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Functional organotypic cultures of prostate tissues: a relevant preclinical model that preserves hypoxia sensitivity and calcium signaling

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ABSTRACT

In prostate cancer research, there is a lack of valuable preclinical models. Tumor cell heterogeneity and sensitivity to microenvironment signals, such as hypoxia or extracellular calcium concentration, are difficult to reproduce. Here, we developed and characterized an *ex vivo* tissue culture model preserving these properties. Prostate tissue slices from 26 patients were maintained *ex vivo* under optimized culture conditions. The expression of markers associated with proliferation, androgen receptor signaling, and hypoxia was assessed by immunostaining. A Macro Zoom System Microscope was used to achieve real-time calcium fluorescence optical imaging. Tissue morphology was successfully maintained without necrosis for 5 days. Compared to native tumors and tissue cultured with androgens, androgen deprivation in the medium led to decreased expression of both androgen receptor and its target gene products, PSA and ERG. *Ex vivo* cultured slices were also sensitive to hypoxia since CAIX and Zeb1 protein levels increased in 1% oxygen. Exposure of slices to supra-physiological extracellular Ca^{2+} concentration induced a robust and rapid Ca^{2+} entry, with a greater response in tumor compared to non tumor tissue. This *ex vivo* model reproduces the morphological and functional characteristics of human prostate cancer, including sensitivity to androgen deprivation and induced response to hypoxia and extracellular Ca^{2+} . It could therefore become an attractive tool for drug response prediction studies.

INTRODUCTION

Although prostate cancer (PCa) is the second leading cause of cancer-related death among men in developed countries, the transfer of scientific findings into medical progress is challenged by the lack of valuable preclinical models. Immortalized PCa cell lines have acquired several mutations in culture, and both their genotype and phenotype are different from those observed in native human cancer cells (1). Moreover, *in vitro* studies with cell lines do not reproduce the interactions with the prostate microenvironment, which is critical for PCa development and progression. Xenografts and genetically engineered mice represent improved models but have inherent advantages and limitations regarding the mimicking of the PCa situation in humans. These models have advanced our understanding of PCa behavior, but differences between species and donors may lead to difficulties in interpreting the results. Additionally, their deviation from the human physiology could result in inaccurate preclinical assessment (2). To address these limitations, the use of *ex vivo* culture of human tissues has been described for different types of cancers (3-6). Culture of tissue slices is a good representative model of the *in vivo* situation in patients since it maintains both cancer cell heterogeneity and an intact microenvironment, which allows stromal-epithelial interactions. In the past, *in vitro* maintenance of prostate tissues has been challenged by rapid degradation and poor survival of epithelial cells (7, 8). However, the use of precision-cut slicing has led to more reproducible models, which have been reported by a few teams in preclinical studies (9-12). However, in these previous studies, the culture conditions did not take into account the specific variations related to the tumor microenvironment. Essential factors such as hypoxia and calcium signaling have been shown to influence tumor progression and resistance to treatment. Hypoxia is a feature characteristic of most solid tumors, and is a major contributor to cancer progression and resistance to treatment, and it mainly exerts its effects through the process of epithelial to mesenchymal transition (EMT) (13-15). Prostate tumors are known to

grow under a hypoxic microenvironment, which comprises oxygen concentrations lower than those found in the normal prostate (16, 17). In addition, Ca^{2+} signaling associated with variations in extracellular Ca^{2+} concentrations has been shown to play a major role in the regulation of cellular processes leading to tumor progression, including proliferation, migration, and apoptosis (18-20). Extracellular calcium concentrations in the tumor microenvironment can reach levels between 8 and 40 mmol/L, and it has been implicated in the development of bone metastasis (21). Moreover, altered expression of specific Ca^{2+} channels and pumps are characteristic features of most cancers including PCa (22). Therefore, the sensitivity of human cancer samples to extracellular calcium concentrations is a major element that needs to be assessed to demonstrate the functional value of an *ex vivo* prostate tissue model. Here, we described a relevant and functional model of cultured benign and malignant human prostate tissues that preserves hypoxia and calcium sensitivity. The development of this model will allow a better analysis of the intrinsic properties of PCa and, possibly, the rapid and accurate identification of targeted cancer treatment.

