Authors: Group 14

Title: The effect of difluoromethylornithine on polyamine levels in colon polyp tissue

Summary

Colon cancer is the third leading cause of cancer death in the United States. A single-site randomized, double-blind, placebo-controlled phase IIb clinical trial was conducted to determine whether difluoromethylornithine (DFMO) would result in transient or sustained suppression of polyamine synthesis in colon polyps. Suppression of polyamine synthesis was assessed by comparing levels of the polyamines putrescine, spermadine, and spermine at different time points between placebo and DFMO treatment groups. Participants previously diagnosed with colon polyps were randomized to receive one of three doses of DFMO (0.075, 0.2, or 0.4 g/sq m/day) for a total of 12 months. Colon biopsies were taken at 0, 6, 12, and 15 months post-randomization to measure polyamine levels. Change in mean putrescine levels tended to decrease across all dosage groups but was significantly different from the placebo group only in the 0.4 g/sq m/day dose group at 6 months post-randomization (-0.32 micromole/ mg protein, 95%CI -0.51 to -0.13, 2-sided p=0.044). Change in mean spermine levels did not significantly differ from the placebo group at 6 or 12 months post-randomization. Change in mean spermidine levels tended to decrease across all dosage groups from basline to 12 months but was statistically different from the placebo group only in the 0.4 g/m^2/day dosage group (-1.76 micromole/ mg protein, 95%CI -2.77 to -0.74, 2-sided p=0.0048). Spermidine:spermine ratio, a measure of cell proliferation, tended to decrease throughout the study but achieved significant difference from placebo only after 6 months of treatment in the 0.075 g/m^2/day dose group. These combined data suggest that statistically significant polyamine suppression is observed only at high relative dosages of DFMO and is transient in nature. As such, oral DFMO at the doses administered in this trial cannot be recommended for antineoplastic therapy in colon tissue.

Comment [A1]: The patients previously had had colon polyps, but the polyamines were measured in flat mucosa.

Comment [A2]: How many, what sex, ages?

Comment [A3]: What about 6 months for spermidine? You rwording for putrescine seemed to imply that you had also considered the 12 month measurements, but your wording here seems to suggest you were only looking at 12 months.

Comment [A4]: I think this is stronger than is known. In any case, we think spd is necessary for cell proliferation, but it is unclear whether it is a good marker of proliferation going on.

Comment [A5]: This seems too strong for me: You observed a decrease in spd at 12 mos. But then DFMO was stopped. You need to define that you mean "transient" in the sense that stopping DFMO removes the effect. I will grant you that you only observed stat aignif in putrescine at 6 mos, but you need to also look at the estimates. Lack of stat signif does not prove equivalence.

Background

Colorectal cancer is the third leading cause of cancer death in both men and women.[1] Polyamines, ubiquitous signaling molecules important in regulating cell proliferation,[2-4] have been found in high levels in malignant colon tissue.[2] Although the precise role for polyamines in carcinogenesis is not understood, manipulation of the polyamine synthesis pathway may represent a means for controlling growth of malignant or premalignant cells.

The enzyme ornithine decarboxylase (ODC) is the rate limiting step in the polyamine synthesis pathway. ODC directly catalyzes the production of the polyamine putrescine. Putrescine is later metabolized downstream by other enzymes into the polyamines spermidine and spermine.[2] Inhibition of ODC modulates response to carcinogens among benign cells, and suppresses cell proliferation among malignant cells. [4] Prior research has found that lower putrescine levels and a lower spermidine: spermine ratio are associated with a attenuated cell proliferation, but whether such findings have implications in human colon mucosa is not known.[2-5]

Interest in controlling ODC activity as a potential anticancer therapy is limited by the absence of an effective enzyme inhibitor. Difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, has shown promise in slowing tumor growth in colon and other tissues in humans and

animals in the laboratory[6,7] and as such may represent a novel antineoplastic agent. It is not known whether safe and tolerable doses of oral DFMO as described in prior phase I trials[8] will result in suppression of ODC or polyamine production *in vivo* in human colon tissue. To assess the effect of oral DFMO on polyamine levels in healthy adults this project measures putrescine, spermine, and spermidine in colon polyp tissue biopsied from subjects randomized to receive DFMO or placebo for 12 months.

Question of Interest

This trial was designed to examine whether 1) administration of DFMO to patients with a history of colon polyps will result in decreased polyamine levels in colon tissue; 2) whether DFMO results in sustained changes in polyamine levels; and 3) whether changes in polyamine levels persist after withdrawal of DFMO.

