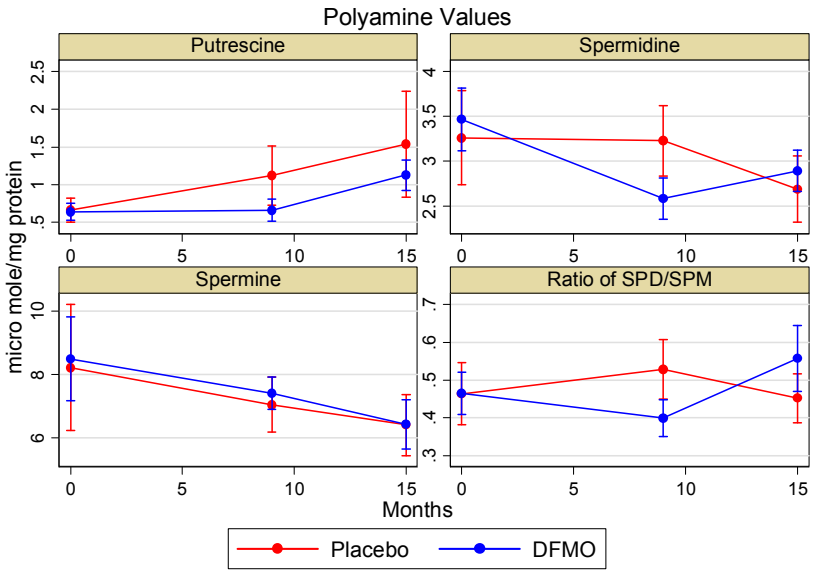

Table 2. Measurements for subjects' baseline, while on-treatment, and post treatment. Values for spermidine (spd), spermine (spm), and putrescine (put) are given in micromole/mg protein.

Comment [A19]: We would want min and max to assess individual level toxicity

		Baseline		Treatment Period		Post Treatment Period	
		Placebo	DFMO	Placebo	DFMO	Placebo	DFMO
age	Mean	65.87	62.68	64.79	62.95	65.14	63.09
	Std Dev.	8.51	7.89	8.48	7.97	8.76	7.78
	Median	66.37	63.45	66.21	63.57	66.21	63.57
sex	Mean	18.8%	13.4%	22.2%	13.4%	22.2%	13.8%
	Std Dev.	-	-	-	-	-	-
	Median	-	-	-	-	-	-
spd	Mean	3.26	3.47	3.23	2.59	2.69	2.89
	Std Dev.	1.45	1.60	0.99	0.94	0.93	0.92
	Median	2.93	2.96	3.12	2.51	2.45	2.78
spm	Mean	8.22	8.49	7.04	7.40	6.39	6.42
	Std Dev.	5.54	6.06	2.21	2.10	2.45	3.17
	Median	7.52	7.48	6.79	7.39	5.79	5.79
put	Mean	0.66	0.64	1.12	0.66	1.54	1.13
	Std Dev.	0.44	0.50	0.99	0.61	1.77	0.81
	Median	0.57	0.57	0.98	0.47	0.80	0.87
	Subjects	32	82	27	67	27	65
	% Missing	0	0	0.16	0.18	0.16	0.21

Table 3. Difference in mean polyamine measurement (micromole/mg protein) between DFMO and placebo groups while on treatment and three months after treatment was stopped. Differences represent DFMO group values minus placebo group values. P values are two-sided.

	Treatment Period		Post-Treatment Period	
	Mean difference (95% CI)	p-value	Mean difference (95% CI)	p-value
spermidine/spermine	-0.13 (-0.22, -0.04)	0.006	0.10 (-0.00, 0.21)	0.053
spermidine	-0.64 (-1.09, -0.20)	0.006	0.20 (-0.22, 0.63)	0.344
spermine	0.35 (-0.65, 1.36)	0.481	0.02 (-1.20, 1.25)	0.968
putrescine	-0.46 (-0.88, -0.04)	0.031	-0.41 (-1.14, 0.31)	0.254

Table 3 shows point estimates and 95% confidence intervals for the differences in mean polyamine measurement between the DFMO and placebo treatment groups while on-treatment and three months after stopping treatment. Difference in means is presented for the ratio of spermidine to spermine as well as the individual polyamines spermidine, spermine and putrescine.

During the treatment period, the DFMO group showed a significantly lower mean spermidine:spermine ratio, as well significantly lower mean levels of spermidine and putrescine, when compared to the placebo group. In the DFMO group, the mean ratio was 0.13 lower (two-sided P=0.006), the mean spermidine level was 0.64 micromole/mg protein lower, (two-sided P=0.006) and the mean putrescine was 0.46 micromole/mg protein lower (two-sided P=0.031). The 95% confidence intervals suggest that the observed data would not be unusual if the true value for the ratio difference was between 0.22 lower to 0.04 lower, the spermidine difference

was between 1.09 micromole/mg protein lower to 0.20 micromole/mg protein lower and the putrescine difference was between 0.88 micromole/mg protein lower to 0.04 micromole/mg protein lower. Applying a Bonferroni correction to adjust for multiple t-tests yields a p-value of <0.006 required to reject our null hypothesis of no difference in means. Based on this conservative correction, we can state that DFMO treatment likely lowers the ratio and spermidine but not putrescine levels.

Three months after stopping treatment, a statistically significant change was not observed in the mean ratio or individual polyamine measurement between the DFMO and placebo group.

Comment [A20]: Discuss whether this is merely lack of precision, or whether this is indicative of a return to "normal"

DISCUSSION

The primary questions of interest in this analysis were whether DFMO treatment decreased colon polyp polyamine levels and whether this effect persisted after stopping treatment. We have shown that treatment with DFMO decreased colon polyp polyamine levels as measured by the ratio of spermidine to spermine as well as the individual polyamine spermidine. The effect of DFMO was no longer evident three months after stopping treatment. DFMO treatment did not have a significant effect on polyp putrescine or spermine levels.

Comment [A21]: I would lead off with a discussion of the missing data on drug. This is potentially biasing.

Comment [A22]: You had no measurements on polyps, only flat mucosa.

This is the first clinical trial of low dose DFMO as an agent for prevention of cancer. Prior trials have used much higher doses of DFMO, typically greater than 5 grams/ m^2/ day, as a therapeutic agent in persons with cancer [7-11]. In those trials the polyamines spermidine and putrescine were decreased. In this study, the effect on putresine did not appear statistically significant after a Bonferroni correction was applied. The reason for a lack of effect on putrescine may be because of the lower doses of DFMO in this trial. Also, the effect on putrescine may occur early after treatment with DFMO. In this analysis we combined measurements made at six and 12 months into one treatment time point and thus may not have been able to detect early changes.

The potential to target a precancerous lesion holds promise for developing novel cancer prevention strategies. However, this study has several limitations. First, clinical endpoints such as polyp growth, new polyp incidence and tumor incidence were not reported. These have to be assessed before we can know whether the observed decrease in polyamine levels is meaningful. Second, we cannot assess the effect of DFMO on normal colon tissue since this was not sampled. If polyamine levels in normal tissue are also affected, we would worry about potential long term effects. Third, toxicity endpoints, including those seen in prior clinical trials, such as ototoxicity and thrombocytopenia, were not reported. This will be important to assess if DFMO will be given for long periods of time.

Comment [A23]: Way too small a study to have much chance of seeing new polyps.

Colon cancer is a leading cause of morbidity and mortality in the United States and novel approaches to prevention are needed. DFMO holds promise as an agent which can be used to target precancerous lesions. Further studies are needed to explore its use as part of cancer prevention strategies.

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