MATERIAL AND METHODS

Patients and tissues

Prostatic tissue samples, including malignant and normal tissues, were obtained from 26 patients undergoing radical prostatectomy for clinically localized PCa. Tumors were classified according to the International Society of UroPathology (ISUP) in group 1 to 5, and according to the pTNM stage as pT2 or pT3 (Table 1). Written informed consent was received from all patients.

Preparation of tissue slices

Radical prostatectomy specimens were obtained immediately after surgery, and 4 to 5 mm samples were dissected aseptically from the peripheral zone within the putative benign or

cancer areas (Fig 1A). Tissue samples were glued on a platen against an agarose block (8% agarose) in stair-step cut and were immersed in RPMI medium supplemented with 10% FBS and 1 % penicillin-streptomycin. Briefly, the samples were immediately cut with a vibratome (Vibratome VT1200, Leica) at 200 to 300 μm thickness, with a total number of slices ranging from 6 to 10 per case. Slices were used for either *ex vivo* culture and biomarkers expression (15 patients) or for *ex vivo* culture and intracellular Ca^{2+} measurements (11 patients).

***Ex vivo* culture of prostate tissue slices**

Each slice was transferred into a 6-well cell culture plate containing DMEM medium (Lonza, Levallois-Perret, France) supplemented with 10% FBS and 1% penicillin-streptomycin, and incubated with or without 1 nM dihydrotestosterone (DHT) (D073, Sigma). Slices were incubated in a 37 °C humidified incubator with 5% CO_2 . Medium was changed daily.

Hypoxia experiments were performed in an hypoxia chamber (SCI-tive, Alliance Bio Expertise) at 1% O_2 at 37 °C in a 5% CO_2 humidified environment. Slices were first placed in normoxia for 24 hours, to avoid additional stress after cutting, and then, were incubated for 24 hours in hypoxia environment. The medium was replaced every day.

Slices were cultured between 3 to 7 days after tissue collection. Analysis of sections after hematoxylin-eosin-saffron (HES) and immunohistochemical staining was performed at baseline (Day 0), then for Day1 (D1) to D3, or D1 to D7, depending on the number of slices available. In four cases the number of slices was sufficient to analyze the morphology from D0 to D7. Analysis of biomarker expression was performed in 15 cases at D0 and after 48H of culture. The effects of DHT deprivation and hypoxia were measured at D2, in 15 samples for DHT deprivation, and in five samples for hypoxia.

Immunohistochemistry

After fixation in 10% formalin, tissue slices were embedded in paraffin, and sections were serially cut at 5 μm with a microtome. Then, sections were spread on a SuperFrost® Plus slide

and dried for one hour at 56 ° C. One section was stained with HES for morphological analysis. The other slides, used for immunostaining, were deparaffinized (histosol and alcohol baths), rehydrated, and heated in citrate buffer pH 6 (citric acid monohydrate and sodium citrate) for antigenic retrieval. After blocking for endogenous peroxidase with 3% hydrogen peroxide, primary antibodies were incubated with the section. The panel of primary antibodies included p63 (Biocare, clone 4A4, dilution 1/100, 30 min), p504S (DakoCytomation, clone 13H4, 1/200, 30 min), androgen receptor (AR) (Abnova, clone 1G3, 1/500, 30 min), PSA (BioSB, clone BSB7, 1/250, 30 min), Zeb1 (Abnova, clone 4C4, 1/500, 1 hour), carbonic anhydrase IX (CAIX) (Novocastra, clone TH22, 1/100, 30 min), ERG (Roche, EPR 3864, 1/1, 30 min), and the proliferation marker Ki67 (DakoCytomation, clone 39-9, 1/50, 30 min). Immunohistochemistry was performed with either the automated BenchMark XT slide stainer (Ventana Medical Systems Inc) using OptiView Detection Kit (Ventana Medical Systems Inc) or manually (for Zeb1) using the streptavidin-biotin-peroxidase method with diaminobenzidine as the chromogen (Kit LSAB, Dakocytomation, Glostrup, Denmark). Slides were finally counterstained with haematoxylin. Negative controls were obtained after omission of the primary antibody or incubation with an irrelevant antibody.