Source of the Data

Data was obtained from a phase IIb clinical trial performed at the University of California, Irvine. A detailed description of the methodology for patient enrollment was not available. Eligible subjects included adults with a history of colon polyps who were willing to undergo repeat colonoscopies over the 15 month study period. Enrolled patients underwent baseline colonoscopy with biopsy and were then randomized to placebo or one of three DFMO doses. Study drug was administered in a double-blinded manner. Patients remained on drug or placebo for 12 months and were followed for 15 months total. Follow-up colonoscopies were obtained at months 6, 12 and 15. Colon biopsies from baseline and at each time point were analyzed for polyamine levels.

Comment [A6]: How many? Age? Sex?

Comment [A7]: What dose?

Statistical Methods

We examined the change in polyamine levels at 6, 12, and 15 months compared to baseline. We performed two-sample t-tests comparing differences in each dose group compared to placebo. We also performed t-tests on the ratio of arithmetic means of spermine to spermidine in each dose group compared to placebo. We observed a trend toward more missing data in the higher dose groups. Since we had no way of knowing if this was informative or non-informative missing data, we ignored it.

Comment [A8]: I would tend to report this in "Results", because we are afraid that it might be toxicity.

To investigate the possibility of effect modification we examined scatterplots of our data at 6, 12 and 15 months stratified by age and sex. Lowess curves on these plots appeared overlapping for all three polyamines at all three times, so we concluded that there was no effect modification in our sample between age or sex and effect of DFMO. Due to small numbers of subjects within subgroups we did not estimate the differences of means between groups using the stratified data. In a randomized controlled trial we would not expect confounding to affect our results, nor did we find evidence of confounding in our plots.

Comment [A9]: This seems to be "results" rather than methods.

Results: Descriptive Statistics

Patient characteristics and polyamine measurements are summarized in Table 1 and Table 2, respectively. A total of 114 patients were represented in the dataset, with 25-32 subjects per treatment group. Approximately 15% of subjects were female. The average age was 63.6 years, with no extreme outliers.

Comment [A10]: You still haven't told us the doses. I would have listed the sample sizes in each dose group.

Patients within each of the four treatment arms were similar with respect to age and sex, except for dose group 0.2g/m^2/d which contained no females, and dose group 0 which contained a slightly older group of patients. Baseline polyamine levels were reported for all 114 subjects. With increasing follow-up time, complete data for polyamine levels were available for fewer subjects: 93% at 6 months, 83% at 12 months and 81% of subjects at 15 months.

Comment [A11]: The trend by dose (which you noted in your Methods) is very, very important. Report it.

Figure 1 illustrates the first-order trends of mean polyamine concentrations stratified by time within each dose group (0, 6, 12, and 15 months post-randomization). Spermine had the highest relative concentration in colon tissue and tended to decrease over time in all dose groups. Spermidine and putrescine had the second and third highest relative concentrations, respectively, and showed transient suppression during the treatment period. Concentrations of both spermidine and putrescine began to rise again after treatment cessation with only spermidine showing an overall decline relative to baseline after 15 months of follow-up.

Results: Main Analyses

Mean putrescine levels in placebo increased 61% over baseline at 6 months and 70% over baseline at 12 months. This represented a significant increase over baseline at 12 months (p = 0.02 for 2-sided t-test) which persisted at 15 months. In all three treatment groups, putrescine levels trended down compared to placebo at 6 months, but the difference in means achieved statistical significance only in the 0.4 g/m^2/ day dose group (-0.32 micromole/ mg protein, 95%CI -0.51 to -0.13, 2-sided p=0.044). Putrescine levels increased at 12 months and 15 months but were not statistically different from placebo in any dose group at 12 or 15 months. There was no dose-dependent reduction in putrescine level with increasing DFMO dose at either 6 or 12 months (Table 3).

Comment [A12]: Your analysis was on differences, rather than ratios. I would not tend to report percentages unless I did an analysis that way. If I did report the percentages, I would use wording that first stressed the differences. Maybe something like: Mean levels were xx.x micromole/mg protein higher at 6 months, which represented an increase of xx% over the mean at baseline.

Mean spermine levels tended to decrease compared to baseline in all groups but did not reach statistical significance compared to placebo after 6, 12, or 15 months. There was a trend toward decreased spermine levels during the 12 month period in all three treatment groups, with the biggest change in the 0.2 dose group (9.03 to 6.08 g/m^2/ day from baseline to 15 months). There was no dose-dependent reduction in spermine with increasing DFMO dose at 6 months, but there was a trend towards one at 12 months (Table 3).