Scoring of antibody staining

Staining for p63, p504, CAIX, and PSA was scored as positive or negative, and staining for AR, ERG, and Zeb1 was evaluated as follows: -, no stained cells; +, focal positivity; ++, diffuse positivity. The number of Ki67 positive cells was expressed as the percentage of total epithelial cells.

Intracellular Ca²⁺ measurements

Intracellular Ca²⁺ measurements were performed on tissue slices after 48 H of *ex vivo* culture. Intracellular calcium variations were assessed with the rhod-2 calcium sensitive dye. All the next steps were done in the darkness. Briefly, human prostate slices were incubated for 30

min with 5 μ M Rhod-2 AM at room temperature. Slices were placed in the bath of Macro Zoom System Microscope MVX10 and were washed for 20 min with a physiological saline solution (PSS) before experiments, using a pump in an open circuit. The dye was excited at 545 ± 20 nm with LED illumination (CoolLED pE-300 white) and fluorescence was collected at 605 ± 40 nm with a sCMOS Zyla 4.2 PLUS camera and a Macro Zoom System Microscope MVX10. Image acquisition rate was 0.1 Hz and exposure time was 600 ms. For each measurement, human prostate samples were incubated with 2 mM CaCl_2 in PSS. After a stabilizing time, CaCl_2 was added to the incubation bath to reach a final concentration of 5 mM Ca^{2+} . Analyses were performed with the ImageJ 1.52a analysis software (<https://imagej.nih.gov/ij/>; last accessed November 29, 2019). The Ca^{2+} fluorescence signal was normalized with the initial fluorescence signal obtained at 2mM CaCl_2 to correct for differences in dye loading across the preparation. Variations of the Ca^{2+} signal were then determined by calculating the difference between the basal value, at 2 mM CaCl_2 , and the maximal Ca^{2+} value after 5 mM CaCl_2 addition.

Statistical analysis

Statistical analyses were performed with StatView, version 5.0, software (Abacus Concepts, Berkeley, CA). Comparison between groups was performed using the χ^2 test for categorical data and non parametric Mann-Whitney U test for continuous data. Paired data were analyzed using the non parametric Wilcoxon test.

RESULTS

Tissue morphology

Tissue morphology after culture under normoxic conditions was compared to the morphology of native tissues (baseline, at D0). For all the tested tissues, the architecture was preserved in both benign and malignant tissues from D1 to D5. No necrosis was observed between D1 and

D5, but at D6 tissue samples started showing some necrosis foci, and at D7, necrosis was extensive (Fig 1).

Biomarkers expression

The expression of biomarkers after culture under normoxic conditions during 2 days was compared to native tissues (baseline, at D0). Biomarkers included markers of normal basal epithelial cells (p63), prostate cancer cells (p504S), proliferation (Ki67), EMT (Zeb1 transcription factor), hypoxia (CAIX), and of the fusion gene *TMPRSS2-ERG* product (ERG). In non tumor tissues (n=7), all benign glands showed preservation of the p63 positive basal cell layer (Fig 2A). In malignant tissue slices (n=8), cancer cells expressed p504S (Fig 2B) in all cases, as observed in the native tumors.

In normal glands at D0, proliferation was limited and restricted to basal cells (median 1% Ki67 positive cells in the epithelial compartment, range 1% to 3%). Basal cell proliferation increased after culture (median 8% Ki67 positive cells in the epithelial compartment, range 6% to 15%) when compared to the baseline status ($P < 0.02$, Mann Whitney test) (Fig 2C, 2D). In tumor tissues, the proliferation rate was identical after culture (median 3%, range 2% to 5%) when compared to native tumors at D0 (median 3%, range 1% to 4%) ($P = 0.8$) (Fig 2E, 2F).

At baseline (D0), prostate cancer cells expressed ERG in four cases out of eight (50%). In all positive cases, ERG expression was preserved in the cultured slices, and ERG negative tumors remained negative after tissue culture. ERG expression was negative in all normal glands, at baseline and after culture. PSA was expressed in all cases of both normal and cancer tissues, before and after culture. In normal tissues at D0, epithelial cells were negative for the Zeb1 transcription factor and for CAIX expression. In cultured slices obtained from normal tissues (DHT-supplemented or not), CAIX staining remained negative, and focal expression of Zeb1 (rare positive epithelial cells) was observed in two of seven cases (without

significant difference compared to baseline, $P = 0.4$). At D0, three malignant samples out of eight showed focal Zeb1 expression in cancer cells, without CAIX staining. After culture under normoxic conditions, CAIX remained negative. Tumors with focal Zeb1 expression at baseline remained unchanged, whereas two out of the five initially Zeb1 negative cases showed focal expression (without significant difference compared to D0, $P = 0.6$).

Effects of DHT deprivation

In both benign and malignant glands, absence of DHT in the medium led to decreased expression of AR (from diffuse to focal staining) and of its target, the PSA gene product, (from positive to negative staining), when compared to slices cultured in medium supplemented with DHT ($P = 0.0001$) (Fig 3A to 3F). In ERG positive tumors, DHT ablation was associated with decreased ERG levels in cancer cells (from diffuse to focal staining) ($P < 0.03$) (Fig 3G to 3I).

Effects of hypoxia

Hypoxic conditions were tested with DHT supplementation for five samples (two benign and three malignant). In normoxia, benign samples were negative for Zeb1 and CAIX, and malignant samples were negative for CAIX (Fig 4A), and focally positive for Zeb1 (Fig 4C). When cultured under hypoxic conditions, all samples (benign and malignant) showed an intense and diffuse membranous staining for CAIX ($P = 0.001$ compared to normoxic conditions) (Fig 4B). In addition, in all cases, hypoxia also led to increased Zeb1 expression, with a diffuse positive staining ($P = 0.007$ compared to normoxic conditions) (Fig 4D). In contrast, hypoxia did not modify ERG expression in cancer cells, as well as AR and PSA staining that remained positive (since hypoxic conditions were tested with DHT supplementation). Cancer cell proliferation was also identical in hypoxic compared to normoxic conditions, with a proliferation rate less than 3% (Fig 4E, 4F).

***Ex vivo* functional Ca^{2+} imaging**

Finally, functional Ca^{2+} signaling was evaluated in prostate tissue slices obtained from tumor and adjacent non tumor tissues from 11 patients. This study was performed by measuring the variations in basal intracellular Ca^{2+} concentration in response to variation of extracellular Ca^{2+} concentrations (from 2 to 5 mM and 5 from 2 mM). Interestingly, as observed in the graphical trace, exposure to supra-physiological extracellular Ca^{2+} concentration (5 mM) induced a robust and rapid increase of basal cytosolic Ca^{2+} concentration resulting from a Ca^{2+} entry followed by a sustained plateau. Interestingly, this increase could be reversed towards basal intracellular Ca^{2+} concentration when returning to a 2 mM extracellular Ca^{2+} concentration (Fig 5A). The comparison of intracellular Ca^{2+} entry between tumor and adjacent non tumor prostate slices shows an enhanced Ca^{2+} entry in malignant compared to benign tissues (for nine cases out of 11) (Wilcoxon test, $P = 0.014$) (Fig 5B). In tumor tissues, intracellular Ca^{2+} entry was greater in PCa from patients at high risk of recurrence (ISUP group 4 or more, or/and pT3) (n=5), compared to PCa from patients at low or intermediate risk of recurrence (ISUP group 1 to 3, and pT2) (n=6) (Mann Whitney, $P = 0.045$) (Table 2).

DISCUSSION

This study demonstrates that prostate tissue slices can be cultured for up to 5 days without necrosis with a preserved morphology and more importantly with unaffected physiological properties. This longevity of prostate tissue is obtained by using precision-cut slicing and daily replacement of medium complemented with DHT, and it is identical to that previously described by others (4). The expression of classical cell type markers was maintained, and they include p63 for basal cells, and AR and PSA for luminal cells. The cancer cell-associated marker p504S was present in all malignant tissue slices. Moreover, ERG expression was maintained after tissue culture in initially positive ERG tumors. Importantly, this finding is in contrast with a previous study that reported a loss of ERG expression (4).

Since ERG positive staining in prostate cancer cells is mediated by the action of androgens on the fusion gene *TMPRSS2-ERG* (23), this result suggests a fully functional AR-dependent signaling pathway in our model.

In benign tissue slices, an increased proliferation of basal cells was observed after culture when compared to the native tissues (D0). This finding, that is likely to reflect an elevated glandular regeneration after culture, was also observed in a previous study (7), but not in another (4). However, in malignant samples, the proliferation rate remained identical in cancer cells after culture, as previously described (4). Since one of the major potential interests of *ex vivo* models is the testing of therapeutic agents, the important point was to maintain an identical proliferation rate in cancer cells before and after culture.