Mean spermidine levels trended down in all treatment groups compared to placebo at 6 months but did not achieve statistical significance in any dose group. After 12 months, the decrease in mean spermidine level reached significance only in the highest dose group, with an estimated 1.71 micromole/mg greater suppression in the treated group (95%CI 0.56 to 2.87, p-value 0.0048). There was a dose-dependent reduction in spermidine with increasing DFMO dose at both 6 and 12 months, put it did not reach statistical significance compared to placebo (Table 3).

The spermine: spermidine ratio tended to decrease after 6 months in all treatment groups but did not show a statistically significant change compared to placebo. After 12 months the ratio in the 0.075 g/m^2/ day group was 0.21 higher compared to placebo (95%CI 0.00 to 0.42, p-value=0.026), and in the 0.4 g/m^2/ day dose group the estimated change was 0.31 higher in placebo than treatment (95%CI 0.08 to 0.54, p-value 0.01). The spermidine:spermine ratio

Comment [A13]: I did not require that you be able to adjust for multiple comparisons for this paper. But I think you can understand the difficulty here. Given all the different comparisons made, do you have any idea of a consistent picture over time and dose? It is very hard to put it all together.

A better approach might have been to just consider the 12 month measurement as your primary analysis, and then look at 6 months to see how early any difference might have shown up, and 15 months to see if it went away went treatment stopped. You would have needed to give that interpretation to your results.

tended to increase at 15 months within the 0.075 g/m^2/ day and 0.4 g/m^2/ day dose groups, but the increase did not reach significance in either group. There was no dose dependent reduction in spermidine: spermine ratio with increasing DFMO dose at either 6 or 12 months (Table 3).

Discussion

Assuming changes in polyamine levels are a reliable marker of cell proliferation, we would expect DFMO to reduce polyamine levels in treatment groups relative to placebo. Evidence for an effect of DFMO would be strengthened by a dose-dependent relationship between DFMO and polyamine levels. DFMO administration would be expected to decrease the spermine: spermidine ratio over time.

Comment [A14]: Sort of reversal of cause and effect. We are in some sense trying to "starve" the cells so they cannot proliferate.

Based on the above hypotheses, our data reveal only weak evidence for an anti-proliferative effect of DFMO, as summarized in Table 3. Examination of the placebo group provided a baseline for polyamine levels during the 15 month study period. Within all three treatment groups there was a tendency towards decreased polyamine levels relative to baseline at 6 months, although this trend only achieved statistical significance versus placebo among subjects in the highest dose group in putrescine. The spermidine: spermine ratio showed no decrease over placebo in any treatment group at 6 months.

Comment [A15]: The primary comparison should not be over time, but instead should be to compare each dose group to placebo. Time is confounded with aging, laboratory drift, dietary trends over time, etc.

By 12 months, the lowering effect of DFMO on polyamine levels had disappeared in putrescine. Spermine and spermidine levels continued to trend down, but were indistinguishable from placebo in all but the highest treatment groups. There was a trend towards a dose-dependent reduction in polyamines with increasing DFMO dose in spermine and spermidine at 12 months, but without a significant difference from placebo in any group. There was a tendency toward a decrease in the spermine: spermine ratio that significantly differed from placebo in both the 0.075 and 0.4 dose groups at 12 months. However, the changes were of low absolute magnitude and thus of unclear biologic significance. By 15 months there was no difference in polyamine levels in any treatment group compared to placebo or compared to baseline.

Comment [A16]: Rather too strong wording. Perhaps you meant it was no longer stat signif and that the estimate relative to placebo was close to zero (though with wide CI)

The reasons behind the variable behavior of the different polyamines may reflect unique kinetics and regulatory mechanisms within each polyamine's synthesis pathway. DFMO in higher doses had a transient effect on putrescine levels at 6 months which in turn could have been reflected in the 12 month spermidine: spermine ratio. It is not clear from this analysis whether changes in polyamines or the spermidine: spermine ratio are better correlates of neoplastic growth. However, the data do not show a reproducible, sustained effect of DFMO on polyamine levels by any of our measures. Spermidine measurements seemed to decrease more with increased dosage, which could indicate trend toward a dose-response effect for this particular polyamine.

The most important limitation this study is small sample size, which may have limited our ability to detect an effect in the lower dose groups even if there truly was one. For example, at 6 months, the average number of people in each dose group was 26, and the average standard deviation of the change in putrescine by dose group was 0.82 micromole/mg. Under these conditions, for a t-test to have 80% power to detect a significant difference in means at the 0.05 significance level there would need to be a difference of 0.65 micromole/mg between the two group means. With baseline mean putrescine measurements (by dose) ranging from 0.61 to 0.65

Comment [A17]: *Post hoc* power analyses are pretty much useless once we have the data. The CI says it all.

micromole/mg, that seems like a very large difference in the effect at 6 months. A larger sample size would allow us to detect smaller between-group differences.

The apparent lack of efficacy for DFMO has several possible explanations. Our study design did not control for ornithine intake among the subjects, which may have provided substrate for ODC and led to increased polyamine synthesis despite the presence of DFMO. Similarly, our design did not control for exogenous polyamine intake from the diet which could have rendered the inhibition of ODC irrelevant. Even if ODC was successfully inhibited initially, its transcription could have been upregulated in colon tissue over time, thereby overcoming the effect of DFMO. In particular, the rebound effect seen in putrescine levels at 12 months compared to 6 months may have been due to this phenomenon. Finally, there are several pathways for polyamine synthesis outside of the ODC pathway which could have compensated for the inhibition of ODC.

Our study population had a large degree of subject attrition, not only reducing our sample size but also introducing the possibility of informative censoring. The trend toward higher attrition was most pronounced among patients in the highest DFMO dose category. Informative censoring could be present if patients withdrew due to development of overt colon cancer, or, alternatively, withdrew due to medication intolerance. In the highest dose group only 64% of patients remained at 15 months, versus 84% in placebo and 89.7% in the 0.075 $g/m^2/d$ dose group ($p = 0.08$ vs placebo; 0.002 vs 0.075 dose group by chi-square test for proportions). This is a concerning trend both for tolerability of DFMO at the highest dose levels as well as the validity of our interpretations from remaining patients. In future studies, more descriptive data pinpointing the causes of attrition would permit analysis for informative censoring.

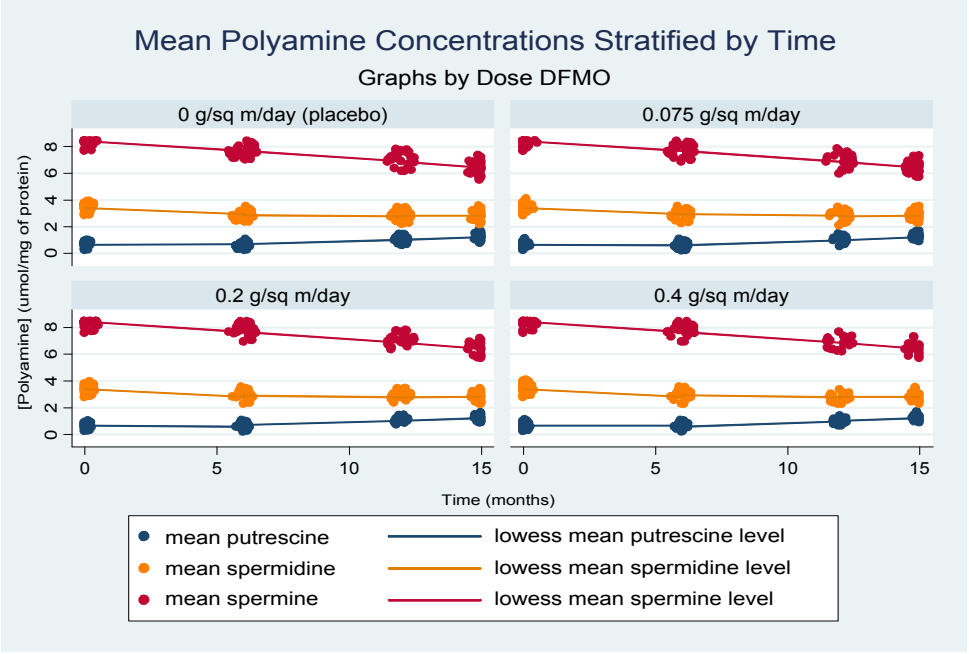
Comment [A18]: Should have been described in the Results

Comment [A19]: This is not "censoring". It is missing data. "Censoring" is a very special kind of incomplete data.