When removing DHT from the medium, decreased levels of proteins encoded by androgen-regulated genes was observed. These proteins include AR, PSA, and ERG, in both malignant and benign tissues. This demonstration of androgen dependence has been reported only in one previous study (4) but without effect on ERG, and it is mandatory to reproduce *ex vivo* the biology of human PCa cells. In fact, the AR is a key molecule that drives both normal prostate development and PCa. To date, the study of androgen action in the human prostate has been hampered by the lack of adequate models, since most PCa cell lines used for *in vitro* studies are generated from long term culture in the absence of androgens (2). The present *ex vivo* model would therefore allow studying the physiological effects of androgens in either normal or malignant human prostate.

Some previous studies have reported the functionality of PCa organotypic culture. Vaira et al have used this model to demonstrate the effects of targeting PI3K signaling on cancer cell proliferation and apoptosis (3). Other reports have shown in *ex vivo* culture of PCa slices that cytotoxic drugs and ionizing radiations can induce DNA damages (9), and that inhibition of PARP-1 can lead to AR signaling alterations (12). However, no previous studies have

analyzed to date the ability of an *ex vivo* PCa model to respond to major signals of cancer microenvironment, hypoxia, and extracellular calcium.

Since the Ca^{2+} signal is a phenomenon well suited for the rapid transfer of information from the microenvironment to cancer cells (22), the functional value of *ex vivo* cultures could also be reflected by the ability of tissue slices to respond to variations in extracellular Ca^{2+} concentrations. We have developed for the first time an *ex vivo* organotypic culture method suitable for the evaluation of live Ca^{2+} signaling in both tumor and adjacent non tumor samples. This method allowed us to follow the variations of basal intracellular Ca^{2+} concentration dynamics in human prostate slices, resulting from Ca^{2+} entry from the extracellular side of the plasma membrane. The functionality of our model was attested by the variations of intracellular Ca^{2+} concentrations in response to variation in extracellular Ca^{2+} . An increased Ca^{2+} entry in malignant compared to benign tissues was also shown. These data are in agreement with previous studies reporting alterations in Ca^{2+} homeostasis in cancer cells compared to normal cells (24). This type of Ca^{2+} entry has already been described in breast cancer cell lines that was named constitutive Ca^{2+} entry (25). Here, we demonstrate for the first time the existence of a constitutive Ca^{2+} entry in PCa and, more interestingly, its differential level in tumor versus non tumor tissues. A greater intracellular Ca^{2+} entry was also found in tissues sampled from high risk PCa compared to tumors with low or intermediate risk of recurrence, suggesting that Ca^{2+} entry may contribute to cancer progression. There is substantial evidence to suggest an important role for Ca^{2+} during tumorigenesis. Many mitogens and tumor growth factors elicit rapid elevations of intracellular calcium. Furthermore, Ca^{2+} is involved in cellular events that regulate cell cycle progression, cell migration, angiogenesis, and apoptosis in various cancer cells (26). Our findings raise the possibility that PCa cells might be particularly susceptible to novel anticancer agents that target Ca^{2+} homeostasis. Elucidating the nature of Ca^{2+} channels involved in dysregulated

constitutive Ca^{2+} entry is therefore essential to better understand the mechanisms contributing to malignant transformation and cancer progression.

Hypoxia is a common phenomenon observed in solid tumors, including PCa. In human tumors, the effects of hypoxia are largely mediated by the hypoxia-inducible factors (HIFs)—transcription factors that induce the expression of several proteins including the carbonic anhydrase IX (CAIX) (27). Immunohistochemical staining of CAIX has been shown to be an endogenous surrogate marker for marked hypoxia in several types of solid tumors (28-30). Here, we demonstrated for the first time that hypoxic conditions could induce the expression of CAIX in *ex vivo* cultures of human prostate even in benign tissues. In PCa, the process of epithelial to mesenchymal transition (EMT) induced by hypoxia is involved in the resistance to both radiotherapy and chemotherapeutic treatment (15, 31). The key transcription factor of EMT, Zeb1, is associated in human PCa with cancer progression, metastases, and decreased survival (32). Here, we showed that, together with CAIX induction, hypoxia led to increased expression of Zeb1 in epithelial cells. Since EMT is a reversible process mostly driven by epigenetic mechanisms (33, 34), the use of organotypic cultures could therefore represent a valuable preclinical model to study the effects of drugs targeting epigenetics, such as histone-modifying enzymes.