Although DFMO did not have a sustained effect on polyamine levels at the doses used in our trial, it did appear to have a transient effect which could potentially have medical applications in short-term settings such as induction chemotherapy or chemoprophylaxis after exposure to a carcinogen. Despite the fact that this trial did not detect a consistent effect for DFMO there could still be a role for future investigation into the effects of ODC inhibition. We did see a modest dose-dependent decline in polyamine levels in spermine and spermidine at 12 months, so it is possible that higher doses of DFMO might amplify this effect, especially since this trial did not use the maximally tolerated doses of the medication, which can be as high as 3.75g/ m^2 every 6 hours. Additionally, since polyamine levels are tightly regulated in normal cells, small changes in levels over time might represent important alterations in cellular metabolism. For this reason, further research evaluating rates of colon cancer or development of polyps in patients taking DFMO might be of interest. Control of ornithine and polyamine intake might be one potential means to reduce the potential confounder of exogenous ornithine or polyamines. Additionally, future studies with a larger sample size might have a better likelihood of detecting statistical significance compared to the current study.

Comment [A20]: wording is too deterministic.

Figure 1.



Comment [A21]: I do not understand this plot. It looks like you are plotting the individual measurements for each individual (else why are there more than one point). But if this were the individual measurements, there would be extremely high statistical significance at every single time.

(Your Table 2 shows that these are not the individual measurements, so what are they?)

Table 1. Patient Characteristics by DFMO dose group assignment

DFMO Dose ($g/m^2/d$)	N Subjects (baseline)	Percent female	Age (years)			N subjects at 15mo (%)
			Mean (sd)	min	max	
0	32	18.8%	65.9 (8.5)	45.5	77.2	27 (84.4)
0.075	29	17.2%	61.3 (7.7)	47.8	76.9	26 (89.7)
0.2	25	0%	62.8 (8.3)	45.4	77.6	21 (84.0)
0.4	28	21.4%	63.9 (7.8)	48.5	81.0	18 (64.3)
Overall	114	14.9%	63.6 (8.2)	45.4	81.0	92 (80.7)

Comment [A22]: Better detail about study drug discontinuation is always desirable, because it is often our only window into drug toxicity.

Table 2. Polyamine levels by DFMO dose group at 6, 12 and 15 months

	0 MONTHS				6 MONTHS				12 MONTHS				15 MONTHS			
DOSE ($g/m^2/d$)	**Mean (SD)**	**Min**	**Med**	**Max**	**Mean (SD)**	**Min**	**Med**	**Max**	**Mean (SD)**	**Min**	**Med**	**Max**	**Mean (SD)**	**Min**	**Med**	**Max**
PUTRESCINE (micromole/ mg protein)																
0	0.66(0.44)	0.06	0.57	1.98	**1.06(1.59)**	0.05	0.72	9.14	**1.16(0.83)**	0.00	0.90	3.18	1.54(1.77)	0.30	0.80	6.51
0.075	0.65(0.52)	0.01	0.54	2.59	**0.47(0.27)**	0.00	0.49	1.05	**1.08(1.03)**	0.04	0.69	4.29	1.19(0.89)	0.00	0.91	3.53
0.2	0.61(0.42)	0.00	0.60	1.96	**0.45(0.54)**	0.00	0.27	2.43	**0.80(0.79)**	0.00	0.51	3.1	1.00(0.71)	0.00	0.87	2.59
0.4	0.65(0.57)	0.00	0.60	2.30	**0.33(0.43)**	0.00	0.19	1.73	**0.88(1.42)**	0.00	0.40	5.48	1.18(0.83)	0.23	0.85	3.20
SPERMINE (micromole/ mg protein)																
0	3.26(1.45)	1.40	2.93	7.05	**3.37(1.53)**	1.51	3.02	6.91	**3.26(1.31)**	1.01	2.82	5.91	2.69(0.93)	1.25	2.45	4.62
0.075	3.47(1.55)	1.51	2.91	7.02	**2.64(0.89)**	1.39	2.46	5.12	**2.92(0.99)**	1.35	2.86	4.92	2.95(0.99)	0.00	2.98	4.83
0.2	9.03(7.04)	2.54	7.53	41.68	**7.18(1.64)**	1.07	1.85	7.84	**7.16(3.15)**	2.96	6.63	13.83	6.08(3.56)	1.93	4.61	12.38
0.4	8.08(5.50)	2.28	6.81	34.04	**8.04(1.43)**	1.06	2.07	6.34	**5.93(2.58)**	0.00	5.97	10.66	6.42(2.53)	2.53	6.00	11.52
SPERMIDINE (micromole/ mg protein)																
0	3.26(1.45)	1.51	3.02	6.91	3.37(1.53)	1.51	3.02	6.91	3.26(1.31)	1.01	2.82	5.91	2.69(0.93)	1.25	2.45	4.62
0.075	3.47(1.55)	1.39	2.46	5.12	2.64(0.89)	1.39	2.46	5.12	2.92(0.99)	1.35	2.86	4.92	2.95(0.99)	0.00	2.98	4.83
0.2	3.35(1.33)	1.07	1.85	7.84	2.58(1.64)	1.07	1.85	7.84	2.71(1.40)	0.29	2.51	6.45	2.98(0.90)	1.81	2.81	4.81
0.4	3.56(1.88)	1.06	2.07	6.34	2.68(1.43)	1.06	2.07	6.34	1.95(0.80)	0.00	1.93	3.42	2.7(0.87)	1.29	2.69	4.47

* Bold values represent significant change from baseline, two-sided $p<0.05$ using two sample t-test

Table 3. Change in polyamine levels from baseline by DFMO dose group at 6, 12 and 15 months

	6 MONTHS		12 MONTHS		15 MONTHS	
DOSE ($g/m^2/d$)	**Mean difference (6mo-baseline) [95% CI]**	**p-value versus placebo**	**Mean difference (12 mo- baseline) [95% CI]**	**p-value versus placebo**	**Mean difference (15 mo-baseline) [95% CI]**	**p-value versus placebo**
PUTRESCINE (micromole/ mg protein)						
0	0.37 (-0.28, 1.03)	--	0.470 (0.15,0.79)	--	0.86 (0.14 – 1.58)	--
0.075	-0.20 (-0.42, 0.026)	0.099	0.39 (-0.049,0.82)	0.75	0.50 (0.12, 0.87)	0.36
0.2	-0.18 (-0.42,0.6)	0.11	0.20 (-0.23, 0.62)	0.29	0.42 (0.053, 0.78)	0.26
0.4	-0.32 (-0.51, -0.13)	**0.044***	0.31 (-0.34,0.97)	0.66	0.50 (0.083, 0.91)	0.37
SPERMINE (micromole/ mg protein)						
0	-0.98 (-3.19, 1.23)	--	-1.98 (-4.24, 0.28)	--	-2.31(-4.60, -0.024)	--
0.075	-0.34 (-2.77, 2.10)	0.69	-0.78 (-3.37, 1.80)	0.48	-1.84(-4.65, 0.96)	0.79
0.2	-2.03 (-5.31, 1.25)	0.59	-2.08 (-6.01, 1.85)	0.96	-3.16 (-6.68, 0.37)	0.68
0.4	-0.23 (-3.46, 2.99)	0.70	-2.57 (-5.41, 0.26)	0.74	-2.52 (-6.02, 0.35)	0.78
SPERMIDINE (micromole/ mg protein)						
0	0.089 (-0.60, 0.78)	--	-0.041 (-0.64, 0.55)	--	-0.78 (-1.32 ,0.23)	--
0.075	-0.72 (-1.31, -0.13)	0.073	-0.41 (-0.97, 0.14)	0.35	-0.38 (-1.15 ,0.39)	0.39
0.2	-0.77(-1.41, -0.13)	0.068	-0.57 (-1.35, 0.22)	0.28	-0.18 (-1.03 ,0.68)	0.22
0.4	-1.03 (-2.08, 0.029)	0.076	-1.76 (-2.77, -0.74)	**0.0048***	-1.20 (-2.25 ,0.15)	0.46
SPERMIDINE/SPERMINE RATIO						
0	0.041 (-0.08, 0.16)	--	0.18 (-0.01, 0.37)	--	0.00 (-0.11, 0.11)	--
0.075	-0.11(-0.20, -0.01)	0.055	-0.03 (-0.14, 0.08)	**0.050***	0.11 (-0.02, 0.24)	0.19
0.2	-0.05 (-0.25, 0.14)	0.41	-0.01 (-0.24, 0.23)	0.21	0.24 (0.00, 0.48)	0.06
0.4	-0.04(-0.28, 0.20)	0.51	-0.13 (-0.29, 0.02)	**0.01***	-0.02 (-0.16, 0.11)	0.81

*2 sided P-values based on two sample t-test with unequal variances

Comment [A23]: Note that our interest in spd:spm ratio is to provide a sort of "internal control". We tended to expect greater precision with this measure, because it would normalize to similar amounts of mucosa in the biopsies.

References

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