In conclusion, we report for the first time an *ex vivo* culture model of human PCa that remains sensitive to androgen ablation, hypoxia, and extracellular Ca^{2+} . This model could be used to test therapeutic agents in viable tumor samples, thereby allowing the development of a precision medicine in clinical practice.

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FIGURE LEGENDS

Figure 1: Tissue morphology and maintenance in culture. Radical prostatectomy specimen cut in a transverse plane: Tissue samples were taken from the peripheral zone in putative benign and cancer zones determined after palpation (**A**). Cancer samples from the same tumor before culture (**B**), after 5 days of culture with preservation of the tissue architecture and without necrosis (**C**), and after 7 days of culture, with areas of necrosis (**D**) (HES). Scale bars = 100 μm .

Figure 2: Biomarker expression in samples cultured 2 days under normoxic conditions. Normal glands show preservation of the p63 positive basal cell layer (**A**), whereas cancer cells are positive for p504s (**B**). When compared to the baseline status (D0) (**C**), the proliferation rate (determined by Ki67 staining) was elevated in the basal cell layer of normal glands, with several positive cells per gland (**D**). In cancer cells the proliferation rate remains identical after culture (**F**), when compared to D0 (**E**), with less than 3% positive cells. Scale bars = 100 μm .

Figure 3: Expression of AR (**A, B, C**), PSA (**D, E, F**), and ERG (**G, H, I**) in native tumors (D0), and cancer samples cultured for 2 days with or without DHT. In presence of DHT, AR, PSA, and ERG expression in cancer cells (**B, E, H**) remains identical to that observed in the baseline status (D0) (**A, D, G**). In contrast, DHT ablation leads to decreased expression of AR (**C**) and ERG (**I**), and PSA staining is abolished (**F**). Scale bars = 100 μm .

Figure 4: Effects of hypoxia on the expression of CAIX, Zeb1 transcription factor, and Ki67. In cancer samples cultured in normoxic conditions, the hypoxia marker CAIX is negative (**A**),

with negative to focal Zeb1 staining (C). After culture in hypoxia, cancer cells show a diffuse and membranous staining for CAIX (B), with a diffuse nuclear staining for Zeb1 (D). In contrast, hypoxia does not modify cancer cell proliferation, with less than 3% of Ki67 positive cells in both normoxic (E) and hypoxic (F) conditions. Scale bars = 100 μ m.

Figure 5: Variations in intracellular Ca^{2+} in human prostate slices. Exposure to supra-physiological extracellular Ca^{2+} concentration (5 mM) induces a robust and rapid Ca^{2+} entry followed by a sustained plateau and a slow return towards baseline by reducing extracellular Ca^{2+} concentration from 5 to 2 mM. Real time fluorescence images were collected every 80 s. Scale bars = 2 mm (A). Comparison of Ca^{2+} entry between tumor and adjacent non-tumor prostate slices: in 6 out of 9 cases, Ca^{2+} entry is more elevated in tumor compared to non-tumor slices. Variations of Ca^{2+} signal were determined by calculating the difference between basal, at 2 mM CaCl_2 , and maximal Ca^{2+} value after 5 mM CaCl_2 addition ($\Delta F/F_0$). * $P < 0.05$ (Wilcoxon). Real time fluorescence images were collected every 10 s. N=11 patients. Scale bars = 2 mm (B).

Table 1: Patients and tissues characteristics.

Characteristic	Value
Age y, median (range)	67 (51-76)
PSA (ng/mL)	9.4 (7-16)
pTNM (n)	
pT2	15
pT3	11
ISUP group (n)	
1	2
2	11
3	8
4-5	5

Table 2 : Differences in calcium entry.

Risk of recurrence (d'Amico criteria)	Median $\Delta F/F_0$ (min-max)	p (Mann- Whitney)
Low	1.08 (0.81-1.25)	0.045
High	1.30 (1.11-2.78)	









